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Increase in Heat Resistance of Listeria monocytogenes

Scott A by Sublethal Heat Shock

by


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Committee Chairman: Merle D. Pierson
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(ABSTRACT)

Log phase cells of Listeria monocytogenes Scott A were heat shocked in Trypticase Soy + 0.6% Yeast Extract broth at 40, 44, and 48°C for 3, 10 and 20 min at each temperature, followed by heating at 55°C for 50 minutes in order to determine an optimum heat shock response. Most heat shock temperatures significantly increased thermal resistance ($p \leq 0.05$). Increasing heat shock temperature and time allowed the organism to survive much longer at 50 to 65°C than non-heat shocked cells. The optimal heat shock condition was 48°C for 20 min where D-values at 55°C increased 2.3 fold in non-selective agar and 1.6 fold in selective agar in comparison to non-heat shocked cells. However, cells heat shocked at 48°C for 10 min gave more consistent results; these cells were heated at 50, 55, 60, and 65°C to determine a z-value. Although D-values notably increased due to heat shocking, z-values remained constant regardless of the plating medium.

When aerobically heat shocked cells (48°C for 10 min) were plated on a non-selective or a selective medium, a 1.4x

increase in D-value was observed when enumerated under strictly anaerobic conditions. Aerobically heat shocked cells (48°C for 10 min) added to shrimp samples retained the increased heat resistance at 55°C when enumerated on a non-selective medium compared to the non-heat shocked cells. Heat shocking conditions may be created in pasteurization or minimal thermal processing of foods allowing increased heat resistance of pathogenic and spoilage microorganisms.

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I. INTRODUCTION

Listeria monocytogenes (L. monocytogenes) is a Gram positive, facultative anaerobic, rod shaped bacteria that has been reported to cause listeriosis from consumption of contaminated foods [Brackett, 1988; Marth, 1988; Pearson and Marth, 1990; Rosenow and Marth, 1987]. This microorganism is able to survive and grow in many different environments which allows L. monocytogenes to be widely distributed in foods. Of special concern to the food industry is the ability of the organism to survive thermal processing of foods and then grow at refrigeration temperatures.

When bacteria are shifted from an ideal growth temperature to a higher temperature outside the normal range for growth, an increase in heat resistance has been reported [Knabel et al., 1990, Mackey and Derrick 1986, 1987; Murano et al., 1990] This shift from a lower to a higher growth temperature is known as heat shocking. It has been proposed that the increase in heat resistance associated with heat shocking may be due to the synthesis of a set of proteins called heat shock proteins. However, this hypothesis has not yet been proven.

Seventeen heat shock proteins have been identified after a shift in temperature from 30-42°C in Escherichia coli K12 strain W3350 [Yamamori and Yura, 1982]. The rate of heat shock protein synthesis increases 20 fold due to

this shift in temperature. More recently, it was shown that when Salmonella typhimurium is pre-incubated at temperatures ranging from 42-48°C, there is a significant increase in heat resistance prior to heating at 50°C and 59°C [Mackey and Derrick, 1986]. Similarly, after Salmonella thompson is shifted to 48°C for 30 min, there is an increase in heat resistance at heating temperatures of 54°C and 60°C [Mackey and Derrick, 1987].

Increasing incubation temperatures can cause L. monocytogenes to acquire a higher heat resistance compared to cells held at normal physiological temperatures. Fedio and Jackson [1989] heat shocked cells of L. monocytogenes at 48°C for 1 hr and reported an increase in thermal tolerance at 60°C in milk and in a non-selective medium. Knabel et al. [1990] observed an increase in heat resistance for cells grown in milk at 43°C compared to cells grown at 37°C. Murano et al. [1990] showed that Escherichia coli 0157:H7 had increased heat resistance at 55°C after heat shocking at 42°C for 5 min.

The increase in heat resistance due to heat shocking has also been demonstrated in a solid food system. Farber and Brown [1990] heat shocked L. monocytogenes in sausage for 2 hrs at 48°C and found that these cells were 2.4x more heat resistant than untreated cells after heating at 64°C.

This phenomenon of heat shocking may have practical

implications within the food industry. Minimal thermal processing methods used to increase the shelf life of food may involve long come up times. Most notable of these processes include vat pasteurization of dairy products and "sous-vide" processed refrigerated foods. If raw milk were permitted to rise slowly in temperature until the final pasteurization temperature is obtained, an increase in heat resistance of L. monocytogenes is conceivable. "Sous-vide" processing involves placing raw or blanched products in a plastic pouch and vacuum sealing the package. The pouch is then placed in a hot water bath at pasteurization temperatures, chilled rapidly after the heat process, and then stored under refrigeration temperatures until consumption [Swientek, 1989]. Some "sous-vide" processed products utilize very low processing temperatures (55°C) for very long periods of time to retain product flavor and texture. These processing conditions may be helpful for improving food quality, but may also cause a heat shock response which may increase heat resistance of certain bacteria in food. Food processors need to consider the increased resistance of heat shocked microorganisms such as L. monocytogenes when evaluating the safety of a thermal process that involves minimal heating temperatures.

This study was undertaken to investigate the heat shock reponse of L. monocytogenes Scott A using different plating

media and different plating environments. The objectives of this study were:

- a) To determine the effect of heat shock on survival of L. monocytogenes Scott A at 55°C.
- b) To study the response of non-heat shocked and heat shocked cells:
 - 1) Non-selective vs. selective enumeration.
 - 2) Aerobic vs. anaerobic enumeration.
- c) To determine the survival at 55°C of non-heat shocked and heat shocked cells in shrimp.

II. LITERATURE REVIEW

A. Characteristics of Listeria monocytogenes

1) Taxonomy and Morphology

L. monocytogenes is a Gram positive, rod shaped bacterium which was first described in 1926. Murray described infection among laboratory rabbits and guinea pigs caused by a bacterium which was named Bacterium monocytogenes [Murray, 1955]. This original name for the organism was chosen because the infection was characterized by monocytosis [McLauchlin, 1987]. A similar bacterium was isolated from a liver of infected gerbils and named Listerella hepatolyticum in honor of Lord Lister, the father of antiseptics [Seelinger and Jones, 1986]. In 1940, Listerella was changed to Listeria for taxonomic reasons and the organism, as we know it today, was named Listeria monocytogenes.

The first report of human infection due to L. monocytogenes was reported by Neyfeldt in 1966 who isolated the organism from the blood of patients who had a disease resembling mononucleosis [Gray and Killinger, 1966]. Early on, the organism was identified in women as a cause of abortion in first trimester pregnancy, stillbirth, or septicemia. Meningitis and encephalitis in the newborn and pregnant women were later found to be caused by the organism.

In 1940, eight species under the genus of Listeria were recognized as L. monocytogenes, L. ivanovii, L. seeligeria, L. innocua, L. welshimeri, L. denitrificans, L. grayi, and L. murrayi. It is now generally agreed that L. denitrificans does not belong as a member of the genus Listeria. DNA hybrid studies suggest that the two species L. grayi and L. murrayi should makeup a new monospecific genus termed "Murraya" [Seelinger and Jones, 1986].

L. monocytogenes is characterized as a short, rod shaped bacterium 0.4-0.5 um in diameter and 0.5-2.0 um in length [Seelinger and Jones, 1986]. In older cultures, rods may appear curved in length and become larger in overall size. The organism is Gram positive and is not acid fast. Listeria monocytogenes is non-sporeforming and is motile at 20-25°C by peritrichous flagella. Colony formation after 48 hrs of incubation at 37°C on nutrient agar appear low convex, dew-drop and are 0.5-1.5 mm in diameter. Colonies appear blue-gray by normal illumination. With obliquely transmitted light (known as the Henry Technique), a blue green sheen is produced from the light reflecting from the colonies.

Antigenic types are distinguished by somatic (O) and flagellar (H) antigens. Based on these antigens, serotypes are divided up into 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 4f, 4g, 5, 6, and 7. Fifty-nine percent of

human listeriosis cases are caused by serotype 4b and 18% by serotype 1/2a [Seelinger and Jones, 1986].

2) Growth Requirements

L. monocytogenes can survive in a very wide range of environments. Growth is best in the temperature range of 30-37°C. The organism can grow in a pH range of 5.0-9.8 with optimum growth at a pH of 7.0 [Brackett, 1988]. L. monocytogenes has been shown to survive at a pH as low as 4.0 in cabbage juice [Beuchat and Brackett, 1986]. The organism is halophilic and is capable of growing in 10% NaCl. At a pH of 6.0, survival has been shown at 16% NaCl for up to a year. Some strains can tolerate up to 20% NaCl and survive for extended time periods under ideal conditions [Seelinger, 1961].

L. monocytogenes grows best in a microaerophilic environment consisting of 5-6% oxygen but can grow aerobically (21% oxygen) and strictly anaerobically (0% oxygen). Catabolism in all environments is by the Embden-Meyerof-Parnas (EMP) pathway. Under anaerobic conditions the end product is primarily lactic acid and aerobically the end products primarily include pyruvate, acetoin, lactic acid and others. No evidence exists for the Entner Doudoroff (ED) pathway, however, glucose-6-phosphate dehydrogenase and 6-phosphoglucanate dehydrogenase, which

are two enzymes involved in the ED pathway, have been reported to be present [Seelinger and Jones, 1986]. The organism possesses a split noncyclic citric acid pathway with an oxidative and reductive portion that is important for synthesis of cell constituents. The energy for the cell is supplied by glycolysis [Pearson and Marth, 1990]. The organism contains a heme-containing catalase that is inhibited by KCN and NaNO_3 [Seelinger and Jones, 1986].

L. monocytogenes is not fastidious, but certain vitamins, amino acids and carbohydrates are essential for growth. Biotin, riboflavin and thiamine are required for growth. Isoleucine, leucine, and valine are required amino acids while other amino acids act as growth stimulants such as arginine, histidine, methionine, and tryptophan. Carbohydrates, especially glucose, are essential for growth of L. monocytogenes. Strains of L. monocytogenes are sensitive to a number of antibiotics including: ampicillin, carbenicillin, cephaloridine, chloramphenicol, erythromycin, furazolidone, neomycin, novobiocin, olecindomycin, ticarallin, azlocillin and less sensitive to chlortetracycline, oxytetracycline, tetracycline, gentamian, kanamyan, nitrofurantoin, penicillin G, streptomycin and methicillin. Cases of listeriosis are usually treated with erythromycin or tetracycline. Strains are resistant to colistin sulfate, nalidixic acid, polymyxin B, and

sulfonamides [Seelinger and Jones, 1986].

3) Distribution of the organism

L. monocytogenes is commonly found in the environment. It is believed that the organism lives a saprophytic life in close association with soil and the bacterium can be isolated from many forms of plant and animal life. The organism has been found in human feces, in cattle, silage and soil [Brackett, 1988]. Because L. monocytogenes is able to survive in many different conditions, and the organism is widespread throughout our environment, it is not surprising that the organism can be found in a wide assortment of food products.

The greatest concern for Listeria in foods in the past decade has been with dairy products. Although only two well documented foodborne outbreaks have been associated with dairy products in the U.S. (milk and cheese), the organism has been isolated from a number of dairy products. A nationwide survey performed in 1986 showed that the incidence of L. monocytogenes in raw milk varied from 0% in California to 7% in Massachusetts. An overall estimation of incidence of L. monocytogenes in raw milk was estimated to be 4.2%. Twenty-five of the 27 strains (93%) isolated from the raw milk were pathogenic to humans. The concentration of L. monocytogenes in raw milk has been shown to be low-

probably <1.0 cell/ml [Lovett et al., 1987].

The organism has been isolated from Brie soft ripened cheese, Camembert cheese, Mozzarella cheese, chocolate milk and ice cream [Rosenow and Marth, 1987]. Several dairy items have been recalled (removed from retail channels) due to contamination by L. monocytogenes. According to the Food and Drug Administration (FDA), a factory making Brie cheese operating under the Soft-Ripened Cheese Agreement (requiring the milk to be pasteurized) was recalled due to Listeria contamination [Pearson and Marth, 1990]. Additional brands of Brie cheese have also been recalled. Ice cream has also been shown to harbor L. monocytogenes. Over 25,000 cases of Polar bar ice cream were recalled due to contamination. No illnesses were confirmed from the contaminated ice cream bars, possibly because the frozen state of the product may have prohibited proliferation of the organism. In another recall, a million gallons of ice cream from a major manufacturer were recalled from 11 midwestern states when L. monocytogenes was found in the product. Additional recalls involved those of Liederkranz cheese in 1985 and soft Mexican-style cheese in Arizona in 1986 [Doyle, 1989].

Listeriosis has also been associated with consumption of meat products. The organism was isolated from an open package of Plantation Brand turkey franks from a patient's refrigerator and from unopened frankfurter packages in a

local grocery store. The isolate from the frankfurter was confirmed by the Center for Disease Control as serotype 1/2a [Doyle, 1989]. This strain was also isolated from the female victim of listeriosis who had cancer and was hospitalized for sepsis at the time of infection.

L. monocytogenes can be isolated from a number of animals which makes it likely that poultry and red meats are contaminated with the bacterium. L. monocytogenes was isolated from 57% of fresh and frozen poultry that were sampled. It has also been reported that 15% of oven-ready poultry samples contained L. monocytogenes [Brackett, 1988]. In another study, 70% of ground beef, 43% of pork sausage, and 48% of poultry were found to be contaminated with the organism [Lee and McClain, 1986; McLauchlin, 1987].

Seafood can also harbor L. monocytogenes. An FDA study of seafood including shrimp, crabmeat, lobster tail, langostinos, scallops, surimi-based imitation seafoods and cold smoked fish found 26% positive samples for L. monocytogenes [Pearson and Marth, 1990].

Prevalence in selected food products was reported as follows: poultry - England 15%, Wales 53%; dry sausage - 22%, sausage - 4.1%, frozen chopped beef - 9.6% and lamb - 14% in France. In a study conducted by the FDA, the incidence of L. monocytogenes was as follows: shrimp 5.1%, cooked lamb 4.9%, cheddar cheese 1.4%, Swiss cheese 0%,

butter 0%, ice cream 3.8% and novelty ice cream 8.9% [Doyle, 1989].

Fruits and vegetables are less frequently reported as sources of L. monocytogenes. The organism has been isolated in fresh lettuce, celery, tomatoes and cabbage. Cabbage has been implicated in an outbreak involving coleslaw in Canada [Beuchat and Brackett, 1986].

In foods, L. monocytogenes can grow in the presence of other microorganisms. Moreover, L. monocytogenes becomes an excellent competitor when stored at refrigerated temperatures. The organism has been shown to compete well in cabbage [Beuchat and Brackett, 1986], various dairy products [Rosenow and Marth, 1987], and in meats [Kahn et al., 1975]. However, lack of effective methods to enumerate and confirm L. monocytogenes in the presence of mixed flora has been a major problem in the food industry.

4) Isolation, Identification, and Enumeration

The isolation, identification, and enumeration of L. monocytogenes has proved challenging for the food microbiologist. The level of L. monocytogenes in foods in most cases is $<10^2$ /g [Lovett, 1988]. The most practiced means to isolate the organism uses a cold enrichment procedure.

A 4°C cold enrichment was first used by Gray and

Killinger [1948]. Cold enrichment was typically used as a pre-enrichment step but can be the only enrichment step. The rationale behind cold enrichment at 4°C is that most other bacteria will be suppressed thus allowing L. monocytogenes to dominate. However, a major disadvantage of cold enrichment is that it may take a few weeks up to a few months to isolate the organism. To speed up the enrichment procedure, antibiotics have been used in conjunction with a 37°C incubation temperature. The use of antibiotics in many cases can reduce enrichment time to only a few days.

Doyle [1989] took advantage of the microaerophilic nature of L. monocytogenes for developing a scheme for isolating the organism from foods. No cold enrichment procedure was utilized. Instead, antibiotics and the microaerophilic environment were used to further select for L. monocytogenes.

Isolation of L. monocytogenes from dairy products has most frequently been done with Modified McBride Listeria (MML) agar, McBride Listeria (ML) agar, and Martain's Listeria agar (MaL) [Lovett, 1988]. Lithium Chloride Polyphenylethanol Moxalactam (LPM) agar has been recommended for isolation of L. monocytogenes from meat and poultry products and is extremely selective for the organism [Lee and McClain, 1986]. Formulations for these four selective media for L. monocytogenes are given in Table 1.

Table 1. Isolation media for L. monocytogenes

Isolation agar and components	Concentration g/L
McBride's <u>Listeria</u> agar:	
Phenyl ethhanol agar	35.5
Glycine anhydride	10.0
Lithium chloride	0.5
Sheep Blood	5.0%
McBride <u>Listeria</u> agar, (modified):	
Phenyl ethanol agar	35.5
Glycine anhydride	10.0
Lithium chloride	0.5
Cycloheximide	0.2
Martain's <u>Listeria</u> agar:	
Typtose broth base	10.0
Nalidixic acid	0.05
MgCl ₂ · 6H ₂ O	0.7
Hydrocollöid gum	8.0
Lithium Chloride Phenylethanol	
Moxalactam:	
Phenylethanol agar	35.5
Glycine anhydride	10.0
Lithium Chloride	5.0
Moxalactam	.02

The use of a stereomicroscope with a light source illuminated towards an agar plate at an angle of 45° can be used for primary identification purposes. This method is known as the Henry illumination method. Colonies of L. monocytogenes on all four isolation media appear blue to blue-gray as long as blood is removed from the media formulation.

The most common method used for the isolation of L. monocytogenes in both the U.S. and in Europe is a procedure developed by the Food and Drug Administration (FDA) (Table 2) [Lovett et al., 1987]. The method uses a single enrichment broth containing Trypticase Soy + 0.6% Yeast Extract (TSYE) broth, 15mg/L acriflavine-HCL, 40mg/L nalidixic acid, and 50mg/L cycloheximide. Samples are taken at day one and day seven and streaked on ML agar. Half of the samples are diluted in an alkalizing solution to reduce competitive microflora in the enrichment broth. Improved recovery has been noted at the seven-day sampling period due to repair of injured cells [Anonymous, 1988].

A shorter method has been devised by the FDA which involves a one and two-day sampling eliminating the alkalizing step by utilizing LPM agar along with MLA agar. LPM is a much more selective medium which reduces the competing microflora in the enrichment broth. The improved procedure has been reported to be equally as sensitive as

Table 2. Current procedures used by federal agencies to detect L. monocytogenes in foods.

