NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

UNIVERSITY OF ALBERTA

FACTORS AFFECTING INCREASED THERMOTOLERANCE IN *LISTERIA MONOCYTOGENES*

BY

EMILIE KATHLEEN BLACK

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

IN

FOOD MICROBIOLOGY

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

SPRING 1992

ISBN 0-315-73195-8

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: **Emilie Kathleen Black**

TITLE OF THESIS: **Factors affecting increased thermotolerance in *Listeria monocytogenes***

DEGREE: **Master of Science**

YEAR THIS DEGREE GRANTED: **1992**

E. Kathleen Black

#305, 10712 University Avenue
Edmonton, Alberta. T6E 4P9

Date: 24/04/92

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **FACTORS AFFECTING INCREASED THERMOTOLERANCE IN *LISTERIA MONOCYTOGENES*** submitted by **EMILIE KATHLEEN BLACK** in partial fulfillment of the requirements for the degree **MASTER OF SCIENCE** in **FOOD MICROBIOLOGY.**

Dr. H. Jackson, Supervisor

Dr. P. Jelen

Dr. R. Whitehouse

Dr. F. H. Wolfe

Date: 23 APRIL 92

Abstract

The phenomenon of tempering, or enhanced thermotolerance, was studied in *Listeria monocytogenes*. The factors investigated were the stability of the tempered state, and the effect of pH and the butterfat content of the tempering menstrua on the acquisition of enhanced thermotolerance.

The results indicate that tempered cultures of *L. monocytogenes* retain their increased thermotolerance for extended periods of time when stored at 0° and 4°C. Incubation at 37°C for 30 to 120 minutes results in a highly significant decrease in the heat resistance of tempered *L. monocytogenes* at concentrations of 10^9 and 10^7 organisms/ mL, in both TSBYE and 2% UHT milk.

Acquired thermotolerance was affected by pH. Tempering at pH 7.5 resulted in the greatest increase in thermotolerance, and tempering occurred over the range of pH 6- 9.

The results of the study on the effect of butterfat content of the tempering and heating menstrua show that there is no significant difference between the D_{60} values of the cells tempered and challenged in 0.1% milk, 2% UHT milk, 10% cereal cream, and 35% whipping cream. Untempered cells challenged in 35% butterfat whipping cream had a significantly greater D_{60} value than untempered cells in 0.1% milk, 2% UHT milk, or 10% butterfat cereal cream. The tempered cells had significantly greater heat resistance than the untempered cells in the same challenging medium.

L. monocytogenes became tempered in a minimal medium, but not in an incomplete medium consisting of essential amino acids, dextrose and magnesium chloride in a pH 7.0 phosphate buffer.

Acknowledgements

I would like to acknowledge the assistance of the faculty, support staff, and students of the Department of Food Science.

My gratitude is extended to Dr. Harry Jackson. His patience, level head, and guidance was greatly appreciated.

I would also like to thank Sal Cooper, for her endeavors to edit this thesis. Despite her frustration with the passive voice, my thesis is much more readable than it would have been without her. Jan Standish made the bad days liveable with her loony sense of humor, and I am glad that we were able to go through this experience at the same time. I'd like to thank Rick Toogood, who also helped me keep everything in perspective.

Finally, I would like to thank the Faculty of Graduate Studies, the University of Alberta, the George Cringle (Palm Dairies) Research Foundation, and the Alberta Agricultural Research Institute, Alberta Agriculture, for financial support.

Table of Contents

List of Tables

Page

List of Figures

Page

1. Introduction

1.1 Background

The link between food consumption and human listeriosis was first made in 1981, following an outbreak of the disease in the Maritime provinces of Canada, associated with coleslaw. Since then, other outbreaks have occurred, notably the 1983 outbreak in Massachusetts associated with the consumption of whole and 2% milk (Fleming *et al.*, 1985), and the outbreak in California associated with the consumption of Jalisco cheese (Linnan *et al.*, 1988). While, in the Jalisco cheese outbreak, unpasteurized milk or post-processing contaminated milk was used to make the cheese, in the Massachusetts milk outbreak, the implicated milk appeared to have been properly pasteurized. These two outbreaks raised questions about the heat resistance of the causative organism, *Listeria monocytogenes*. One of the possible explanations for the presence of the organism in pasteurized milk products is tempering, the increased heat resistance in microorganisms resulting from a sub-lethal heat treatment.