F.D.A.	U.S.D.A
a) 25 g or 25 ml sample into 225 ml enrichment broth (EB). Stomached 2 min Incubated at 30°C 1, 7 days	a) 25 g sample into 225 ml primary enrichment broth (PEB). Incubated 30°C, overnight.
b) Cultures of 1 and 7 day incubation in EB, streaked on Modified McBride <u>Listeria</u> (MML) agar. Samples are streaked directly and after a 1/10 dilution in KOH. MML agar incubated 35°C, 48hrs.	b) 0.1 ml sample from PEB into secondary enrichment broth (SEB). Incubated 30°C, overnight.
c) MML agar plates examined by Henry technique.	c) From SEB, 1 ml samples streaked directly and after a dilution in 4.5 ml of 0.25% KOH. onto Lithium chloride Phenylethanol Moxalactam (LPM) agar.
d) Colonies from MML agar streaked onto Trypticase Soy + 0.6% Yeast Extract (TSYE) agar, incubated 30°C until colony formation.	d) LPM agar incubated 30°C, overnight. Examination by Henry technique.
e) Confirmation done with various biochemical tests using colonies from TSYE agar. [Lovett et al. 1987]	e) Confirmation done with various biochemical tests using colonies from LPM agar. [McClain and Lee, 1988]

the current method used by the FDA. These methods have been most successfully used for isolation in dairy products.

Because the traditional cold enrichment was too long and the methods used by the FDA were unable to detect 20-2500 L. monocytogenes/ml in previously inoculated raw ground beef samples, the United States Department of Agriculture (USDA) developed a quicker method to identify L. monocytogenes. Beta-hemolytic Listeria colonies from meat products can be identified in only 3-4 days [McClain and Lee, 1988]. According to the USDA, intense competition from inherent microflora in the raw ground beef did not allow for recovery of L. monocytogenes when the FDA recovery methods were used.

The method developed at the USDA involves a two stage enrichment procedure that can be used to detect Listeria in meats (Table 2). The primary enrichment broth contains nalidixic acid and acriflavine for selectivity. After incubation of the primary enrichment broth, the culture is transferred into a second enrichment broth containing an identical formulation as the primary enrichment broth except for an additional 13 mg/L of acriflavine. Colonies are streaked onto the very selective LPM agar and observed using Henry illumination for blue or blue-gray colony formation.

A major concern with procedures used by the FDA and USDA is that of recovery of injured Listeria. The use of highly selective media may not give the organism sufficient time to

recover and form colonies on the plate. Unrecovered and undetected injured cells could recover in a food and proliferate to high numbers. The USDA scheme deserves the most amount of scrutiny because it uses LPM agar which is highly selective against heat-injured L. monocytogenes. The extent of growth and the ability of the organism to recover in refrigerated foods from thermal injury of L. monocytogenes is not known.

All of the isolation methods described involve lengthy procedures to complete confirmation of the organism. The procedures also may not enable recovery of the injured population. To shorten identification time, rapid methodology has been developed including: flow cytometry, an enzyme linked immunosorbant assay (ELISA) and a beta-hemolysin gene probe.

Donnelly, et al. [1988] developed a method of flow cytometry. Flow cytometry characterizes a bacterial cell population by morphology, nucleic acid profile, and surface antigens as the cell passes through a laser beam. The species need to be labeled with polyvalent antibodies for cell surface antigens and propidium iodide for cellular DNA [Pearson and Marth, 1990]. Unfortunately, current methods involving flow cytometry of L. monocytogenes provide many false positive and false negative results. In addition, the method may be impractical because of very high costs.

An ELISA technique using monoclonal antibodies against flagellar antigens of L. monocytogenes has been described [Farber and Speirs, 1987]. In addition, a monoclonal antibody procedure specific for L. monocytogenes, L. innocua, and L. welshimeri has been developed. All strains of L. monocytogenes were positive using this monoclonal antibody assay [Siragusa and Johnson 1990]. This technique using antibodies against L. monocytogenes holds great promise for a variety of foods, but further laboratory and field research needs to be conducted before this methodology can be applied on an industrial scale.

In 1987, Datta et al. developed a beta-hemolysin gene probe. All virulent strains of L. monocytogenes have been found to contain a beta-hemolysin. The DNA probe contains a 500 base pair complementary to the beta-hemolysin gene and has been used primarily for research purposes and for naturally contaminated raw milk samples. Current concerns with the gene probe are that contaminated product must have >10 L. monocytogenes/g and not all strains of L. monocytogenes react with the probe.

B. Listeriosis - The Disease

1) Disease Characteristics

Listeria monocytogenes is an opportunistic intracellular pathogen that primarily affects four subsets of the population; a) immunocompromised individuals (such as those on chemotherapy, corticosteroids or infected with the AIDS virus), b) pregnant women, c) fetuses and young children, and d) the elderly. These hosts are at the greatest risk, however, under the proper circumstances, anyone can be infected by L. monocytogenes.

The symptoms associated with listeriosis can be drastically different depending on the host. A healthy host is generally well equipped to fight the disease. Depending on the condition of the healthy host, symptoms may vary from unnoticeable to flu-like. If detected in time, a healthy host is usually cured by the administration of antibiotics.

Listeriosis for the immunocompromised host can occur in many different forms. Meningocystic listeriosis generally occurs in newborns or adults over 50 years old. A 70% mortality rate is observed in untreated individuals or victims treated too late [Seelinger and Jones, 1986]. Meningoencephalitic listeriosis is a combination of meningitis and encephalitis. Changes occur in the blood including accelerated sedimentation rate, leukocytosis with predominant granulocytosis (an increase in the number of

cells within the cytoplasm that contain granules usually with an increase in white blood cell count). Spinal fluid appears turbid with appearance of monocytes and elevated pressure [Seelinger and Jones, 1986]. Treatment usually consists of antibiotics such as ampicillin, penicillin, or erythromycin.

Cutaneous listeriosis is a form most common for people who are in direct contact with animals such as veterinarians and farmers. Skin lesions start out as skin nodules increasing from a pin hole size to the size of a pea including pus formation in about 24 hrs time. Septicemic listeriosis is a form of the disease characterized by fever, pharyngitis, and leukocytosis accompanied by mononucleosis (an increase in number of agranulocytes and leukocytes in cytoplasmic granules in circulating blood). Often accompanying this form of listeriosis is oculoglandular listeriosis which is an infection of the eye causing conjunctivitis. These are the most common forms of the disease in an immunocompromised host, however, the disease has been described in many other forms.

Common symptoms of pregnant female host include fever, chills, backache, headache and discolored urine. Meningitis can be formed in the pregnant host but is very uncommon. The organism can be isolated from blood, umbilical cord blood, lochia (discharge from uterus and

vagina following delivery of a baby), tissue obtained by curettage, vaginal mucus, urine and placental tissue [Seelinger and Jones, 1986].

Infection of the fetus may occur via the transplacental route or during delivery. After the fetus is infected by the organism, fetal urine is discharged into the amniotic fluid and then aspirated by the fetus causing the bacteria to spread to respiratory and gastrointestinal tracts [Marth, 1988]. Symptoms of listeriosis in newborns are extremely variable including respiratory distress, heart failure, convulsions, vomiting, cyanosis, refusal to drink, mucus stools, early discharge of meconium (greenish mass that accumulates in the bowel during fetal life) and pronounced leukocytosis (an increase in leukocytes in red blood cells).

Virulence of L. monocytogenes may be due to two toxins - hemolysin and cytotoxic toxins. The hemolysin is a cytotoxin capable of lysing both tissue and red blood cells. Once ingested, L. monocytogenes is first taken up by the macrophages where the organism has the ability to survive and multiply causing the macrophages to burst. Once inside the macrophage, it is thought that a bacteria-coded hemolysin acts to dissolve the membrane of the macrophage. After the macrophage is broken down, L. monocytogenes uses the cytoskeleton of the macrophage for its own purposes. As the bacterium enters the cytoplasm of the macrophage, it is

surrounded by a protein called actin. L. monocytogenes uses actin to aid in cell division and to form a pseudopod-like extension. This extension allows the bacterium to be propelled out of the host macrophage membrane and engulfed by another host macrophage which starts the cycle again. Each time this occurs, one bacterium is left in the original host macrophage and, due to bacterial cell division, another is engulfed by another host macrophage [Donelson and Fulton, 1988].

2) Foodborne Outbreaks

Between March and September, 1981, in the Maritime Provinces of Canada, 34 cases of perinatal (comprising mothers and newborn children) and 7 cases of adult listeriosis occurred. The organism was isolated in blood and stool cultures as well as other areas in both the mother and fetus. The food vehicle in this outbreak was thought to be coleslaw that was probably contaminated by sheep manure used to cultivate the cabbage fields. L. monocytogenes was not isolated from the cabbage or from the field, but, sheep from the field had recently died of listeriosis [Twedt, 1986]. In addition, the cabbage was held at refrigeration temperatures for an extended period of time before distribution which may have allowed for proliferation of the psychrotrophic pathogen. L. monocytogenes serotype 4b was

isolated from coleslaw and from patients with listeriosis. The mortality rate for this outbreak was 44% [Pearson and Marth, 1990].

Between June and September, 1983, another large outbreak of human listeriosis occurred in Massachusetts including 7 perinatal and 42 non-pregnant immunocompromised adult cases [Twedt, 1986]. The vehicle was 2% fat pasteurized milk from a single dairy plant. This outbreak alerted and concerned researchers that L. monocytogenes may have increased thermal tolerance when compared to other vegetative foodborne pathogens. The organism was never recovered from the dairy plant which was reported to have pasteurized the milk at 169°C for 19.5 sec. [Twedt, 1986].

After the Massachusetts outbreak in 1985, 121 raw milk samples associated from the outbreak were taken from 3 different sources: trucks that transported the milk from the farms to the cooperative, trucks that transported raw milk from the cooperative to the dairy plants, and bulk tanks on the dairy farms which the milk originated from. The method for recovery that consisted of a cold enrichment period of 1 month at 4°C revealed 15/121 (12%) raw milk samples positive for L. monocytogenes [Hayes et al., 1986].

In another outbreak, Mexican style fresh cheese was determined to be the cause of a widespread multistate outbreak involving 142 cases of listeriosis between January

and June, 1985 [Twedt, 1986]. Ninety-three perinatal and 49 immunocompromised cases were reported with a 31% and 37% mortality rate, respectively. The outbreak strain was serotype 4b which was recovered from unopened soft cheese packages, and also from many areas in the processing plant environment. Some cheese samples were phosphatase-positive indicating that the milk used in the cheese was improperly pasteurized. It has been proposed that producers of the cheese may have mixed raw or post-pasteurized contaminated milk with pasteurized milk during the making of the cheese.

A food related outbreak in Vaud, Switzerland between 1983-1987 was associated with consumption of Vacherin Mont d'Or cheese. Sixty-one adult cases and 61 maternal cases were diagnosed with a mortality rate of 27% [Doyle, 1989]. The source of contamination was thought to originate from ripening cellars where the cheese was stored.

C. Thermal Resistance

1) Heat Resistance Studies

When subjected to moist heat, bacteria most often die in a logarithmic fashion. A general equation of a survival curve (plotted as log number of survivors vs. time) is given by $t = D (\log a - \log b)$ in which t is time in minutes, D is time in minutes to kill 90% of the cells (spores or vegetative) in the population of a given organism, a is the

initial number of viable cells in the population and b is the number of viable cells in the population after time t . A survival curve is usually plotted as log number of survivors vs. time in minutes at a heating temperature. The D-value is equal to the negative reciprocal of the slope of the survivor curve [Stumbo, 1973].

In addition to D-values, z , and F values are frequently given to indicate the heat resistance of an organism. A "phantom" thermal death time curve is a plot of log D vs. temperature. A z -value is defined as degrees (C or F) required for the thermal death time curve to transverse one log cycle (or the negative reciprocal of the slope). An F value is equivalent to time in min at a given temperature, of all heat considered, to completely destroy spores or vegetative cells of a particular organism. A plot of F versus temperature is called a thermal death time curve; a z -value can be calculated from this plot [Stumbo, 1973].

Thermal resistance studies are usually performed in one of three ways: sealed thermal death time (TDT) tubes, capillary tube method, and the flask method. The sealed TDT tube method [Bigelow and Esty, 1920] involves placing the inoculated menstruum in small diameter test tubes that are sealed and completely submerged in a thermostatically controlled heating medium such as water, mineral oil, etc. Sample TDT tubes are withdrawn, rapidly cooled in ice, and

plated. In the capillary tube method, [Stern and Proctor, 1954] very rapid heating and cooling is possible because small sample sizes are placed in capillary tubes, heated in a heating bath, withdrawn at the appropriate times, and cooled prior to plating. The flask method, which was chosen for this study, is ideal for studying resistance of non-spore forming bacteria below the boiling point of water. The method involves a pre-heated heating menstruum that is circulated (usually by a magnetic stir bar). The flask is located in the bath in such a way that the surface of the heating medium will be well above the substrate surface. When the desired heating temperature is achieved, the inoculum can be introduced. At different time intervals, samples are withdrawn using a sterile pipette and placed in a sterile tube prior to cooling in ice and plating. When the method is properly employed, heating and cooling lags are negligible. These methods can be used to determine D, z, and F values [Stumbo, 1973].

The heat resistance of microorganisms in foods is affected by both the composition of the food and the physiological condition of the microbial cells or spores. Cells in stationary phase of growth are more tolerant to heat than cells in log phase. Also, heat resistance of bacteria tends to increase as the temperature of incubation increases. Log phase cells grown at 35°C of Salmonella

senftenberg 775W in Trypticase Soy broth (TSB) had a D=4.8 min while cells grown at 44°C had a D=12.5 min at 55°C. Stationary cells of S. senftenberg 775W grown at 35°C in TSB have a D=14.6 min, compared to a D=42.0 min at 55°C for cells grown at 44°C [Ng et al., 1969].

2) Heat Resistance of Listeria monocytogenes

A major problem in the food industry is the ability of pathogens and/or spoilage organisms to survive thermal processes and then proliferate at refrigerated storage temperatures prior to consumer use of the food. L. monocytogenes is a major concern to food processors because it is psychrotrophic and it is believed that the thermal tolerance may be higher than other vegetative food-borne pathogens. Some of the published D-values for L. monocytogenes are presented in Table 3. A range of z-values from 4.3 to 9.9°C have been published with an average z of 6.7°C. These z values are considerably higher than other non-sporeforming bacteria [Mackey et al., 1989]. Although conflicting data has appeared in the literature because of differing heating methods and test strains, most data does conform well to a straight line plotted log-D versus temperature given as: $\log D = 10.888 - 0.14519t$ (D=decimal reduction time in seconds, t = temperature °C, and z = 6.9°C [Mackey et al., 1989].

Table 3. Thermal resistance of L. monocytogenes.

Strain		Product	Process Temp °C	D-value (seconds)	Method
9-127	(1)	Sterile Milk	61.7	648.0	HOLD
9-128	(2)	Sterile Milk	61.7	648.0	HOLD
9-71	(4a)	Sterile Milk	61.7	636.0	HOLD
9-138	(1)	Sterile Milk	61.7	630.0	HOLD
9-63	(3)	Sterile Milk	61.7	630.0	HOLD
9-137	(4b)	Sterile Milk	61.7	570.0	HOLD

[From Bearns and Girard, 1958]

Scott A (4b)		Raw Milk	52.2	1683.7	ST TDT
Scott A (4b)		Raw Milk	57.8	289.6	ST TDT
Scott A (4b)		Raw Milk	63.3	19.9	ST TDT
Scott A (4b)		Raw Milk	66.1	7.3	ST TDT
Scott A (4b)		Raw Milk	68.9	3.0	ST TDT
Scott A (4b)		Raw Milk	71.7	0.9	ST TDT
Scott A (4b)		Raw Milk	74.4	0.7	ST TDT

[From Bradshaw et al., 1985]

Scott A (4b)	I	Raw Milk	52.2	3171.8	ST TDT
Scott A (4b)	F	Raw Milk	52.2	2289.3	ST TDT
Scott A (4b)	I	Raw Milk	57.8	490.1	ST TDT
Scott A (4b)	F	Raw Milk	57.8	445.0	ST TDT
Scott A (4b)	I	Raw Milk	63.3	33.3	ST TDT
Scott A (4b)	F	Raw Milk	63.3	31.9	ST TDT
Scott A (4b)	I	Raw Milk	66.1	ND	ST TDT
Scott A (4b)	F	Raw Milk	66.1	15.1	ST TDT
Scott A (4b)	I	Raw Milk	52.2	7.0	ST TDT
Scott A (4b)	F	Raw Milk	52.2	6.4	ST TDT
Scott A (4b)	I	Raw Milk	52.2	1.6*	ST TDT
Scott A (4b)	F	Raw Milk	52.2	1.3	ST TDT

[From Bunning et al., 1986]

Table 3. (Cont.)

Strain	Product	Process Temp °C	D-value (seconds)	Method
F5069 (4b)	TP Broth	62.0	24.0	SL TDT
F5069 (4b)	TP Broth	62.0	24.0	SL TDT
F5069 (4b)	TP Broth	62.0	6.0	SL TDT

[From Donnelly et al., 1987]

Scott A (4b)	Beef	60.0	499.2	HOLD
Scott A (4b)	Chicken	60.0	317.4	HOLD
NCTC 11994 (ND)	Beef	60.0	376.2	HOLD
NCTC 11994 (ND)	Chicken	60.0	301.2	HOLD

[From Mackey and Bratchell, 1989]

TP = Tryptose Phosphate

I = L. monocytogenes in an intracellular state.

F = Freely suspended L. monocytogenes.

* = extrapolated value

ND = Not determined

SL TDT = Sealed Thermal Death Time Tube

HOLD = Holding

The high heat resistance and ability of L. monocytogenes to survive refrigeration temperatures could be hazardous with minimally thermal processed foods such as "sous-vide" foods or pasteurized dairy products. In both minimal thermal processes, low temperatures are used for inactivation of inherent pathogenic microorganisms. The dairy industry is so concerned that a "zero" tolerance rule is in effect for all Listeria in pasteurized dairy products. Survival of a single cell of L. monocytogenes after a minimal thermal process is threatening because growth of the organism at refrigerated storage is likely.

3) Pasteurization Debate

The Massachusetts outbreak of 1983 involving pasteurized milk led many researchers to believe that L. monocytogenes may have enough thermal tolerance to survive current pasteurization of milk. Based on results from experiments with the sealed tube heating method, a 39-D reduction in L. monocytogenes should occur during vat pasteurization of milk (62.8°C for 30 min). A much smaller level of safety, 5.2-D reduction, is predicted for High Temperature Short Time (HTST, 71.7°C for 15 sec.) [Mackey et al., 1989].

The question of whether or not L. monocytogenes can

survive pasteurization of milk is still debated.

Conflicting data has been published due to a) different heat resistance methodology, b) the strain of L. monocytogenes used in the study and c) the physiological state of the microorganism during heating. Because of the Massachusetts outbreak involving pasteurized milk as the food vehicle, strains isolated from this outbreak are currently used to determine thermal resistance in milk. Of these strains, Scott A is believed to be the most heat resistant [Bradshaw et al., 1987].

Bearns and Girard [1958] were the first to provide evidence that L. monocytogenes may be able to survive pasteurization used in the dairy industry. Using the holding technique (61.7°C, 35 min), it was shown that when initial inoculum levels of 5×10^4 or higher were added to the raw milk, survivors could be detected after heating. D-values at 61.7°C were greater than 10 min. for most strains (Table 3). The heating method used for heating involved using unsealed test tubes submerged slightly below the water level in a pre-heated hot water bath followed by spread plating and 37°C incubation for 48 hours. Many researchers are skeptical of these findings since the methodology used in this study may have allowed cells to adhere to the neck of the test tube. If this occurred, these cells would not have received proper heat treatment.

In later investigations involving inoculated raw milk (1×10^8 L. monocytogenes/ml) that was subjected to a pilot plant size HTST pasteurizing unit, no cells were detected immediately after thermal treatment. However, after the pasteurized milk was allowed to incubate at refrigerated storage (4°C), for 5 days, 2500 L. monocytogenes/ml could be detected [Garazabal et al., 1987]. Authors of this study explained that the cells surviving the heat treatment were thermally injured cells and required a cold enrichment procedure to be recovered for detection.

L. monocytogenes is an intracellular parasite and is commonly found within leukocytes of milk contaminated with L. monocytogenes. Some investigators believe that when in an intracellular state the leukocytes may confer enough added heat resistance to survive the heating process. Doyle et al. [1987] using a HTST plate heat exchanger pasteurization unit showed that the organism could survive pasteurization of milk when contained within polymorphonuclear leukocytes (PMNL's). Holstein cows were inoculated with L. monocytogenes and raw milk containing 1×10^8 cells/ml was collected. This milk was given heat treatments of 71.7-73.9°C for 16.4 sec. Survivors were observed after six of the nine pasteurization trials.

Conflicting evidence supports that the organism will not survive pasteurization of milk. Bradshaw et al. [1985]

heated milk containing a mixture of strains Scott A and Murray B (1×10^5 cells/ml) at seven different temperatures ranging from 52.2 to 74.4°C. D-values ranged from 1683.7 to 0.7 sec respectively (Table 3). The D-value for HTST pasteurization (71.7°C) yielded a D-value of 0.9 sec for the more resistant Scott A strain suggesting that L. monocytogenes could not survive the process. Bunning et al., [1986] further investigated the possibility of enhanced thermal resistance when the cells were in an internalized condition. They compared freely suspended bacteria with bacteria contained in phagocytes. There were no significant differences in mean D-values for each condition. The suspensions were heated at 52.2 to 71.7°C for various time periods in a combined tube and slug-flow heat exchanger. The D-value was 1.6 sec at 71.7°C for freely suspended bacteria which did not significantly differ from those bacteria in an intracellular state (Table 3).

Donnelly et al. [1987] appeared to settle the pasteurization debate when they compared two methods of thermal inactivation. Three strains of L. monocytogenes were compared using the unsealed test tube method (as used in Bearns and Girard, 1958) and the sealed test tube method (as used by Bradshaw et al., 1985). All strains were rapidly inactivated at 62°C when survival was measured using the sealed test tube method, however, thermal inactivation

could not be accomplished even after 30 min using the unsealed test tube method. It was shown that the unsealed method of thermal inactivation was not linear but quadratic [Donnelly et al., 1987]. Two explanations for the tailing off are: a) condensate and splashed cells could have collected on the sides or cap of the unsealed test tubes and drip back into the heating menstrua, and b) upon mixing of the heating menstrua, cells might have adhered and coated to the sides of the test tubes and therefore not received proper heating. Conversely, the sealed tube method of thermal inactivation did show linearity and after only 6-24 sec no survivors were noted by an initial inoculum level of 10^8 - 10^9 cells/ml (Table 3).

The FDA currently supports these findings and concludes that only the presence of extraordinarily high numbers of the organism in the raw milk could result in survivors of the pasteurized product. It is further concluded that the presence of L. monocytogenes in pasteurized dairy products is most probably due to incomplete pasteurization or post-pasteurization contamination. Since raw milk usually contains from 1-100 L. monocytogenes/ml, most researchers also agree that it seems highly unlikely that the bacteria will survive current pasteurization (vat or HTST) conditions for milk. But other minimally processed methods such as "sous-vide" refrigerated foods have yet to be extensively

investigated for possible survivability of L. monocytogenes.

4) Heat Resistance in Other Foods

Other than dairy products, only a limited number of studies have been conducted on the heat resistance of L. monocytogenes in other foods. Depending on the strain used, D-values at 60°C can be nearly 500 sec in beef and over 300 sec in chicken (Table 3) [Mackey and Bratchell., 1989]. Exceptional heat resistance has been reported in chicken breasts heated to an internal temperature of 65.5-71°C and 82°C where large numbers and small numbers of L. monocytogenes remained, respectively [Carpenter and Harrison, 1988]. The organism has also been isolated from grilled meatballs cooked to an internal temperature of 78-85°C with an initial inoculum of 1×10^3 /ml. Possible reasons for such survivability may be due to fat protection and lowered water activity. Addition of salt, spices, and curing salts to sausage meat increased D-values at 60-62°C four to five fold [Farber et al., 1988a].

D. Cell Injury

1) Injury Due to Stress

When microorganisms are subjected to stressful conditions, many times the cell will become injured rather than die. There are several types of stress that can cause

bacterial cell injury. Examples include exposure to: higher or lower temperatures outside the normal range for growth, lowered water activity, salts, antibiotics and toxic forms of oxygen [Smith and Archer, 1988a].

Cell injury is experimentally determined by the inability of bacterial cells, which have been exposed to stressful conditions, to form colonies on a medium containing selective agents (salts, bile salts, antibiotics etc..). Whereas, cells not subjected to these stressful conditions produce colonies on such a medium [Smith and Archer, 1988a]. On a medium not containing selective agents (non-selective medium), even stressed cells can form colonies. Many microbiologists believe that when the cell undergoes changes in order to survive hostile environments, cell injury results. A concern is that an injured population of cells may be able to recover and multiply in foods.

Exposure to temperatures above the optimum growth temperature of a microorganism is a common form of stress that can lead to cell injury. L. monocytogenes and most other bacteria are injured when subjected to a sublethal heating stress. To determine the extent of cell injury due to thermal stress, cells are enumerated with both non-selective and selective media. The total colony count on the non-selective medium minus the total count on the

selective medium results in the number of injured cells. For some microorganisms, addition of solutes to the heating menstrum may protect bacterial cells from heat injury. Smith and Hunter [1988b] reported that salts, polyols, and sugars protected L. monocytogenes Scott A against heat injury. However, the mechanism by which solutes exert their protective effects on bacteria is unknown.

Another type of injury may be due to the formation of toxic forms of oxygen when the organism is grown in an aerobic environment. Under normal oxygenated atmospheric conditions, oxygen spontaneously generates toxic derivatives which may reduce counts. This is a problem for most aerobic bacteria as it is with L. monocytogenes. Toxic forms of oxygen such as singlet oxygen, superoxide radicals, hydrogen peroxide, and hydroxyl radicals can be formed spontaneously in microbiological media. In the case of L. monocytogenes, superoxide radicals, hydrogen peroxide, and hydroxyl radicals pose the greatest threat to cell survival.

A superoxide radical is generated when an oxygen molecule gains an electron which pairs with an antibonding electron. This molecule is a radical because both bonding electrons are not paired and is designated as O_2^- . Superoxide radicals themselves may not be very toxic to the cell; however, their apparent toxicity may be due to the fact that they give rise to hydrogen peroxide. Hydrogen

peroxide is formed when oxygen gains two electrons (or when superoxide radicals gain a single electron), each bonding with an antibonding electron of the oxygen molecule. This molecule is designated as O_2^{2-} and is not a free radical since electrons are paired. Hydrogen peroxide is known to damage the DNA of a bacterial cell. Repair of DNA may be difficult even after addition of catalase. When superoxide radicals are present, hydroxyl radicals are also present. The hydroxyl radical is the most reactive, strongest oxidizing agent, and shortest lived derivative of oxygen. It is capable of damaging almost any biological molecule including DNA [Krieg, 1990].

2) Recovery of Injured Cells

Microorganisms that are injured due to stress may have the ability to survive and return to normal metabolic functions. These cells that can regenerate normal metabolic functions may be on resuscitated on microbiological media with ideal conditions for growth.

Resuscitation of heat injured cells can be very difficult especially with L. monocytogenes, but can be improved with cold enrichment procedures. Enumeration of heat injured cells on a non-selective medium was improved 10-fold when incubated 25°C for 7 days compared to a 37°C, 48 hr incubation [Bunning et al., 1988]. Resuscitation of

heat injured cells can also be enhanced utilizing a 24 hr/30°C incubation, followed by a 21 day/5°C incubation in turn, followed by incubation at 30°C for 48 hrs [Beuchat and Brackett, 1986]. Addition of 2-8% NaCl has been reported to inhibit resuscitation of heat injured cells. But addition of lesser levels of NaCl or addition of KCl, glycerol and sugars appear to enhance resuscitation of heat injured cells. [Golden et al., 1988; Smith and Hunter, 1988b] Therefore, the composition of the resuscitation medium and incubation procedures significantly influence the resuscitation of injured cells.

Under optimal growth conditions, superoxide dismutase (converts two molecules of superoxide radicals to hydrogen peroxide and water) and catalase (converts two molecules of hydrogen peroxide to two molecules of water and two molecules of non-toxic oxygen) are present in L. monocytogenes and act to combat toxic forms of oxygen. Under aerobic growing conditions, these enzymes reduce the levels of toxic oxygen. However, during thermal processing of foods that incorporate temperatures near 55°C, these enzyme systems are inactivated allowing toxic forms of oxygen to have a dramatic effect on the survivability of the organism and hence, recovery is much more difficult on plating media containing these toxic forms of oxygen. These injured undetected cells can pose a problem in the food

industry because the cells may have the ability to repair themselves and proliferate under the right set of conditions. Microbiologists are developing better recovery methods so that the injured population of microorganisms in foods can be more accurately estimated.

To aid in recovery of injured cells due to toxic forms of oxygen, agents such as sodium pyruvate or 3,3'-thiodipropionic acid can be incorporated into recovery media to quench toxic forms of oxygen [Byczkowski and Gessner, 1988]. However, if cells are grown in a strictly anaerobic system, no available oxygen is present, hence, no toxic forms of oxygen. Anaerobic plating rather than aerobic plating may be a solution for improved recovery of thermally stressed organisms lacking proper enzyme systems to combat toxic forms of oxygen.

In a study which determined the catalase and superoxide dismutase activities after heat injury in L. monocytogenes, it was reported that strains Scott A and LCDC produced the greatest amounts of these two enzymes. Superoxide dismutase activity sharply declined after heat treatment of 45°C for 45-60 minutes and catalase activity decreased rapidly between 55-60°C for a 50-60 minute heat treatment. It was also determined that the addition of pyruvate, the addition of exogenous catalase, or anaerobic incubation of cells did not increase the number of uninjured cells plated

out on Trypticase Soy or Trypticase Soy + 5.0% salt agar [Dallmier and Martain, 1988].

E. The Heat Shock Response:

1) Heat Shock and Heat Shock Proteins:

One possible explanation for the survival of L. monocytogenes at high heating temperatures may be due to a heat shock phenomenon. When bacteria are subjected to temperatures slightly higher than optimal conditions for growth, these "heat shocked" cells may have increased heat resistance compared to "non-heat shocked" cells. Neidhardt et al. [1984] were able to increase the heat resistance of Escherichia coli K-12 by raising the cell suspension from 0-50°C at various rates prior to heating at 50°C. More recently, it was shown that when Salmonella typhimurium was pre-incubated at temperatures ranging from 42 to 48°C, there was a significant increase in thermal tolerance at 50°C to 59°C [Mackey and Derrick, 1987]. Likewise, there was a significant increase in heat resistance prior to heating at 54°C and 60°C for Salmonella thompson when cells of this microorganism were incubated at 48°C for 30 min. before heating [Mackey and Derrick, 1986].

The reason for increased heat resistance of microbial cells upon incubation at elevated temperatures is not clearly understood. One possible explanation is the

synthesis of proteins produced in response to heat shocking cells. The synthesis of these "heat shock proteins" (HSPs) is strongly stimulated by a few degrees change in temperature above the normal physiological temperature. The gene coding for HSPs contains a conserved sequence of 14 base pairs in the 5' noncoding region, the Pelham box. This sequence serves as the promotor for heat shock protein mRNA synthesis. Heat shock proteins are believed to be synthesized in all living things from bacteria to man. A major function of heat shock proteins is thought to somehow enable the cell to survive and recover during stressful conditions [Schlesinger, 1986].

In Escherichia coli K12 strain W3350, 17 HSPs have been identified by pulse labeled experiments of log phase cells [Yamamori and Yura, 1982]. When cells were shifted from incubation temperatures of 30°C to 42°C, the rate of HSP synthesis increases 5 to 20 fold, respectively [Grossman et al., 1984] An htpR gene located on the chromosome of E. coli stimulates transcription and is a positive regulator required for a normal heat shock response, causing newly synthesized proteins to be produced. A dnaK gene (66 kilodaltons) coding for the major heat shock protein limits and regulates the synthesis of heat shock proteins. Presumably, the synthesis of HSPs in bacteria helps the cell overcome temperature-induced damage and, therefore, creates

more resistance to heat compared to non-heat shocked cells.

Heat shock proteins have also been found in Bacillus subtilis SR22. Pulse labeling experiments revealed that at least 12 proteins were synthesized in response to a heat shock treatment of 48°C for 10 min. Densitometer tracings from gel-electrophoresis revealed that the 66K protein comprised about 30% of the protein composition of the cell and about 67% of the heat shock related proteins. After heat shocking, the researchers returned the cells to normal plating temperatures and examined the level of heat shock proteins. Within 10 min after heat shocking and re-incubation at 37°C, the bacterial cells resumed normal protein synthesis and the 66K protein synthesized in response to heat shocking diminished rapidly [Streips and Polio, 1985].

Heat shock proteins synthesized in different organisms seem to be at least partially homologous. The dnaK gene which is responsible for turning off the synthesis of heat shock protein synthesis in E. coli was shown to be 57% analogous to the Drosophila melanogaster hsp 70 gene [Bardwell and Craig, 1984]. Log phase cultures of Caulobacter crescentus produce 20 heat shock proteins after heat shocking at 40°C for 5-40 min. Two of the major heat shock proteins produced were hsp62 and hsp 70. Using immunological cross-reactivity procedures, antibodies

against heat shock proteins in E. coli were shown to be analogous to heat shock proteins in C. crescentus. Hsp 62 from C. crescentus was analogous to the most abundant heat shock protein formed in E. coli which was the GroEL protein. Hsp 70 from C. crescentus was analogous to the dnaK gene formed in E. coli and is most likely a regulator of heat shock protein synthesis in C. crescentus [Gomes et al., 1986].

2) Heat Shock Studies with Listeria monocytogenes:

The effect of mild preheating or "tempering" cells of L. monocytogenes has been investigated. Fedio and Jackson [1989] exposed cells of L. monocytogenes Scott A to a preheating treatment of 48°C for 1 hr in Trypticase Soy + 0.6% Yeast Extract (TSYE) broth followed by heating at 60°C. Both heating methods involved stationary phase cells of L. monocytogenes inoculated into a preheated and circulating heating menstrua. Samples were withdrawn and spread plated onto two media: a) TSYE agar (non-selective) and b) Lithium Chloride Phenylethanol Moxalactam (LPM) agar. Cells that were preheated and plated on a non-selective medium showed more than a 3-log difference in survivors after 20 min of heating at 60°C compared to untreated cells. Lower survival was also noted after heating at 60°C and plated on the selective medium demonstrating that the organism is

subject to thermal injury as a result of the heating process. In the same study, cells were pre-incubated for 48°C for 1 hour and heated at 60°C in ultra-high-temperature (UHT) milk. Plating on non-selective and selective media yielded similar results to the cells preheated in TSYE broth. Bunning, et al. [1986] grew cells of L. monocytogenes at 35°C and exposed them to preheating temperatures of 35-52°C for 5-60 min prior to heating at 57.8°C using the sealed tube method (Table 4). Although heat shocking at 42-48°C for 5-60 min consistently increased the D-values at 57.8°C 1.1 to 1.4 times, this data was not significantly different from non heat-shocked cells. When cells were held at a 42°C preheating temperature, cells maintained an increase in thermal tolerance for up to 4 hrs. However, if the cells, after prior preheating, were then incubated at 35°C, the increased thermal tolerance was short-lived, lasting less than an hr. In this same study, cells that were preheated at 48°C for 30 min and then heated at 52°C, were 1.3 times more resistant to heat when compared to the control cells [Bunning et al., 1986].

The induction of the heat shock response and its effect on the thermal resistance of stationary cells of L. monocytogenes was investigated by Knabel et al. [1990]. Cells of L. monocytogenes F5069 preheated at 43°C had a higher heat resistance than cells held at 37°C. Using

Table 4. Thermal resistance of L. monocytogenes as a result of heat shocking.