1.2 The genus *Listeria*

Within the genus *Listeria*, there are 8 species: *monocytogenes, ivanovii, seeligeri, welshimeri, innocua, grayi, murrayi,* and *denitrificans*. All are described in Bergey's Manual of Systematic Bacteriology (7th edition) as Gram-positive, nonsporing rods, which are motile by peritrichous flagella at 20 to 25°C, and are aerobic and facultatively anaerobic. They are able to grow between 1 and 45°C, and pH 6 and 9. When stabbed into tubes of Motility Test Medium (Difco Laboratories, Detroit, MI), members of the genus display umbrella motility. Fermentation of glucose results in the production of mainly L-lactic acid, and fermentation of other sugars produces acid but not gas. The most common pathogenic species of the genus is *L. monocytogenes*. It was the first described species of the genus and is identified by a weak zone of beta haemolysis on 5-10% sheep blood agar plates (SBAP), a positive

CAMP test with *Staphylococcus aureus* but not *Rhodococcus equi* (the exception being *L. monocytogenes* ATCC 15313, which is non-haemolytic on horse, sheep and bovine blood agar plates, and which has a negative CAMP test using both *S. aureus* and *R. equi*), and its ability to produce acid from alpha-methyl-D-mannoside and L-rhamnose but not mannitol. The serotypes of *L. monocytogenes* are: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7.

Most outbreaks of food borne listeriosis are caused by *L. monocytogenes*. The organism infects its host by penetrating the lining of the gastrointestinal wall. Once within the bloodstream, the organism initiates endocytosis by non-activated phagocytes. Within the phagosomes, *L. monocytogenes* disrupts the phagosomic membrane and thus becomes a part of the cytoplasm of the phagocyte. Within 2 hours of phagocytosis, *L. monocytogenes* becomes coated with actin filaments. The actin filaments migrate to the ends of the bacteria and may assist in intracellular movement and spreading of the bacteria among non-activated macrophages. The organism is carried to various parts of the body this way, particularly to the cerebro-spinal fluid. Symptoms of listeriosis include: meningitis (inflammation of the spinal column), encephalitis (inflammation of the brain), and/ or spontaneous abortion, since the organism is able to cross the placental membrane. *L. monocytogenes* may cause granulomatosis infantiseptica in a foetus, the widespread presence of abscess and/ or granulomas in multiple internal organs, including the liver, spleen, kidney, lungs and brain. Some cases of endocarditis (inflammation of the lining of the heart) have been reported due to *L. monocytogenes*. Listeriosis is associated most frequently with persons who are immunocompromised; however, the disease does occur in healthy individuals.

L. ivanovii displays very wide or double zones of haemolysis on SBAP, a positive CAMP test with *R. equi*, but a negative one with *S. aureus*. *L. ivanovii* is considered a pathogen, however few cases of listeriosis are caused by this species.