Strain	Heating medium	Plating medium	Process Temp °C	D-value (minutes)	Method
F5069 (4b)	TSYEB	TSYEA			
	Heat Shocked	35°C			
	5 min		57.8	9.9	SL TDT
	15 min		57.8	9.5	SL TDT
	30 min		57.8	9.4	SL TDT
	60 min		57.8	8.0	SL TDT
	Heat Shocked	42°C			
	5 min		57.8	11.8	SL TDT
	15 min		57.8	11.3	SL TDT
	30 min		57.8	11.1	SL TDT
	60 min		57.8	10.9	SL TDT
	Heat Shocked	48°C			
	5 min		57.8	11.5	SL TDT
	15 min		57.8	11.7	SL TDT
	30 min		57.8	10.3	SL TDT
	60 min		57.8	9.2	SL TDT
	Heat Shocked	52°C			
	5 min		57.8	9.3	SL TDT
	15 min		57.8	7.8	SL TDT
	30 min		57.8	7.0	SL TDT
	60 min		57.8	6.9	SL TDT
F5069 (4b)	TSYEB	TSYEA			
	Heat Shocked	35°C			
	30 min		52.0	37.9	SL TDT
	Heat Shocked	48°C			
	30 min		52.0	49.8	SL TDT

[From Bunning et al., 1986]

Table 4. (Continued)

Strain	Heating medium	Plating medium	Process Temp °C	D-value (seconds)	Method
F5069 (4b)	TSYEB (37°C, 18hr incubation, aerobic plating)	TSYEA	62.8	36.0	SL TDT
F5069 (4b)	TSYEB (43°C, 18hr incubation, anaerobic plating)	TSYEA	62.8	243.0	SL TDT

[From Knabel et al., 1990]

Mixed (4,1)	Sausage TA Non-heat shocked Heat shocked 48°C		64.0	198.0	HOLD
	30 min		64.0	252.0	HOLD
	60 min		64.0	282.0	HOLD
	120 min		64.0	480.0	HOLD

[From Farber and Brown., 1990]

TA = Tryptose Agar

TSYEA = Trypticase Soy + 0.6% Yeast Extract Agar

TSYEB = Trypticase Soy + 0.6% Yeast Extract Broth

SL TDT = Sealed Thermal Death Time Tube

HOLD = Holding

sealed thermal death time tubes, D-values in milk at 62.8°C for L. monocytogenes heat shocked at 43°C and plated anaerobically were six-fold higher than cells held at 37°C heated at 62.8°C and plated aerobically. Under the conditions of this study, low levels of L. monocytogenes survived the HTST heating process. Addition of catalase and superoxide dismutase to the plating medium improved recovery, but recovery was still best under strictly anaerobic conditions. Best fitting linear thermal death time curves were noted under strictly anaerobic conditions rather than plating aerobically.

The increase in heat resistance due to prior preheating has also been demonstrated in a model food system. Farber and Brown [1990] inoculated a sausage mix with 1×10^7 L. monocytogenes/g and subjected the food to a preheat treatment of 48°C for 30, 60 and 120 min. Although cells preheated at 30 and 60 min did not show significant increases in thermal tolerance, cells preheated at 48°C for 120 min significantly increased the thermal tolerance when compared to the non-heat shocked cells (Table 4). D-values at 64°C were 198 sec for control cells and 480 sec for heat shocked cells. This results in a D-value ratio of heat shocked to non-heat shocked cells of 2.4. After heat shocking, samples of the inoculated meat were held at 4°C for 24 hrs and then tested for heat resistance at 64°C.

Heat resistance decreased slightly for heat shocked and non-heat shocked cells but the D-value ratio of heat shocked to non-heat shocked remained consistent. It has also been shown that cells of L. monocytogenes Scott A grown at 37°C are far more resistant during a thermal process of 52°C compared to cells grown at 28, 19, or 10°C. This indicates that temperature abuse could significantly affect heat resistance [Smith and Archer, 1988a].

During a heat shock of 48°C for 30 min in TSYE broth with L. monocytogenes (SV 1/2a, SLCC5764), heat shock protein synthesis was determined by radioactive (S-35) labeling of synthesized proteins during heat shocking. Twelve to 14 heat shock proteins were shown to be synthesized in the range of 20 to 120 kilodaltons. All but one of the heat shock proteins produced under these conditions appeared to be intracellular. Listeriolysin was the only extracellular protein to be produced by heat shocking at 48°C for 30 min [Sokolovic and Goebel., 1988]. Listeriolysin is inherent to all virulent strains of Listeria and allows the bacterium to escape the phagosome by lysing the phagosomal membrane. Listeriolysin production as well as other heat shock proteins synthesized during stress conditions such as heat shocking may allow the organism to survive extremely hostile environments until more suitable growth conditions occur.

F. Experimental Rational

The wide-spread distribution of L. monocytogenes, the infectious nature of the organism and its ability to survive in many hostile environments including very high and very low temperatures, and strictly aerobic and anaerobic conditions, pose many potential problems to the food industry. Thermal processing can be effectively used for destroying L. monocytogenes in foods. However, a heat shock response that has been shown to cause elevated thermal resistance in microorganisms, may allow cells to survive during and after thermal processing of foods.

This study was undertaken to investigate the heat shock response and its effect on thermal tolerance of L. monocytogenes Scott A in microbiological media and in a food system. Selective and non-selective microbiological media using aerobic and anaerobic plating methods were evaluated for recovery of heat shocked and non-heat shocked cells.

III. MATERIALS AND METHODS

A. Culture and Culture Conditions

Listeria monocytogenes Scott A (obtained from Dr. R. E. Brackett, University of Georgia) was maintained on Trypticase Soy agar (BBL Microbiological systems - Cockeysville, MD) + 0.6% Yeast Extract (Difco Laboratory - Detroit, MI) at 7°C. Cultures were grown in Trypticase Soy + 0.6% Yeast Extract (TSYE) broth at 37°C and transferred daily. This strain was chosen because it is a foodborne isolate from the 1985 Massachusetts outbreak involving pasteurized milk. It is also believed that L. monocytogenes Scott A is one of the most heat resistant foodborne strains of Listeria.

B. Determination of Log Phase Cell Culture

Log phase cells are characterized by an exponential increase in viable cell numbers and when the metabolic activity of the organism is at its greatest. Log phase cells, although less resistant than stationary phase cells, were chosen for this study. It is believed that the synthesis of heat shock proteins is greatest in the log phase of growth.

For determination of log phase cells, the batch culture system was used as described by Gerhardt et al. [1981]. One ml of a 24 hour culture of L. monocytogenes grown in

TSYE broth was inoculated into a side arm flask containing 250 ml of preheated TSYE broth at 37°C. At time zero absorbance at 600 nm was determined using a spectrophotometer (Spectronic 20, Bausch and Lomb - Milton Roy Co. - Rochester, New York) and calibrated to zero. The flask was then allowed to incubate and agitated slowly (100 rpm, Controlled Environment Incubator Shaker, New Brunswick Scientific - Edison, New Jersey) at 37°C and the absorbance was read hourly. Log absorbance vs. time in hrs was plotted to determine a growth curve.

C. Aerobic Heat Shock, Aerobic Enumeration

Aerobic heat shock was performed at various heat shocking time and temperature parameters to determine the optimal condition for increasing thermal tolerance of L. monocytogenes.

One ml of a 24 hr culture was transferred into a 250 ml Erlenmeyer flask of TSYE broth and incubated at 37°C. After a 7.5 hr incubation, log phase cells (as determined by previous growth curve experiments) were heat shocked in the following procedure: 10 ml of log phase culture was pipetted into 90 ml of pre-heated TSYE broth contained in a round bottom flask that was submerged in a preheated circulating water bath (Haake E52-Rochelle Park, NJ) and cells were heat shocked for various times. Circulation of

the flask contents was accomplished using a magnetic stir bar with the flask in the water bath which was fixed on top a multi-magnastir plate (Lab-Line Instruments-Melrose Park, IL). To ensure that the desired heating temperature was obtained, a sterile thermometer was placed in the flask.

Heat shock was at 40, 44, and 48°C for 3, 10, and 20 min at each temperature. After heat shocking, the cell suspension was immediately cooled in ice water for 5 min. A low temperature treatment of 40°C was chosen because heat shock protein synthesis is thought to be optimal at a few degrees above the normal physiological temperature of 37°C. The upper temperature limit of 48°C was chosen because this temperature is above normal limits for growth of the organism [Doyle, 1988]. A 10 ml sample of heat shocked cells was then transferred into 90 ml of preheated TSYE broth and heated at 55°C for 50 min in an identical water bath system used for heat shocking. Samples were withdrawn every 12.5 min and immediately cooled in an ice water bath (Fig. 1). There were two controls for each experiment. A negative control consisted of untreated cells held at 37°C and then processed at 55°C for 50 min. The second control was identical to the negative control, except cells held at 37°C were cooled in ice water for 5 min prior to heat processing at 55°C for 50 min to determine the effect, if any, of cold shocking cells. Both control cells were

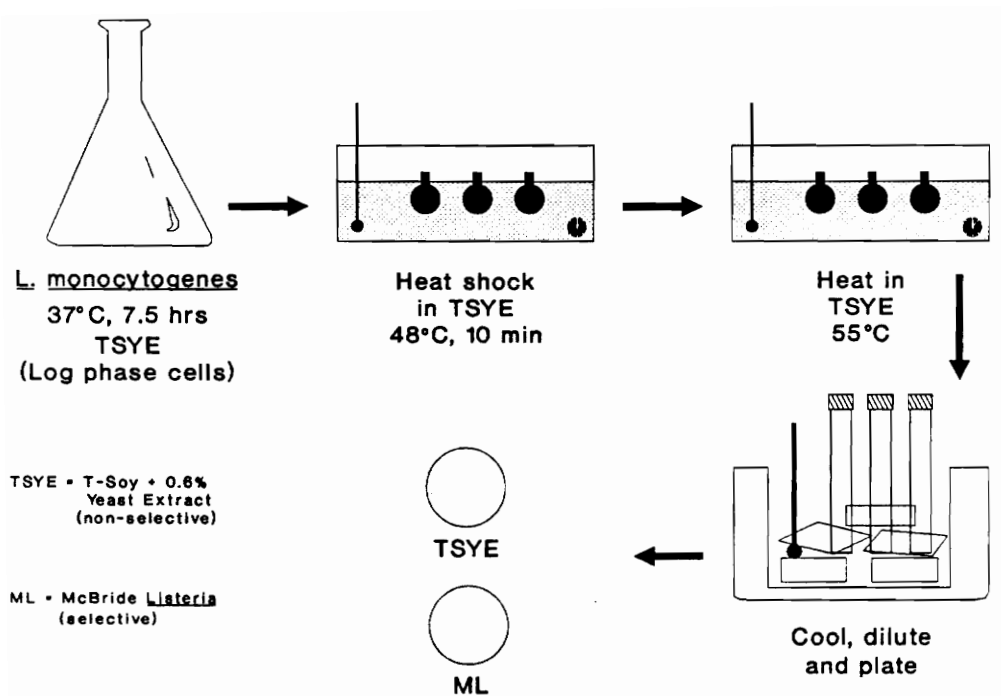


FIGURE 1. Aerobic heat shock, aerobic enumeration.

withdrawn at time zero and every 12.5 min during heating at 55°C and cooled immediately in ice.

D. Enumeration of Surviving Bacteria

Heat processed samples were diluted in 0.1% Trypticase peptone (BBL) and spread plated onto two different media: TSYE agar and McBride Listeria (ML) agar. TSYE agar medium is non-selective and was used to enumerate both injured and non-injured cells. ML agar is selective for Listeria and was used for enumeration of non-injured cells. The difference in these two media gave an indication of cell injury during the heat treatments (Fig. 1).

E. Biochemical Confirmation

Inoculated ML and TSYE agar were incubated at 37°C for 48 hrs. Typical colonies were confirmed using the following tests: (a) Gm(+), short rods; (b) TSI-acid slant, acid butt, no H₂S, no gas; (c) MR/VP(+,+); (d) acid but no gas from glucose; (e) catalase(+); (f) oxidase(-); and (g) urease (-).

F. Determination of D-values and Z-values

Mean values of bacterial counts from duplicate plate samples were converted to log numbers for each heating time and temperature and then log number of survivors were

plotted versus time in min. For each heat shock treatment, and for both controls, $D_{55^{\circ}\text{C}}$ values were calculated. Z-values were calculated for non-heat shocked cells (cells held at 37°C) and for cells heat shocked at 48°C for 10 min. D-values from heating temperatures including 50, 55, 60, and 65°C were used to determine z-values for selective and non-selective media.

G. Cell Injury and Heat Shock Ratio Calculations

Cell injury for those cells of L. monocytogenes given the same heat treatment were calculated as follows:

$$\text{CELL INJURY} = \text{SURVIVORS ON TSYE} - \text{SURVIVORS ON ML}$$

Cell injury was calculated for both heat shocked and non-heat shocked cells.

A heat shock ratio was determined so that different heat shocking parameters could be easily compared. A heat shock ratio (given equal treatment and an identical plating medium) was determined as follows:

$$\text{HEAT SHOCK RATIO} = \text{HEAT SHOCK D-VALUE} / \text{NON-HEAT SHOCK D-VALUE}$$

A heat shock ratio was calculated for both non-selective and selective plating media. A calculated heat shock ratio greater than 1.0 indicated that heat shocking increased the thermal tolerance. A calculated heat shock ratio less than or equal to 1.0 indicated that heat shocking did not increase the thermal tolerance.

H. Aerobic Heat Shock, Anaerobic Enumeration

Aerobically heat shocked cells of L. monocytogenes were also enumerated using anaerobic conditions. Anaerobic enumeration was performed to determine if D-values of heat shocked and non-heat shocked cells differed and/or if aerobic vs. anaerobic enumeration differed after an aerobic heat shock.

For comparison of aerobic and anaerobic enumeration, heat shocking and heating at 55°C was done aerobically followed by aerobic and anaerobic plating (Fig. 2). Aerobic plating, and incubation of plates was performed as previously described. Cells were only heat shocked at 48°C for 10 min since this was the optimum aerobic heat shocking condition for increasing thermal tolerance as determined by prior experiments. Aerobic heat shocked samples that were plated anaerobically were diluted in anaerobic peptone diluent (Bacto peptone (Difco), then plated onto TSYE agar and ML agar using the roll tube method [Holdeman et al. 1977]. Diluent, and both plating media included L-15 Cysteine hydrochloride (Sigma Chemical, St Louis, MO) to reduce the media, and rezazurin to indicate the reduction potential of the anaerobic media (Allied Chemical, Morristown N.J.). During inoculation, the tubes were continuously flushed with oxygen-free CO₂ by means of a cannula, re-stoppered, and incubated. A control for this

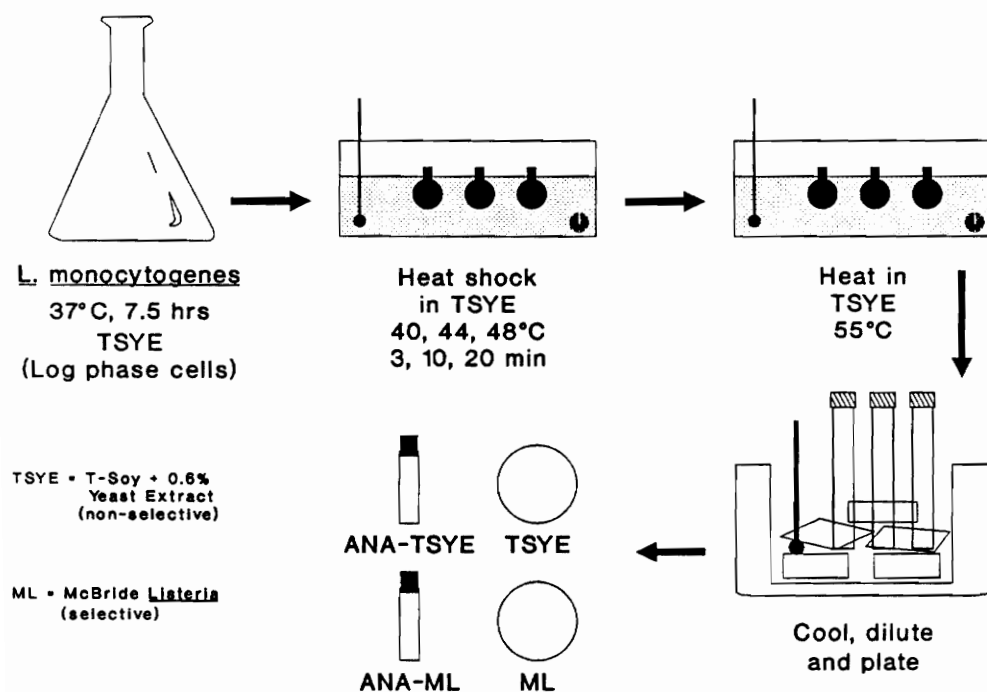


FIGURE 2. Aerobic heat shock, anaerobic enumeration.

procedure consisted of non-heat shocked cells. No cold shock control was performed. Determination of D-values, cell injury, and heat shock ratios were performed as previously described.

To compare the effect of anaerobic enumeration vs. aerobic enumeration an anaerobic/aerobic ratio of the D-values was calculated as follows:

ANAEROBIC/AEROBIC RATIO = ANAEROBIC D-VALUE/AEROBIC D-VALUE

This ratio was determined for both heat shocked and non-heat shocked cells on TSYE medium.

I. Aerobic Heat Shock, Inoculated Shrimp

Frozen headless shrimp with intact exoskeletons were obtained from Dr. Cameron Hackney and Mr. Thomas Rippen at the Seafood Agricultural Experiment Station in Hampton, Virginia. The shrimp (250-350 count/lb.) contained a mixture of species and was held at -20°C until use. Original level of microbial contamination of the shrimp was approximately 1×10^5 cfu/g as determined by a standard plate count.

The frozen shrimp were placed within a sterile bag and placed in a water bath at 20°C until thawed. Once thawed, the shrimp were surface dip inoculated for 5-10 sec in TSYE broth containing either heat shocked or non-heat shocked log phase cells (10^8 - 10^9 L. monocytogenes/ml) (Fig. 3). There

were two controls and one experimental sample. A negative control consisted of non-heat shocked log phase cells and another control consisted of uninoculated shrimp. The experimental sample consisted of log phase cells aerobically heat shocked for 48°C for 10 min (as previously described).

After dip inoculation, 11 g shrimp samples were placed within a sterile non-oxygen permeable bag (EVA/Saran/EVA Cryovac-Duncan, SC) vacuum packaged (Multivac, West Germany), and subjected to a heating process at 55°C in a hot water heating bath for up to 100 min. Vacuum packaged samples were withdrawn at time 0, 25, 50, 75, and 100 min during the 55°C thermal process. Samples were cooled rapidly in an ice water slurry for 5 min. The entire 11 g shrimp samples were stomached in 99 ml of peptone diluent for 2 min (Stomacher Lab-Blender 400 - London, England). Shrimp samples were diluted and plated both aerobically and anaerobically as described.

Additional vacuum packaged samples were held at 3.0-5.0°C and sampled at day 3, 10, and 21 for the presence of L. monocytogenes. Vacuum packaged samples were diluted aerobically and anaerobically and enumerated on TSYE and ML agar as previously described. A holding temperature of 3.0-5.0°C was chosen to simulate possible storage temperatures of minimally thermal processed refrigerated foods such as sous-vide.

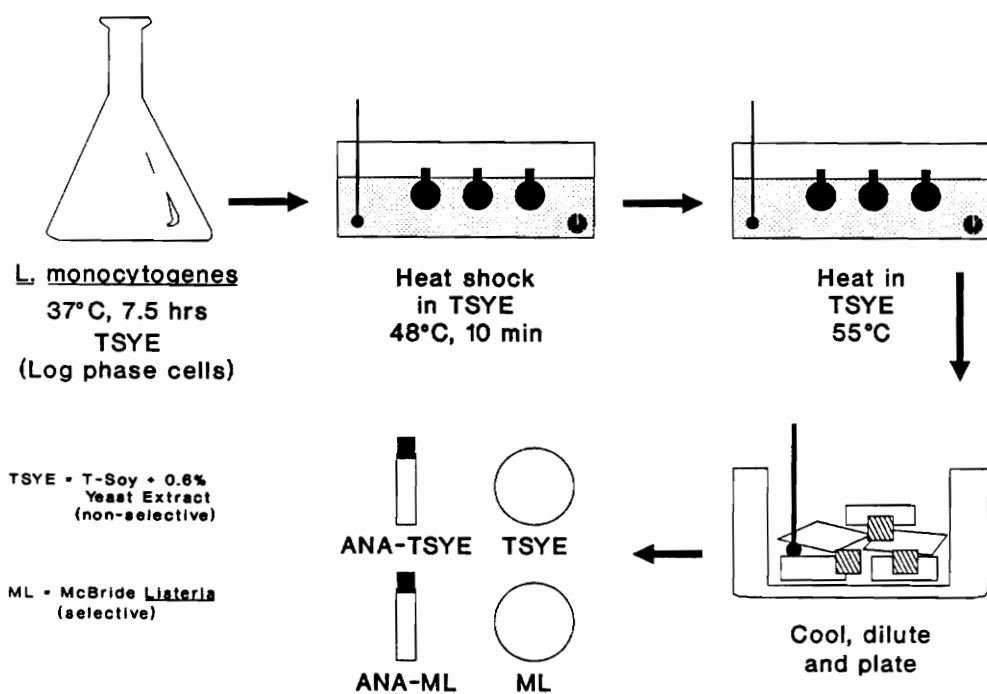


FIGURE 3. Aerobic heat shock, inoculated shrimp.

J. Statistical Analysis

Statistical analysis was performed using a general linear model to obtain the best fit line to determine D and z values for each condition described. Duncan's multiple range test was used to compare means of D-values and z-values. A test of significance at the 5% level was performed for each treatment. All statistical analyses were performed using Statistical Analysis Systems (SAS Institute, Inc., Box 8000, Cary, NC 27511).

IV. RESULTS AND DISCUSSION

A. Log Phase Cells

At 37°C, L. monocytogenes grew rapidly in TSYE broth with stationary phase cell growth obtained at approximately 9-10 hrs. The log (or exponential) phase of growth, as characterized when cells are dividing at a steady rate and the metabolic activity is highest, appeared between 2-9 hrs. Mid to late log phase cells, which were used throughout the heat shocking experiments, was determined to be at 7.5 hrs of incubation at 37°C (Fig. 4). The cell concentration at 7.5 hrs of incubation was 10^7 - 10^8 L. monocytogenes/ml.

B. Optimum Aerobic Heat Shock Condition

The effect of thermal tolerance of L. monocytogenes was examined using three trials of each heat shock treatment. As previously described in the materials and methods section, the heat shock conditions consisted of 40, 44, and 48°C, for 3, 10, and 20 min each.

The increase in heat resistance for both enumeration media were determined by a heat shock ratio. All heat shocking conditions with the exception of 40°C for 3 min, increased thermal tolerance of L. monocytogenes significantly ($p < 0.05$) when plated on the non-selective TSYE medium as compared to non-heat shocked cells. When

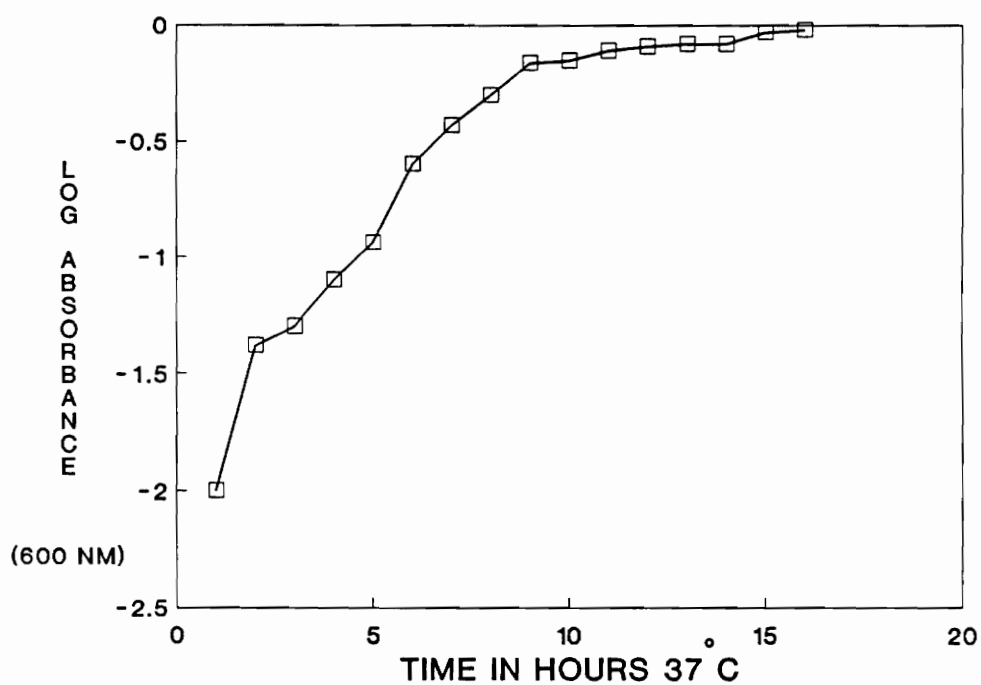


FIGURE 4. Aerobic growth curve for *L. monocytogenes* Scott A at 37°C in TSYE broth.

plated on the selective ML medium, heat shocking for 10 and 20 minutes at 44°C and heat shocking at 48°C for all times significantly ($p < 0.05$) increased the heat resistance of L. monocytogenes as compared to non-heat shocked cells. The increase in heat resistance on both a non-selective and selective medium indicates that non-injured as well as injured cells may acquire increased thermotolerance due to prior sublethal heat shock.

The optimum heat shock condition, as determined by heat shock ratios, for increasing the thermal tolerance of L. monocytogenes was at 48°C for 20 min (Fig. 5, Table 5). However, more consistent results were obtained at a treatment of 48°C for 10 min (Fig. 6) which was not significantly different from the 48°C, 20 min treatment (Table 5). Therefore, the optimum heat shock condition for increasing the thermal tolerance of L. monocytogenes in this study was considered 48°C, 10 min where the heat resistance was increased 2.2x on TSYE and 1.4x on ML. All results from other heat shock conditions are given in the appendix (Figs. 13-19).

In previous work done by Mackey et al. [1986], a 4 log difference in survivors between heat shocked (48°C, 30 min) and untreated cells after 40 min at a heat shock challenge of 55°C was found for stationary cells of Salmonella

typhimurium in T-Soy broth. Similarly, under optimum heat shock conditions (48°C, 10 min) in this study, a 3 log difference in survivors between heat shocked and untreated cells after 40 min at a heat challenge of 55°C was found for log phase cells of L. monocytogenes Scott A (Fig. 6). It appears as though the heat resistance of both log and stationary phase cells may be influenced due to a prior sublethal heat shock in bacteria.

Bunning et al. [1989] has also investigated the heat shock response of in stationary cells of L. monocytogenes 5069. An optimum heat shock condition of 42-48°C for 5-15 min in TSYE broth yielded a heat shock ratio of 1.3 and 1.2 when challenged at 52°C and 57.8°C, respectively. In that study, both of these heat shock ratios were not significantly different than untreated cells. Our study indicated a much higher increase (heat shock ratio of 2.2) when cells of L. monocytogenes Scott A were heat shocked at 48°C, 10 min and plated onto TSYE. Reasons for the differences in heat shock ratios between the two studies are unknown, but may be due in part to strain differences and/or growth phase of cells.

Previous heat shock studies [Bunning, 1989; Fedio, 1989; Farber, 1990] confirmed an optimal heat shock temperature of 48°C to increase the thermal resistance of

stationary cells. This is consistent with our study in which a heat shock temperature of 48°C was optimal for increasing the heat resistance of log phase cells. Previous studies using a heat shock temperature of 48°C for stationary cells needed longer time periods compared to the 10 min at 48°C found in our study for log phase cells. Stationary cells are known to be more heat resistant than those cells in the log phase [Jay, 1986]. Because log phase cells are undergoing more metabolic activity, a greater synthesis of heat shock proteins may be occurring allowing bacterial cells to increase their heat resistance in less time.

C. Cell Injury:

Cell injury for non-heat shocked and heat shocked cells after heating at 50, 55, 60, and 65°C was examined. At each heating temperature, the percentage of cell injury was high regardless of if the cell was heat shocked or non-heat shocked (Fig. 5-9, 11-19). At the optimal heat shock treatment of 48°C for 10 min followed by heating at 55°C, the degree of cell injury was 91.7% for non-heat shocked cells and 99.8% injury for heat shocked cells (Fig. 6). This represents a very substantial difference, almost 2 logs, between injured and non-injured cells. Cell injury

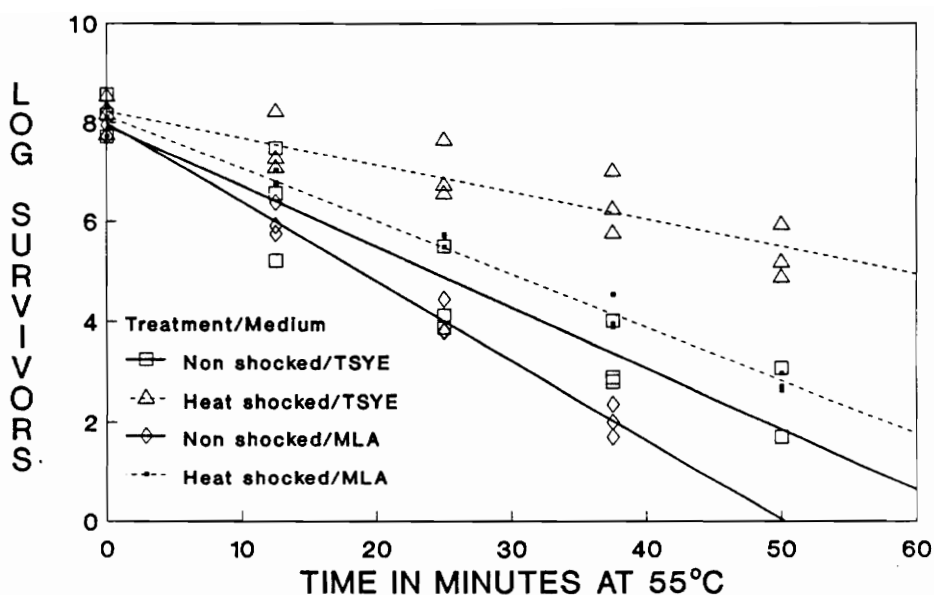
Table 5. Effect of heat shock on thermal resistance of L. monocytogenes at 55°C in TSYE broth.

Treatment		D-value ratio ^a	
Temp. (°C)	Time (min)	Non-selective ^b medium	Selective ^c medium
40	3	1.2 Aa	1.0 Aa
40	10	1.3 ABa	1.1 Aa
40	20	1.5 Ba	1.2 Aa
44	3	1.4 Aa	1.1 Ab
44	10	1.7 Aa	1.4 Ba
44	20	2.0 Ba	1.4 Bb
48	3	1.8 Aa	1.5 Ab
48	10	2.2 Ba	1.4 Ab
48	20	2.3 Ba	1.6 Ab

^a Values are the ratio of [heat shock D-value]/[non-heat shock D-value] for cells heated at 55°C. D-value ratios in the same column within each heating temperature with different upper case letters are significantly different ($p \leq 0.05$). D-value ratios in the same row with different lower case letters are significantly different ($p \leq 0.05$)

^b Trypticase Soy + 0.6% Yeast Extract agar.

^c McBride Listeria agar.



Non Heat Shock D-value on TSYE ^{a,d}	8.26 min
Non Heat Shock D-value on ML ^{a,e}	6.28 min
Heat Shock D-value on TSYE ^a	18.6 min
Heat Shock D-value on ML ^a	9.79 min
Heat Shock Ratio on TSYE ^b	2.25
Heat Shock Ratio on ML ^b	1.56
Non Heat Shock Cell Injury ^c	>99.99%
Heat Shock Cell Injury ^c	99.7%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

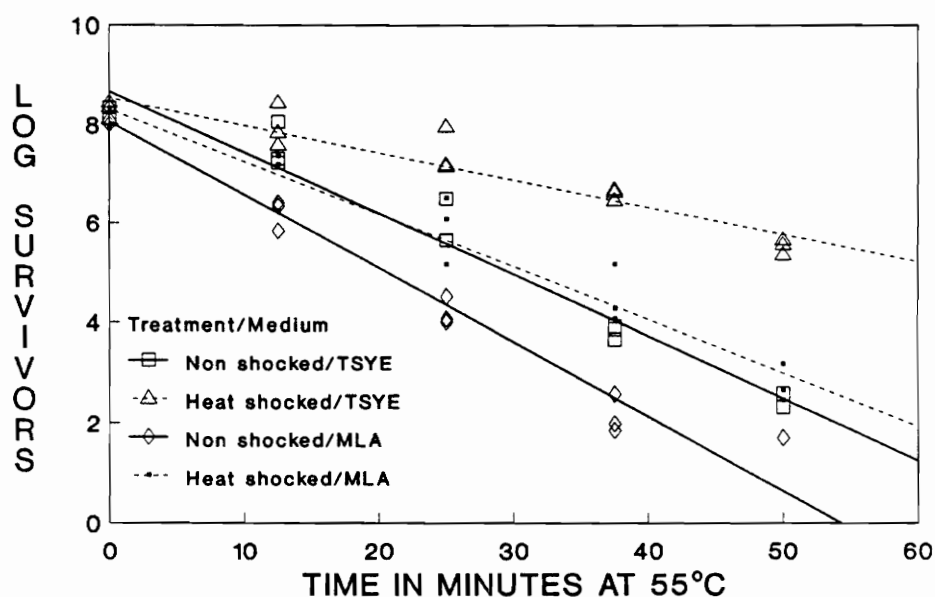
^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

FIGURE 5. Survivor curve at 55°C for cells heat shocked 48°C, 20 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	8.10 min
Non Heat Shock D-value on ML ^{a,e}	6.59 min
Heat Shock D-value on TSYE ^a	18.0 min
Heat Shock D-value on ML ^a	9.46 min

Heat Shock Ratio on TSYE ^b	2.23
Heat Shock Ratio on ML ^b	1.44

Non Heat Shock Cell Injury ^c	91.7%
Heat Shock Cell Injury ^c	99.8%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

FIGURE 6. Survivor curve at 55°C for cells heat shocked 48°C, 10 min and plated aerobically on TSYE and ML agar.

was consistently higher for cells that were heat shocked compared to non-heat shocked cells after heating at 55°C (Fig. 5-8, 11-19).

Fedio and Jackson [1989] found that for stationary cells of L. monocytogenes Scott A, a heat shock response of 48°C for one hour was optimal for increasing thermal tolerance. Using TSYE as a plating medium, the difference in survivors at a heat challenge of 60°C between heat shocked and non heat shocked cells after 8 min was 1 log. Using LPM agar as a plating medium, the difference in survivors at a heat challenge of 60°C between heat shocked and non heat shocked cells after 8 min was 1.5 logs. In our study, the difference in survivors at a heat challenge of 60°C between heat shocked and non heat shocked cells after 8 min was 2.5 logs for TSYE and 3.5 logs for ML agar. The heat resistance of both log phase and stationary phase cells of L. monocytogenes appears to be greatly influenced by sublethal heat shock.

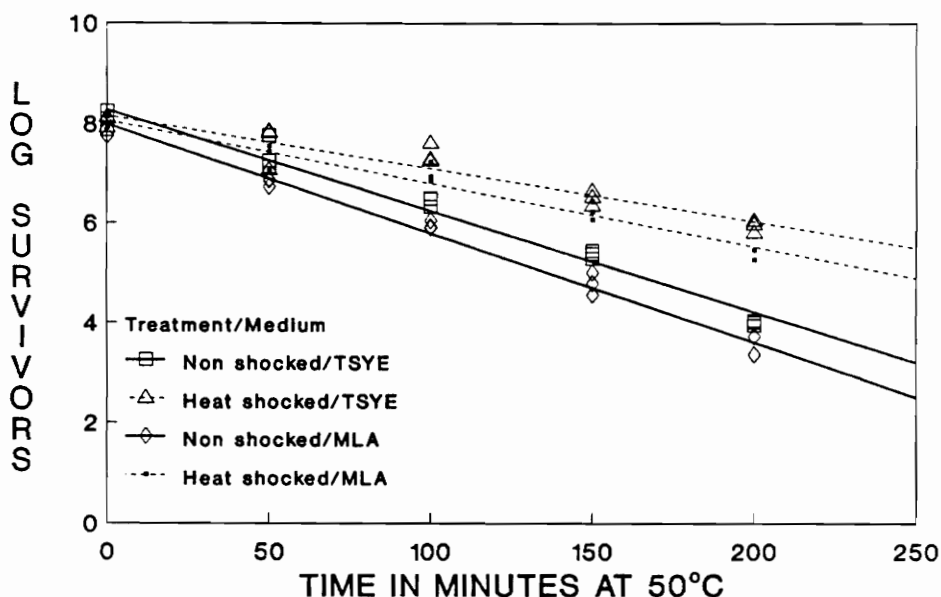
The percentage of injured stationary cells was not calculated in the study by Fedio and Jackson [1989]. However, a survival curve at 60°C showed that after 8 min approximately a 0.5 log difference between the non-selective TSYE and the selective LPM medium for heat shocked (48°C, 1 hr) stationary cells and approximately a 1.0 log difference between the non-selective TSYE and the selective LPM medium

for untreated stationary cells. In our study, a difference between TSYE and ML agar was 1 log for heat shocked cells and 2 logs for non-heat shocked cells after 8 min at 60°C (Fig. 8). Injury seems to be more prevalent in our study, which is no surprise, since our study involves the more fragile log phase cells. However, if LPM had been the choice for a selective medium in our study, a greater amount of injury would be expected since LPM is a highly selective medium for L. monocytogenes.