The species produces acid from D-xylose, but not from L-rhamnose, alpha-methyl-D-mannoside or mannitol. All isolated strains of *L. ivanovii* belong to serovar 5. *L. seeligeri* is positive for beta haemolysis on SBAP and has a positive CAMP test with *S. aureus*, but a negative one with *R. equi*. Despite being positive for beta haemolysis and the CAMP test with *S. aureus*, *L. seeligeri* is not pathogenic for mice. The species produces acid from D-xylose, but not from L-rhamnose and mannitol. Most strains do not produce acid from alpha-methyl-D-mannoside. Strains of *L. seeligeri* belong to serovars 1/2b, 4c, 4d, and 6b. *L. welshimeri* is another non-pathogenic species of *Listeria*. It is differentiated from other species by its ability to produce acid from alpha-methyl-D-mannoside and D-xylose. Some strains are able to ferment L-rhamnose and the serotypes are all within group 6 (serovars 6a and 6b). *L. innocua* is non-pathogenic, does not show beta haemolysis on SBAP, or a positive CAMP test with either *S. aureus* or *R. equi*. It produces acid from alpha-methyl-D-mannoside and most strains produce acid from L-rhamnose. It does not produce acid from mannitol or D-xylose. Most strains of *L. innocua* are of serogroup 6 or serovar 4ab. *L. grayi* is a *species incertae sedis*, based upon DNA-DNA hybridization studies with *L. monocytogenes*. This species is not haemolytic and gives a negative CAMP test on SBAP. It does not produce acid from L-rhamnose, or D-xylose, but acid is produced from mannitol. *L. murrayi* is also a *species incertae sedis* based on DNA-DNA hybridization studies with *L. monocytogenes*, and is considered to be very similar to *L. grayi*. Like *L. grayi*, *L. murrayi* does not produce beta haemolysis on SBAP, nor does it give a positive CAMP test with either *S. aureus* or *R. equi*. It ferments mannitol, some strains produce acid from L-rhamnose, and no acid is produced from D-xylose. One characteristic differentiating the two species is that *L. murrayi* is able to reduce NO_3^- to NO_2^-, while *L. grayi* is not.

Most authors now agree that *L. denitrificans* should be removed from the genus *Listeria*, based upon "morphological, biochemical, serological, chemical and nucleic

acid studies" (Seeliger and Jones, 1986). *L. denitrificans* does not produce beta haemolysis on SBAP, and has a negative CAMP test using *S. aureus* or *R. equi*. Unlike other species of *Listeria*, it is able to produce acid from L-arabinose, glycogen, and melibiose and it is able to hydrolyse cellulose. Its molecular G+C percentage is higher than any other species of *Listeria*, its major peptidoglycan diamino acid is L-lysine, and its major menaquinone is MK-9, while every other species of *Listeria* has *meso*-diaminopimelic acid as the major peptidoglycan diamino acid, and MK-7 as the major menaquinone.

1.3 Food-borne outbreaks of human listeriosis

In 1981, an outbreak of human listeriosis was caused by the consumption of coleslaw that had been made from cabbage harboring *L. monocytogenes*. The cabbage had been fertilized with manure from a sheep flock suspected of having listeriosis. In this outbreak there were 41 cases. The adult mortality rate was 28.6%, and the mortality rate of infected infants was 27%. In Massachusetts in 1983, 49 persons became ill after consumption of 2% or whole milk. Although in this incident the pasteurization procedure appeared to be adequate, various authors hypothesized that *L. monocytogenes* was present in such high numbers that the pasteurization treatment was inadequate, or that the pathogen was protected from destruction by existing within leukocytes. In 1985, 142 individuals were infected after consumption of a Mexican-style cheese from a Californian plant. *L. monocytogenes* was isolated from the "pasteurized" milk, dried milk, the cheese, and from plant environmental sources. In this case, it developed that the cheese was made from raw milk. Other examples of outbreaks of listeriosis associated with cheese consumption occurred in the United Kingdom in 1986, and in Switzerland in 1987. In both cases *L. monocytogenes* was carried in soft cheese. Several authors indicate that *L. monocytogenes* is a frequent

contaminant (Johnson *et al.*, 1986, Doyle and Schoeni, 1986), and may proliferate in soft cheese (Northolt *et al.*, 1988, Ryser, Marth and Doyle, 1985).

1.4 Incidence of *L. monocytogenes* in foods

L. monocytogenes has been found in a large variety of food products. They include meats, poultry, seafood, vegetables, and dairy products. Outbreaks of listeriosis have been associated with the consumption of foods from each of these groups.