D. Determination of z-values

To determine whether heat shock at 48°C for 10 min would induce an increased heat resistance of L. monocytogenes over a range of normally lethal temperatures, cells were heat shocked and their survival was determined during subsequent heat challenges of 50 (Fig. 7), 60 (Fig. 8), and 65°C (Fig. 9). At all heating temperatures, heat shocked (48°C, 10 min) cells were much more resistant to heat than control cells incubated at 37°C prior to the heat shock challenge. Heat shocked cells (48°C, 10 min) that were enumerated on TSYE agar were consistently about 2x more heat resistant to heating than non-heat shock cells.

The average log D-values for untreated cells plated on TSYE agar obtained from this study were 3.47 at 50°C, 2.75 at 55°C, 1.98 for 60°C and 1.23 for 65°C. According to a



Non Heat Shock D-value on TSYE ^{a,d}	49.4 min
Non Heat Shock D-value on ML ^{a,e}	45.9 min
Heat Shock D-value on TSYE ^a	96.9 min
Heat Shock D-value on ML ^a	79.3 min
Heat Shock Ratio on TSYE ^b	1.96
Heat Shock Ratio on ML ^b	1.73
Non Heat Shock Cell Injury ^c	67.3%
Heat Shock Cell Injury ^c	71.5%

^a D-values are at 50°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 50°C. Ratios represents mean of three replicates.

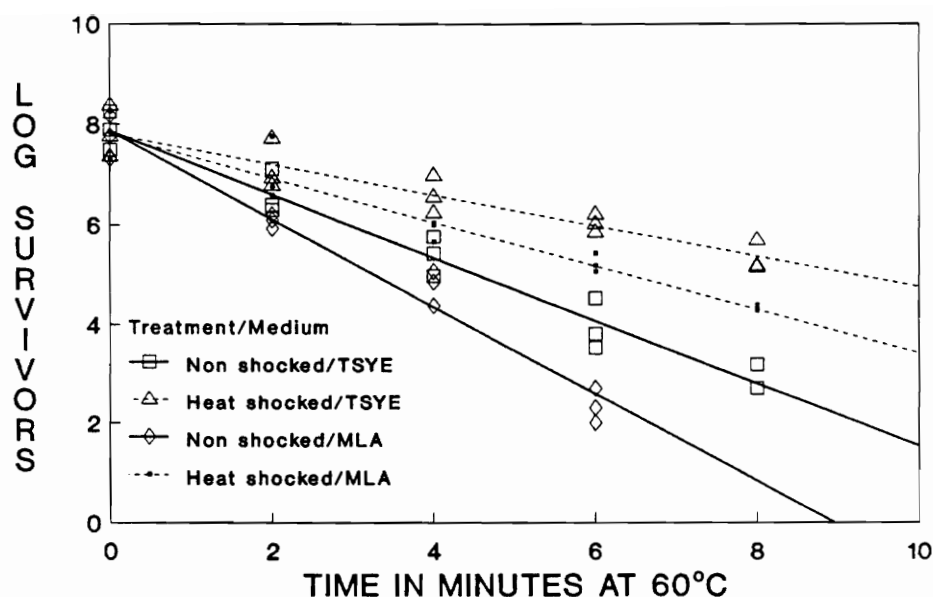
^c Values are determined after 100 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride *Listeria* agar.

FIGURE 7. Survivor curve at 50°C for cells heat shocked 48°C, 10 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	1.58 min
Non Heat Shock D-value on ML ^{a,e}	1.14 min
Heat Shock D-value on TSYE ^a	3.32 min
Heat Shock D-value on ML ^a	2.35 min

Heat Shock Ratio on TSYE ^b	2.10
Heat Shock Ratio on ML ^b	2.06

Non Heat Shock Cell Injury ^c	>99.99%
Heat Shock Cell Injury ^c	88.3%

^a D-values are at 60°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 60°C. Ratios represents mean of three replicates.

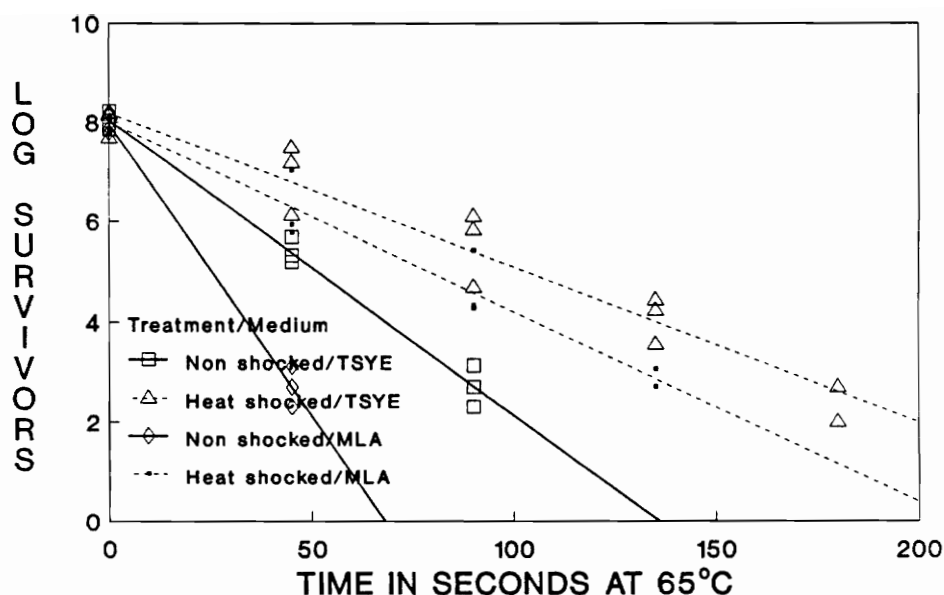
^c Values are determined after 8 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

FIGURE 8. Survivor curve at 60°C for cells heat shocked 48°C, 10 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	0.28 min
Non Heat Shock D-value on ML ^{a,e}	0.14 min
Heat Shock D-value on TSYE ^a	0.54 min
Heat Shock D-value on ML ^a	0.39 min
Heat Shock Ratio on TSYE ^b	1.93
Heat Shock Ratio on ML ^b	2.79
Non Heat Shock Cell Injury ^c	>99.99%
Heat Shock Cell Injury ^c	92.5%

^a D-values are at 65°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 65°C. Ratios represents mean of three replicates.

^c Values are determined after 3 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

FIGURE 9. Survivor curve at 65°C for cells heat shocked 48°C, 10 min and plated aerobically on TSYE and ML agar.

review article which combines much of the thermal resistance data for L. monocytogenes, these values are comparable to other published results [Mackey et al., 1989]. A similar comparison for thermal resistance of untreated cells plated on ML agar or heat shocked cells plated on TSYE or ML agar was not available in the literature. The average log D-values for cells heat-shocked at 48°C for 10 min plated on TSYE agar were 3.76 at 50°C, 3.03 at 55°C, 2.30 for 60°C, and 1.51 for 65°C.

Log D-values were plotted vs. heating temperature to determine z-values for non heat shocked and heat shocked cells on both TSYE and ML media (Fig. 10). The z-values were not significantly different regardless of the conditions of the cells or the plating medium (Table 6). Z-values ranged from 6-6.7°C (Table 6) which is consistent with the literature. Many separate estimates of z have been published ranging from 4.3-9.4°C with a mean z of 6.7°C [Mackey et al., 1990]. These z-values are considered high when compared to other food-borne vegetative bacteria which generally have a z-value near 5.0°C.

E. Anaerobic vs. Aerobic Enumeration of Heat Shocked Cells

Since L. monocytogenes is a facultative anaerobe, the investigation of anaerobic vs. aerobic enumeration

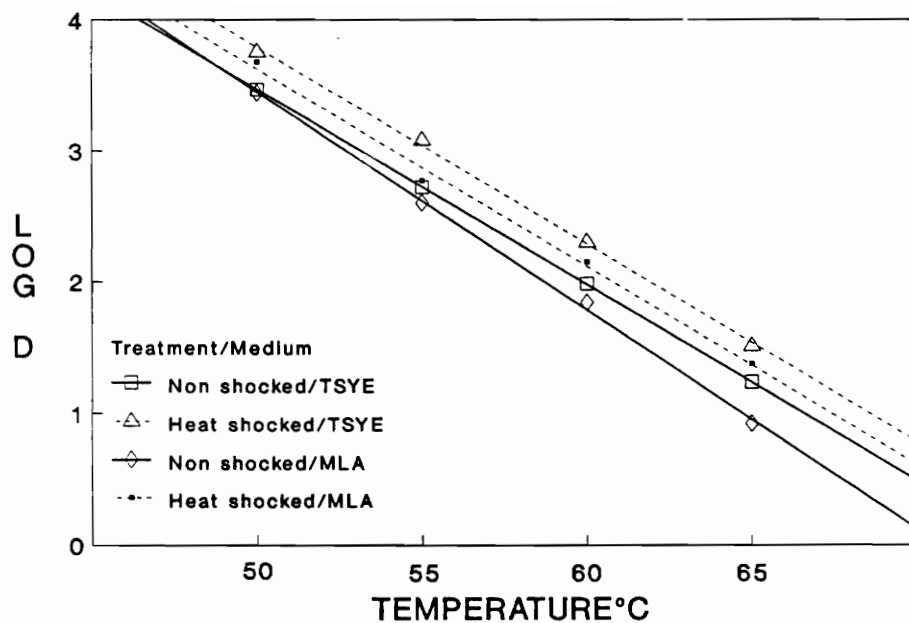


Figure 10. Phantom thermal death time curve for cells heat shocked 48°C, 10 min and plated aerobically on TSYE and ML agar.

Table 6. Heat resistance of heat shocked and non-heat shocked (48°C, 10 min) cells of L. monocytogenes Scott A and plated aerobically on TSYE and ML agar.

D-values ^a (min)				
Temp. (°C)	TSYE ^b agar		ML ^c agar	
	Heat Shocked ^d	Non-Heat Shocked ^e	Heat Shocked ^d	Non-Heat Shocked ^e
50	96.9	49.4	79.3	45.9
55	20.0	8.81	9.85	6.61
60	3.32	1.58	2.35	1.14
65	0.54	0.28	0.39	0.14
Z values ^{f,g}	6.7	6.6	6.0	6.6

^a D-values represent means of three replicates.

^b Trypticase Soy + 0.6% Yeast Extract.

^c McBride Listeria.

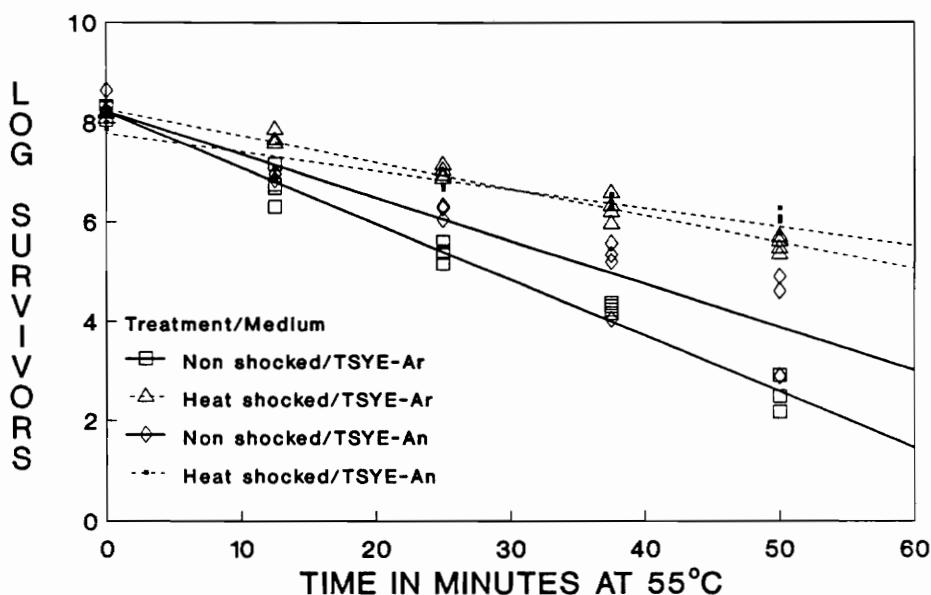
^d Heat shocked at 48°C for 10 min.

^e Cells held at 37°C.

^f Z-values determined using simple linear regression.

^g Z-values not significantly different ($p \geq 0.05$).

procedures for untreated and heat shocked cells was done (48°C, 10 min). Heat shocked and non-heat shocked cells had equal levels of L. monocytogenes/ml at time zero, but higher counts were found at all other sampling times during heating at 55°C when enumerated on TSYE agar anaerobically (Fig. 11). D-values for cells enumerated with TSYE were higher for heat shocked and non-heat shocked cells that were enumerated using anaerobic methods (Fig. 11). D-values on TSYE media at 55°C for untreated cells enumerated anaerobically were 12.0 min compared to a D-value of 8.89 min using the aerobic spread plate technique (Fig. 11). This is a 1.4x increase in D-value when plated anaerobically. Heat shocked cells had a D-value at 55°C anaerobically of 26.4 min versus an aerobic D-value of 18.7 min when plated on TSYE agar (Fig. 11). Again this is a 1.4x increase when plated anaerobically. Non-heat shocked cells as well as heat shocked cells were recovered in greater numbers under anaerobic conditions. The heat shock anaerobic (26.4)/non-heat shock aerobic (8.89) value obtained from the non-selective medium was 3.0. Knabel, et al. [1990] also reported an improvement in non-selective anaerobic enumeration. In this study, a heat shock anaerobic/non-heat shock aerobic ratio of 6.0 was reported. The heat shock ratios in the study reported here were very



Non Heat Shock D-value on TSYE ^{a,c}		
Aerobically ^d		8.89 min
Anaerobically ^e		12.0 min
Heat Shock D-value on TSYE ^{a,c}		
Aerobically ^d		18.7 min
Anaerobically ^e		26.4 min
Heat Shock Ratio on TSYE ^b		
Aerobically ^d		2.10
Anaerobically ^e		2.19
Anaerobic/Aerobic Ratio on TSYE ^f		
Non Heat Shocked Cells		1.35
Heat Shocked Cells		1.41

^a D-values are at 55°C and represent mean of four replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of four replicates.

^c Trypticase Soy + 0.6% Yeast Extract agar.

^d Aerobic spread plating method.

^e Anaerobic roll tube method.

^f Values are the ratio of [Anaerobic D-value]/Aerobic D-value].

FIGURE 11. Survivor curve at 55°C for cells heat shocked 48°C, 10 min and plated aerobically and anaerobically on TSYE and ML agar.

similar using aerobic or anaerobic methods. An aerobic heat shock ratio was 2.1 and an anaerobic heat shock ratio was 2.2 on TSYE agar (Fig. 11). Murano et al., [1990] found similar results with Escherichia coli 0157:H7 where the heat shock anaerobic/non-heat shock aerobic ratio was 2.3.

A possible explanation for increased enumeration using the anaerobic roll tube method may be due to the absence of toxic forms of oxygen. Toxic forms of oxygen such as hydrogen peroxide and superoxide radicals are spontaneously formed in aerobic media. Catalase and superoxide dismutase usually reduce the levels of toxic forms of oxygen in aerobic conditions. If these enzyme systems are decreased the formation of toxic forms of oxygen can severely decrease the bacterial population. Anaerobic culturing, which is absent of toxic forms of oxygen, may be a solution to improved and more efficient enumeration of L. monocytogenes as well as other facultative anaerobic bacteria. Catalase activity and superoxide dismutase activity in Escherichia coli 0157:H7 was eliminated by a heating treatment of 55°C after 20 min in aerobically grown heat shocked and non-heat shocked cells [Murano et al., 1990]. Our study also used a heating treatment of 55°C.

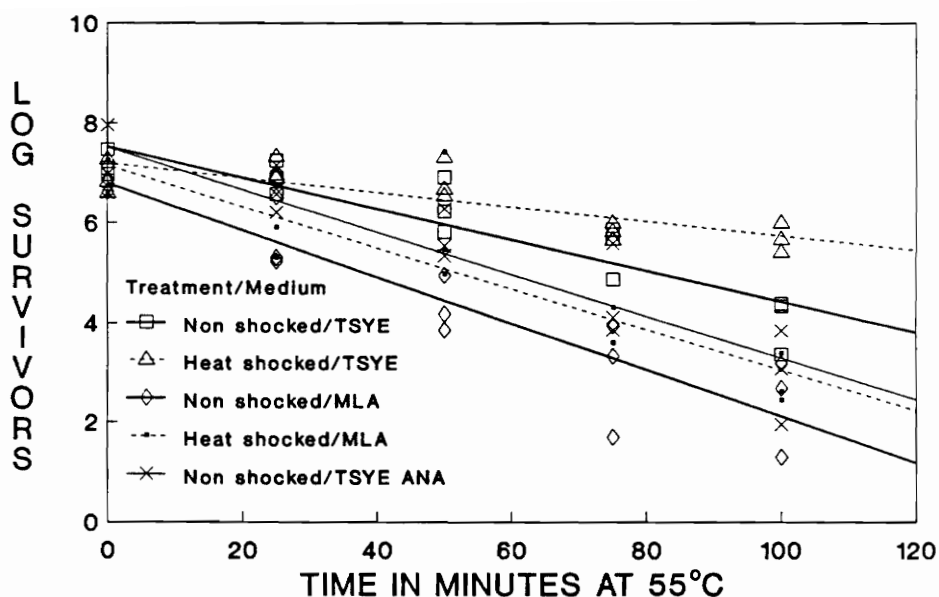
F. Heat Shocked Cells in Shrimp

Other researchers have demonstrated that heat shock

resulting in an increase in heat resistance can occur in foods [Farber and Brown 1990, Fedio and Jackson 1989, Knabel et al., 1990]. This part of the work was designed to determine if an increased heat resistance could be observed for L. monocytogenes in shrimp. Shrimp was chosen since L. monocytogenes has been isolated from it and shrimp is often given a mild heat treatment before consumption.

A negative control consisting of uninoculated shrimp had an initial microbial load near 1×10^5 cfu's/ml. Other samples were dip inoculated into either untreated or heat shocked (48°C, 10 min) log phase cells (10^7 - 10^8 cells/ml). Recovery was evaluated using aerobic and anaerobic techniques and TSYE and ML media.

D-values at 55°C were much higher in shrimp samples when compared to broth experiments (Fig. 12 vs. Fig. 6). This may be due to the constituents (fats, sugars, proteins) inherent to the shrimp that aid to protect bacterial cells from heat. Non-heat shocked cells plated aerobically on TSYE had a D-value at 55°C of 33.0 min and a heat shock D-value at 55°C was 66.9 min (Fig. 12). This calculated to a heat shock ratio of 2.0 which is very similar to results obtained in broth. On the other hand, heat shock ratios from aerobic enumeration were much lower on ML agar with a heat shock ratio of 1.12 which corresponds to D-value at 55°C = 21.9 min for non-heat shocked cells and 24.5 min for



Non Heat Shock D-value on TSYE ^{a,d}	33.0 min
Non Heat Shock D-value on ML ^{a,e}	21.9 min
Heat Shock D-value on TSYE ^a	66.9 min
Heat Shock D-value on ML ^a	24.6 min
Heat Shock Ratio on TSYE ^b	2.03
Heat Shock Ratio on ML ^b	1.12
Non Heat Shock Cell Injury ^c	96.7%
Heat Shock Cell Injury ^c	99.9%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

^c Values after 100 min are determined by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

Figure 12. Survivor curve at 55°C in shrimp for cells heat Shocked 48°C, 10 min and plated aerobically on TSYE and ML agar.

heat shocked cells.

Aerobic injury values in shrimp, as in broth, were very high for untreated and heat shocked cells with 96.7% and 99.9% injury, respectively (Fig. 12). Biochemical tests for colonies of aerobic enumeration were positive for L. monocytogenes on both plating media.

Because the anaerobic roll tube method was more time consuming, only non-heat shocked cells were evaluated anaerobically on TSYE and ML agar for shrimp samples. The D-value for non-heat shocked cells plated on TSYE anaerobically was 23.9 min. Interestingly, the anaerobic D-value was lower than the aerobic D-value on TSYE (33.0 min) (Fig. 12). This conflicts with earlier experiments in broth where anaerobic techniques for enumeration on TSYE agar were superior to aerobic methods. This may have been due to the possible presence of competitive microflora such as anaerobic or facultative anaerobic spore-forming organisms initially inherent in the shrimp. Many spore forming organisms could have survived a heating challenge of 55°C.

The D-value at 55°C for non-heat shock cells plated anaerobically on ML agar could not be calculated for either the broth or shrimp thermal death time studies. The selective ingredients in the medium along with the anaerobic environment did not allow for recovery, in most cases, of L.

monocytogenes. When recovered, counts on anaerobic roll tubes were inconsistent among tubes with identical treatment and dilution.

Vacuum packaged shrimp inoculated with non-heat shocked or heat shocked L. monocytogenes were analyzed after a storage temperature of 3.0-5.0°C after the thermal process at 55°C. After 21 days of storage, shrimp samples were confirmed positive for 10^7 - 10^8 L. monocytogenes/ml when plated aerobically on either plating medium. Because nearly all of the colonies were confirmed positive for L. monocytogenes when plated aerobically, this indicates that L. monocytogenes competed well with other inherent facultative anaerobic microflora within the food at refrigeration temperatures.

Equally as high cfu's/ml were obtained on TSYE agar anaerobically; however, many biochemical conformation tests were not positive for L. monocytogenes. The negative conformation results for L. monocytogenes in thermally processed shrimp plated anaerobically may be due to the survival of naturally occurring microorganisms in the food. Few anaerobic bacteria, except spore-formers, are able to survive after a 55°C heat challenge of 100 min and then survive/proliferate at refrigeration storage. A vacuum packaged food like those used in this study should contain very low concentrations of oxygen, if any. Anaerobic and

facultative anaerobic should be the majority of surviving microflora. Recovery, once again, was not consistent when plated on ML agar anaerobically.

Recently, the first heat shock study was reported for a food system other than milk. Farber and Brown [1990], heat shocked a mixture of 10 strains of L. monocytogenes (Table 4) in fermented sausage. An optimum heat shock treatment for increased heat resistance was at 48°C for 2 hrs where the heat shock ratio was 2.4 after a heating challenge at 64°C. These results compare well with our study with shrimp where the heat shock ratio was found to be 2.0 after heating at 55°C.

V. SUMMARY AND CONCLUSIONS

As with many other bacteria, this study has illustrated that increasing incubation temperatures caused L. monocytogenes Scott A to acquire a higher heat resistance compared to cells held at normal physiological temperatures. The largest increase in heat resistance of the organism was obtained after a heat shock of 48°C for 10 min. At this heat shock treatment, an increase in heat resistance at 55°C was observed on non-selective and selective media and under aerobic and anaerobic plating conditions. In addition, cells heat shocked at 48°C for 10 min also showed an increase in heat resistance at 50, 60, and 65°C when plated aerobically on a non-selective and a selective medium. Cells that were heat shocked at 48°C for 10 min also retained their increased heat resistance when inoculated into shrimp and given a thermal process at 55°C.

The highest D-value at 55°C for L. monocytogenes Scott A was 26.4 min. This value was for those cells heat shocked at 48°C for 10 min and plated anaerobically on a non-selective medium. The lowest D-value at 55°C for L. monocytogenes Scott A was 6.59 min. This value was for non-heat shocked cells plated aerobically on a selective medium. Depending on the methods chosen for enumeration and the condition of the cells, about a 4-fold change in D-value at 55°C could be observed. Regardless of the conditions of

the cells, recovery was always improved on a non-selective medium using anaerobic techniques.

The important conclusions of this study are: a) the heat resistance of log phase cells of L. monocytogenes Scott A can be significantly increased when exposed to sublethal temperatures and, b) enumeration procedures significantly affected the recovery of the organism.

This phenomenon of increased heat resistance due to heat shock may be extremely important to the food industry. Additional heat resistance gained during a minimally thermal processed refrigerated food could potentially create a health hazard. Survival of only a few cells of L. monocytogenes is unacceptable because this microorganism can grow quite well at refrigeration temperatures with or without oxygen. Because lengthy come-up times may actually create an ideal heat shock environment, process temperatures of thermally processed foods should be obtained as soon as possible to avoid an increase in heat resistance of microorganisms. When a thermal process is developed for a food, consideration should be given to increased heat resistance due to heat shock.

Since recovery was improved using anaerobic techniques rather than aerobic techniques, further studies involving anaerobic techniques should be done to investigate and develop improved methods for recovering L. monocytogenes

from foods. Anaerobic conditions may be a step in the right direction for developing better recovery methods for L. monocytogenes from foods.

VI. REFERENCES

- Anonymous. 1988. Revised FDA Listeria method takes 5-6 days. Food Chem. News. 3036:30
- Bardwell, J.C., and E.A. Craig. 1984. Major heat shock genes of Drosophila and the E. coli heat inducible dnaK gene are homologous. Proc. Natl. Acad. Sci. USA. 81:848-852.
- Bearns, R.E., and K.F. Girard. 1958. The effect of pasteurization on Listeria monocytogenes. Can. J. Microbiol. 4:55-61.
- Beuchat, L.R., R.E. Brackett, D.Y. Hao, and D.E. Conner. 1986. Growth and thermal inactivation of Listeria monocytogenes in cabbage and cabbage juice. Can. J. Microbiol. 32:791-795.
- Bigelow, W.D., and J.R. Esty. 1920. Thermal death time in relation to time of typical thermophilic organisms. J. Infect. Diseases. 27:602.
- Brackett, R.E. 1988. Presence and persistence of Listeria monocytogenes in food and water. Food Technol. 42:162-164.
- Bradshaw, J.G., J.T. Peeler, J.J. Corwin, J.M. Hunt, J.T. Tierney, E.P. Larkin, and R.M. Twedt. 1985. Thermal resistance of Listeria monocytogenes in milk. J. Food Prot. 48:743-745.
- Bradshaw, J.G., J.T. Peeler, J.J. Corwin, J.M. Hunt, and R.M. Twedt. 1987. Thermal resistance of Listeria monocytogenes in dairy products. J. Food Protection. 50:543-544.
- Bunning, V.K., R.G. Crawford, J.G. Bradshaw, J.T. Peeler, J.T. Tierney, and R.M. Twedt. 1986. Thermal resistance of intracellular Listeria monocytogenes cells suspended in raw bovine milk. Appl. Environ. Microbiol. 52:1398-1402.
- Bunning, V.K., R.G. Crawford, J.T. Tierney, and J.T. Peeler. 1989. Thermotolerance of Listeria monocytogenes and Salmonella typhimurium after sublethal heat-shock. Presented at Annual Meeting American Society of Microbiology.

- Byczkowski, J. Z., and T. Gessner. 1988. Biological role of the superoxide ion-radical. *Int. J. Biochem.* 20:569-580.
- Carpenter, S.L. and M.A. Harrison. 1988. Survival of Listeria monocytogenes on processed chicken. Presented at IFT Annual Meeting. New Orleans.
- Dallmier, A.W., and S.E. Martin. 1988. Catalase and superoxide dismutase activities after heat injury of Listeria monocytogenes. *Appl. Environ. Microbiol.* 54:581-582.
- Datta, A. R., B. A. Wentz, D. Shook, and M. W. Truckess. 1988. Synthetic oligodeoxyribonucleotide probes for detection of Listeria monocytogenes. *Appl. Environ. Microbiol.* 54:2933-2937.
- Donelson, J.E., and A.B. Fulton. 1988. The pushy ways of a parasite. *F.D.A. Consumer.* 342:615-616.
- Donnelly, C.W., and E.H. Briggs. 1986. Psychrotrophic growth and thermal inactivation of Listeria monocytogenes as a function of milk composition. *J. Food Prot.* 49:994-998.
- Donnelly, C.W., E.H. Briggs, and L.S. Donnelly. 1987. Comparison of heat resistance of Listeria monocytogenes in milk as determined by two methods. *J. Food Prot.* 50:14-17.
- Donnelly, C.W., G.J. Baigent, and E.H. Briggs. 1988. Flow cytometry for automated analysis of milk containing Listeria monocytogenes. *J. Assoc. Off. Anal. Chem.* 71:655.
- Doyle, M.P., K.A. Glass, J.T. Beery, G.A. Garcia, D.J. Pollard, and R.D. Schultz. 1987. Survival of Listeria monocytogenes in milk during high-temperature, short-time pasteurization. *Appl. Environ. Microbiol.* 53:1433-1438.
- Doyle, M.P. 1988. Effect of environmental and processing conditions on Listeria monocytogenes. *Food Tech.* 42:169-171.
- Doyle, M.P. Personal communication - Carolina-Virginia IFT meeting, May 4, 1989.

- Farber, J. M., and J. I. Speirs. 1987. Monoclonal antibodies directed against the flagellar antigens of Listeria species and their potential in EIA-based methods. J. Food Prot. 50:479-484.
- Farber, J.M., A. Hughes, and R. Hully. 1988a. Thermal resistance of Listeria monocytogenes in sausage meat. In abstracts for the 10th International Symposium on Listeriosis, Hungary. p 99.
- Farber, J.M, and B.E. Brown. 1990. Effect of prior heat shock on heat resistance of L. monocytogenes in meat. Appl. Environ. Microbiol. 56:1584-1587.
- Fedio, W.M., and H. Jackson. 1989. Effect of tempering on the heat resistance of Listeria monocytogenes. Lett. Appl. Microbiol. 9:157-160.
- Garayzabal, J.F., L.D. Rodriguez, J.A. Boland, E.F. Ferri, V.B. Dieste, J.L. Cancelo, and G.S. Fernandez. 1987. Survival of Listeria monocytogenes in raw milk treated in a pilot plant size pasteurizer. J. Appl. Bacteriol. 63:533-537.
- Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips. 1981. Manual of Methods for General Bacteriology. American Society for Microbiology. Washington D.C. pp. 65-220.
- Golden, D.A., L.R. Beuchat, and R.E. Brackett. 1989. Inactivation and injury of Listeria monocytogenes as effected by heating and freezing. Food Microbiol. 5:17-23.
- Gomes, S.L., M.H. Juliani, J.C. Maia, and A.M Silva. 1986. Heat shock protein synthesis during development in Caulobacter crescentus. Jour. of Bacteriol. 168:923-930.
- Gray, M.L., and A.H. Killinger. 1966. Listeria monocytogenes and Listeria infections. Bacteriol. Rev. 30:309.
- Grossman, A.D., J.W. Ericson, and C.A. Gross. 1984. The htpR gene product of E. coli is a sigma factor for heat shock promoters. Cell. 38:383-390.

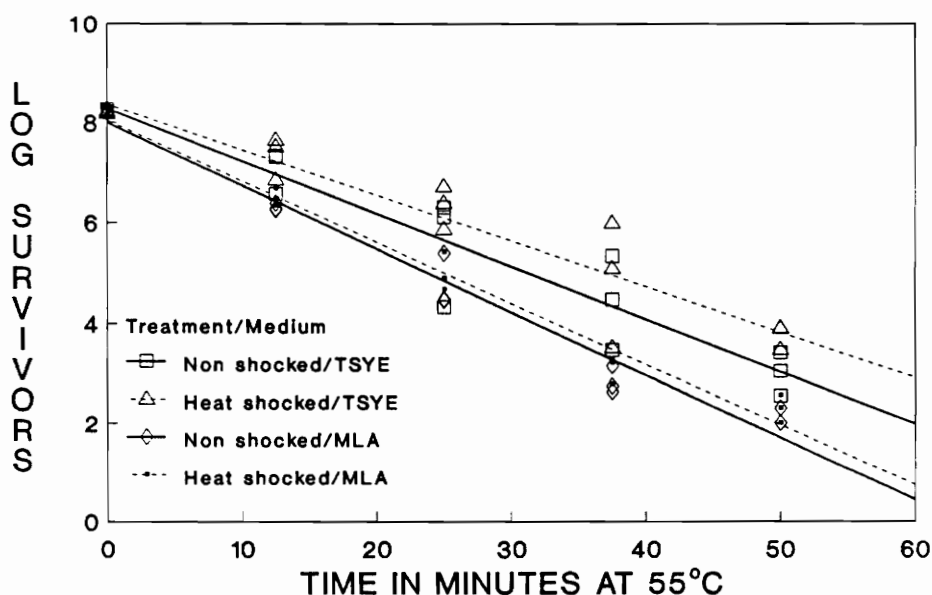
- Hayes, P.S., J.C. Feely, L.M. Graves, G.W. Ajello, and D.W. Fleming. 1986. Isolation of Listeria monocytogenes from raw milk. Appl. Environ. Microbiol. 51:438-440.
- Holdeman, L.V., E.P. Cato, and W.E.C. Moore. 1977. Anaerobe Laboratory Manual 4th ed. Virginia Polytechnic Institute and State University. Southern Printing Co. Blacksburg, VA.
- Jay, J.M. 1986. Modern Food Microbiology 3rd ed. New York: Van Nostrand Reinhold Co. pp.331-345.
- Kahn, M.A., A. Seaman, and M. Woodbine. 1974. Synergistic drug treatment in experimental listeriosis. Page 52 in Problems of listeriosis. M. Woodbine ed. Heicester UMV Press, Surrey UK.
- Knabel, S.J., H.W. Walker, P.A. Hartman, and F. Mendonca. 1990. Effects of growth temperature and strictly anaerobic recovery on the survival of Listeria monocytogenes during pasteurization. Appl. Environ. Microbiol. 56:370-376.
- Krieg, N.R. December 15, 1989. Personal Communication.
- Lee, W.H., and D. McClain. 1986. Improved Listeria monocytogenes selective agar. Appl. Environ. Microbiol. 52:1215-1217.
- Lovett, J., D.W. Francis, and J.M. Hunt. 1987. Listeria monocytogenes in raw milk: Detection, incidence, and pathogenicity. J. Food Prot. 50:188-192.
- Lovett, J., 1988. Isolation and enumeration of Listeria monocytogenes. Food Technol. 42:172-175.
- Mackey, B.M., and C.M. Derrick. 1986. Elevation of the heat resistance of Salmonella typhimurium by sublethal heat shock. J. of Appl. Bacteriol. 61:389-393.
- Mackey, B.M., and C.M. Derrick. 1987. The effect of prior heat shock on the thermoresistance of Salmonella thompson in foods. Lett. Appl. Microbiol. 5:115-118.
- Mackey, B.M., and N. Bratchell. 1989. The heat resistance of Listeria monocytogenes. Lett. Appl. Microbiol. 9:89-94.

- Mackey, B.M., C. Pritchett, A. Norris, and G.C. Mead. 1990. Heat resistance of Listeria: Strain differences and effects of meat type and curing salts. *Letters Appl. Microbiol.* 10:251-255
- Marth, E.H. 1988. Disease characteristics of Listeria monocytogenes. *Food Technol.* 42:165-168.
- McClain, D., and W.H. Lee. 1988. Development of USDA-FSIS method for isolation of Listeria monocytogenes from raw meat and poultry. *J. Assoc. Anal. Chem.* 71:660-664.
- McLauchlin, J. 1987. Listeria monocytogenes, recent advances in the taxonomy and epidemiology of listeriosis in humans. *J. Appl. Bacteriol.* 63:1-11.
- Murano, E. A., M. D. Pierson, J. R. Bishop, G. W. Claus, J. K. Palmer, and A. A. Yousten. 1990. The effect of heat shock, growth atmosphere, and recovery atmosphere on the survival of Escherichia coli 0157:H7 to heat. PhD dissertation, Virginia Polytechnic Institute and State University.
- Murray, E.G.D. 1955. A characterization of listeriosis in man and other animals. *Can. Med. Assn. J.* 72:99.
- Neidhardt, F.C., R.A. VanBogelen, and V. Vaughn. 1984. Genetics and regulation of heat shock proteins. *Ann. Rev. Genet.* 18:295-329.
- Ng, H., H.G. Bayne, and J.A. Garibaldi. 1969. Heat resistance of Salmonella: The uniqueness of Salmonella senftenberg 775W. *Appl. Microbiol.* 17:78-82.
- Pearson, L.J., and E.H. Marth. 1990. Listeria monocytogenes - A threat to the food supply: A review. *J. Dairy Sci.* 73:912-928.
- Rosenow, E.M. and E.H. Marth. 1987. Listeria, listeriosis and dairy foods: A review. *Cult. Dairy Prod. J.* 22:4.
- Schlesinger, M.J. 1986. Heat shock proteins: The search for functions. *J. Cell Biol.* 103:321-325.
- Seelinger, H.P.R., 1961. Listeriosis. Hafner Publ. Co., Inc., New York, New York.

- Seelinger, H.P.R., and D. Jones. 1986. pp. 1235-1245.
In P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G.
Holt (eds.) Bergey's Manual of systematic bacteriology,
vol. 2. Williams and Wilkins, Baltimore, MD.
- Siragusa, G.R., and M.G. Johnson. 1990. Monoclonal
antibody specific for Listeria monocytogenes,
Listeria innocua, and Listeria welshimeri. Appl.
Environ. Microbiol. 56:1897-1904.
- Smith, J.L., and D.L. Archer. 1988a. Heat-induced injury
in Listeria monocytogenes. J. Ind. Microbiol.
3:105-110.
- Smith, J.L., and S.E. Hunter. 1988b. Heat injury in
Listeria monocytogenes: Prevention by solutes.
Lebensm.Wiss.u.Technol. 21:307-311.
- Sokolovic, Z. and W. Goebel. 1988. Synthesis of
Listeriolysin in Listeria monocytogenes under heat
shock conditions. Infect. Immun. 57:295-298.
- Speck, M.A. 1984. Compendium of Methods for the
Microbiological Examination of Foods. 2nd Ed.
American Public Health Association. Washington D.C.
pp. 62-83.
- Stern, J.A., and B.E. Proctor. 1984. A micro-method and
apparatus for the multiple determination of rates of
destruction of bacteria and bacterial spores subjected
to heat. Food Technol. 17:460.
- Streips, U.N., and F.W. Polio. 1985. Heat shock proteins
in bacilli. J. of Bacteriol. 162:434-437.
- Stumbo, C.R. 1973. Thermalbacteriology in Food
Processing, 2nd ed. New York: Academic Press.
- Swientek, R.J. 1989. Sous vide - Refrigerated upscale food
in a vacuum pouch. Food Processing. 50(13):34-39.
- Twedt, R.M. 1986. Listeria. Progress in Food Safety.
(Preceedings of a symposium entitled "Progress in our
knowledge of foodborne disease during the life of the
Food Research Institute, University of Wisconsin,
Madison). pp. 113-120.

Yamamori, T., and T. Yura. 1982. Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in Escherichia coli K12. Proc. Nat. Acad. Sci. USA. 79:860-864.

VII. APPENDIX



Non Heat Shock D-value on TSYE ^{a,d}	9.61 min
Non Heat Shock D-value on ML ^{a,e}	7.96 min
Heat Shock D-value on TSYE ^a	11.1 min
Heat Shock D-value on ML ^a	8.24 min
Heat Shock Ratio on TSYE ^b	1.16
Heat Shock Ratio on ML ^b	1.04
Non Heat Shock Cell Injury ^c	83.1%
Heat Shock Cell Injury ^c	94.7%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

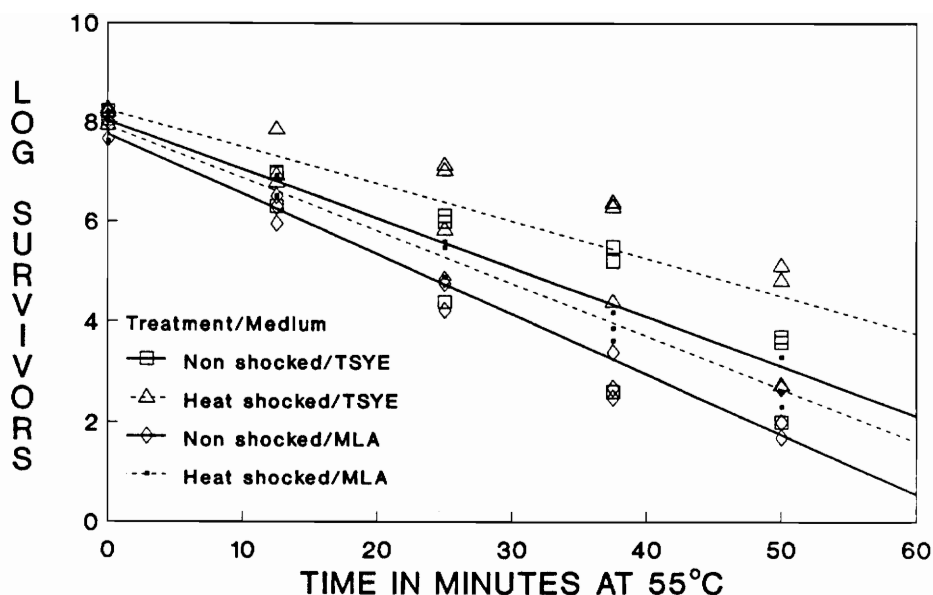
^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

Figure 13. Survivor curve at 55°C for cells heat shocked 40°C, 3 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	10.5 min
Non Heat Shock D-value on ML ^{a,e}	8.39 min
Heat Shock D-value on TSYE ^a	14.3 min
Heat Shock D-value on ML ^a	9.56 min
Heat Shock Ratio on TSYE ^b	1.35
Heat Shock Ratio on ML ^b	1.14
Non Heat Shock Cell Injury ^c	79.2%
Heat Shock Cell Injury ^c	87.2%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

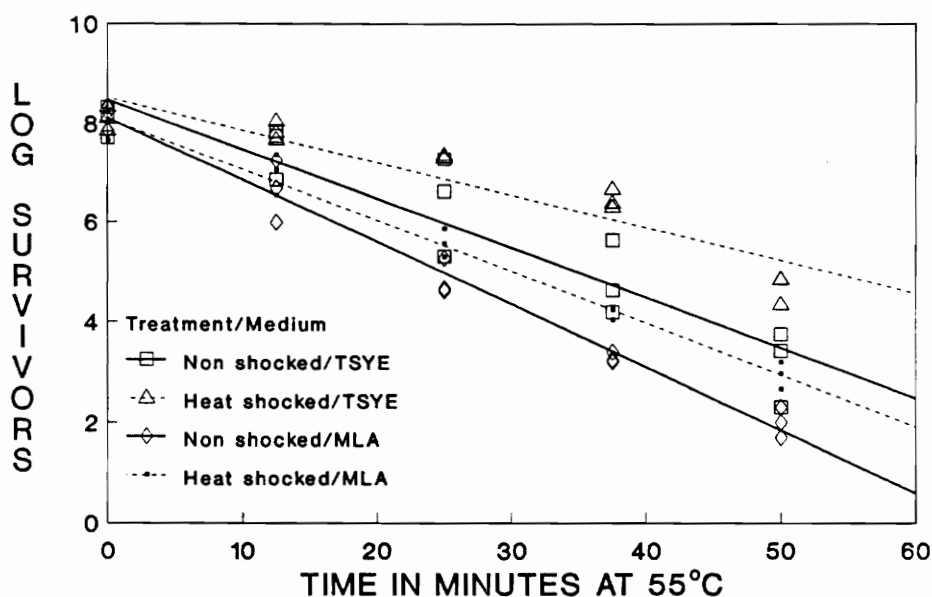
^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

Figure 14. Survivor curve at 55°C for cells heat shocked 40°C, 10 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	10.0 min
Non Heat Shock D-value on ML ^{a,e}	8.00 min
Heat Shock D-value on TSYE ^a	15.2 min
Heat Shock D-value on ML ^a	9.71 min
Heat Shock Ratio on TSYE ^b	1.52
Heat Shock Ratio on ML ^b	1.21
Non Heat Shock Cell Injury ^c	89.2%
Heat Shock Cell Injury ^c	98.8%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

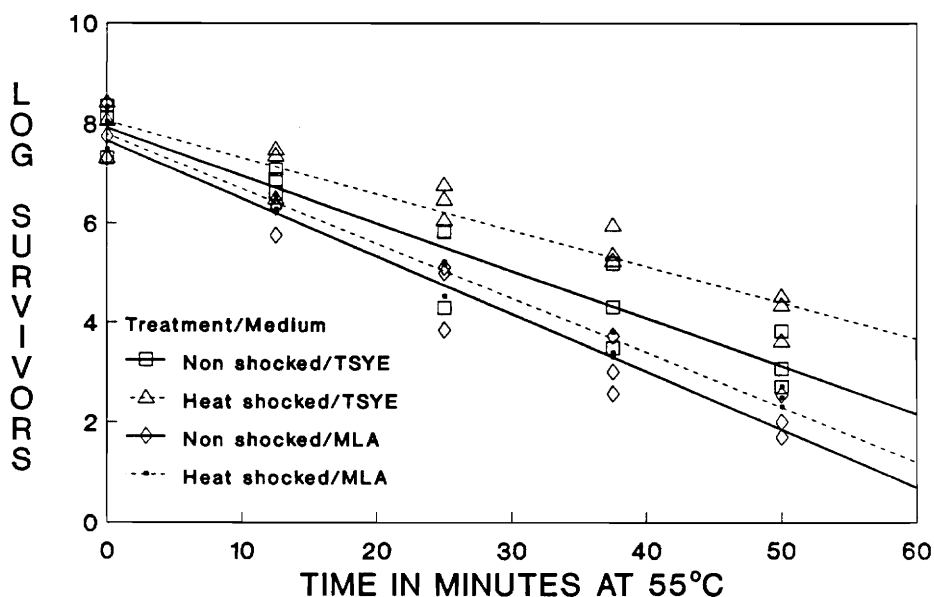
^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride *Listeria* agar.

Figure 15. Survivor curve at 55°C for cells heat shocked 40°C, 20 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	10.5 min
Non Heat Shock D-value on ML ^{a,e}	8.61 min
Heat Shock D-value on TSYE ^a	14.7 min
Heat Shock D-value on ML ^a	9.10 min
Heat Shock Ratio on TSYE ^b	1.39
Heat Shock Ratio on ML ^b	1.06
Non Heat Shock Cell Injury ^c	92.0%
Heat Shock Cell Injury ^c	96.6%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

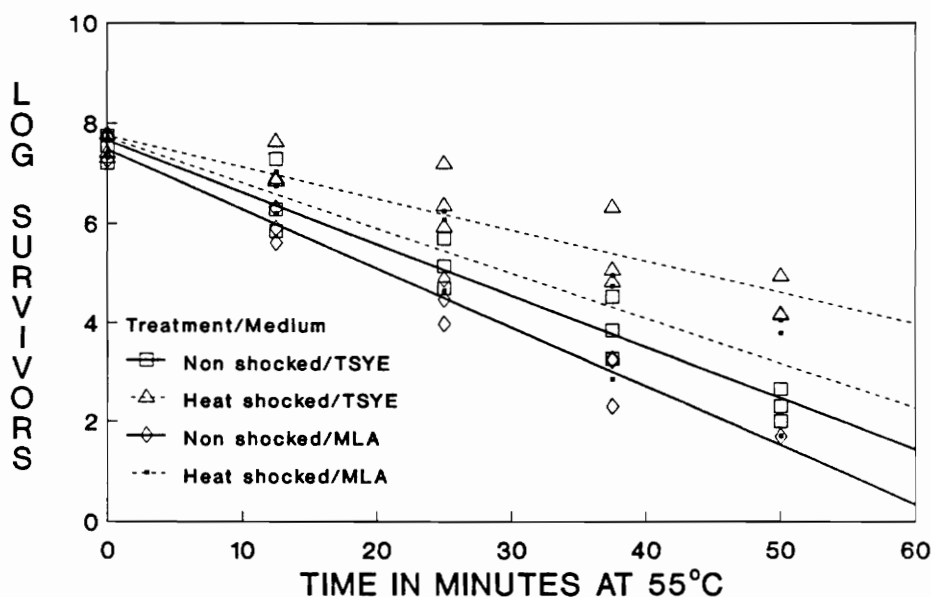
^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

Figure 16. Survivor curve at 55°C for cells heat shocked 44°C, 3 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	9.67 min
Non Heat Shock D-value on ML ^{a,e}	7.92 min
Heat Shock D-value on TSYE ^a	16.0 min
Heat Shock D-value on ML ^a	11.4 min

Heat Shock Ratio on TSYE ^b	1.66
Heat Shock Ratio on ML ^b	1.44

Non Heat Shock Cell Injury ^c	79.6%
Heat Shock Cell Injury ^c	81.9%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

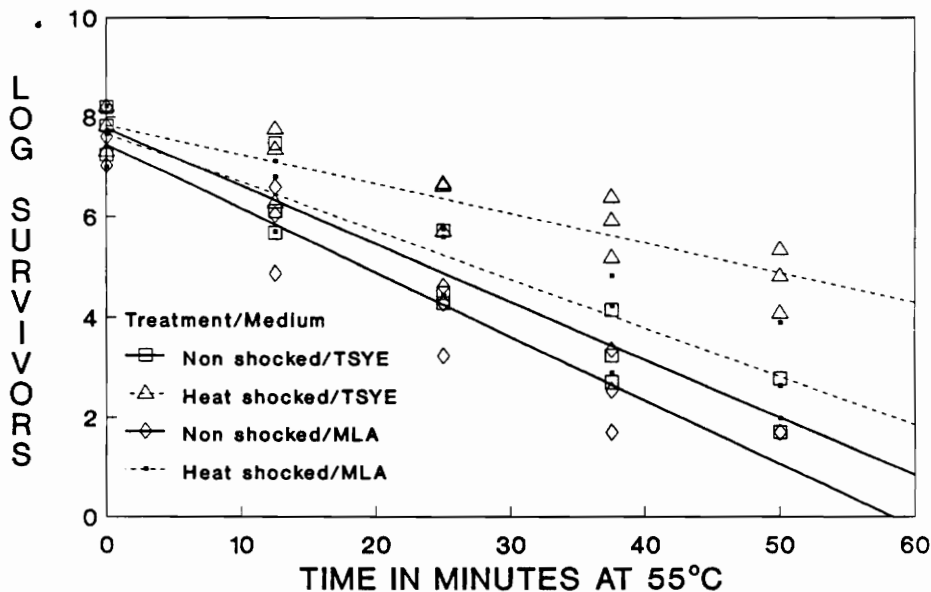
^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

Figure 17. Survivor curve at 55°C for cells heat shocked 44°C, 10 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	8.68 min
Non Heat Shock D-value on ML ^{a,e}	7.22 min
Heat Shock D-value on TSYE ^a	17.1 min
Heat Shock D-value on ML ^a	10.4 min
Heat Shock Ratio on TSYE ^b	1.97
Heat Shock Ratio on ML ^b	1.44
Non Heat Shock Cell Injury ^c	97.2%
Heat Shock Cell Injury ^c	98.5%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

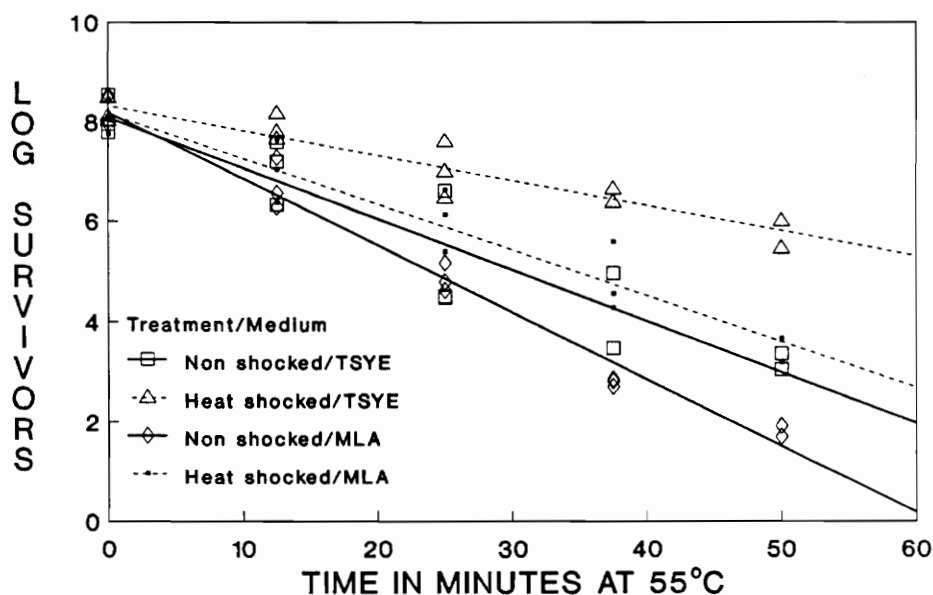
^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

Figure 18. Survivor curve at 55°C for cells heat shocked 44°C, 20 min and plated aerobically on TSYE and ML agar.



Non Heat Shock D-value on TSYE ^{a,d}	9.31 min
Non Heat Shock D-value on ML ^{a,e}	7.75 min
Heat Shock D-value on TSYE ^a	17.3 min
Heat Shock D-value on ML ^a	11.5 min

Heat Shock Ratio on TSYE ^b	1.86
Heat Shock Ratio on ML ^b	1.49

Non Heat Shock Cell Injury ^c	97.9%
Heat Shock Cell Injury ^c	99.7%

^a D-values are at 55°C and represent mean of three replicates.

^b Values are the ratio of [heat shock D-value]/[non heat shock D-value] for cells heated at 55°C. Ratios represents mean of three replicates.

^c Values are determined after 50 min by the equation:

$$[(1 - \text{colony forming units on TSYE} / \text{colony forming units on ML}) \times 100]$$

^d Trypticase Soy + 0.6% Yeast Extract agar.

^e McBride Listeria agar.

Figure 19. Survivor curve at 55°C for cells heat shocked 48°C, 3 min and plated aerobically on TSYE and ML agar.

VITA

Richard Howard Linton, the author of this thesis, was born March 18, 1966 in Scotch Plains, New Jersey. He is the son of Howard and Doris Linton. He received his high school diploma from Concord High School in Wilmington, Delaware. He received his Bachelor of Science degree in Biology in May, 1988 from Virginia Polytechnic Institute and State University. He decided to remain at Virginia Polytechnic Institute and State University and is currently pursuing the Master of Science degree in Food Science and Technology.

Richard is a member of Institute of Food Technologists and American Society of Microbiology.

Richard H. Linton