1.4.1 Incidence of *L. monocytogenes* in meats and meat products

L. monocytogenes has been isolated from many meats and meat products. Table 1.1 gives a summary of the percentage positive samples of meats and meat products for *L. monocytogenes*. Wide variations in the numbers of positive samples may be accounted for by differences in the number of samples, meat type differences, sampling locations (retail sampling versus commercial sampling), and isolation methods used for *Listeria*. At the farm level, the organism has been found in soil, water, insects, silage, faeces, and plants. Thus, farm animals may carry the bacteria externally. As well, the animal may be an asymptomatic carrier of *L. monocytogenes*, or may be suffering from listeriosis. In the abbatoir, it is possible that the meat may become contaminated with *L. monocytogenes* from the environmental sources carried on the hide of the animal, from contamination by faeces, from carcass to carcass transmission, or from workers, who may be carriers of the organism, handling the carcass. The organism is able to grow in a variety of meats and meat products, although growth is dependent upon the temperature and pH of the meat, the species of animal, the cut of meat, and the background microflora already on the meat (Farber and Peterkin, 1991). Fenlon (1985) reports that wild birds are a common reservoir of *L. monocytogenes*.

Table 1.1: Incidence of *L. monocytogenes* in meats and meat products

Beef	% positive samples
Raw	6.2
	16.7
	92.0
	6.9
	6.5
Minced/ ground	28.0
	52.4
	46.0*

Pork	% positive samples
Raw	12.9
	68.0
Minced/ ground	80.0
	40.0
	11.8
	40.0*
	16.7

Ham	% positive samples
Uncooked	0.0
Slices, canned	1.5

Mixed, minced meat	% positive samples
Minced meat	22.4
	36.0
Pork and beef	20.8
Pork, beef, veal	15.8

Sausages	% positive samples
Fresh mettwurst	59.0
Dry and fresh	12.7
Salami and pressed pork	0.2
Beef, salami, mettwurst	4.0
Seasoned mix	12.8
Salami	6.3
Smoked	5.5
Mettwurst	21.1
	23.0
Onion mettwurst	9.0
Fermented	5.2

Miscellaneous	% positive samples
Frozen beef patties	26.2
Air-dried meat	9.1
Boneless beef	20.0
Boneless lamb	60.0
Paté	50.0
Jerky	0.0

*= *L. monocytogenes* and *L. innocua*
Adapted from: Farber and Peterkin, 1991

1.4.2 Incidence of *L. monocytogenes* in raw poultry and seafood

Poultry and seafood have been shown to harbor *L. monocytogenes*. Despite the implication of these food types in several outbreaks of listeriosis, neither poultry nor seafood has been examined as extensively as the red meats. Table 1.2 shows a summary of the types of poultry and seafoods examined for the presence of *L. monocytogenes* and the reported percentages of samples found to be positive for the organism. Transmission routes in poultry are suspected to be the same as for red meats. Thorough examinations on the origin of *L. monocytogenes* in fish and seafood are lacking, and no reports on this subject were found in the literature.

Table 1.2: Incidence of *L. monocytogenes* in raw poultry, fish and seafood

Chicken	% positive samples	Other Poultry	% positive samples
Cook-chill	24.0	Not known (N.K)	25.0
Fresh chicken parts	14.6	Portions, N.K	48.0
	49.3	Precooked, ready to eat	12.0
Fresh and frozen	57.0		
Legs	56.3	Chilled meats (mainly poultry)	18.0
Marked broilers (damaged)	23.0	N.K	14.7
Chicken	25.0	Turkey parts, fresh	15.0
Fresh	66.0		
Frozen	54.0		
	15.0		
Precooked	26.5		

Fish and Seafood	% positive samples
Shrimp, crab meat, lobster tail, fin fish, surimi based seafood	26.3
Smoked and marinated fish	12.5
Smoked	24.0
Tropical fish and fish products	0.0

Adapted from: Farber and Peterkin, 1991

1.4.3 Incidence of *L. monocytogenes* in raw vegetables

Table 1.3 shows the reported incidence of samples found positive for *L. monocytogenes* in some vegetables. The highest incidence levels of *L. monocytogenes* have been reported in potatoes and radishes, while in some vegetables, such as chopped carrots, *L. monocytogenes* does not thrive, probably due to the production of a bacteriostatic agent or a bacteriocide by the vegetable (Nguyen-the and Lund, 1991). Transfer between vegetables can occur, and *L. monocytogenes* has been found in 8 of 42 mixed salads examined by Velani and Roberts (1991). These authors hypothesized that the organism was transferred between ingredients during salad preparation. Outbreaks of listeriosis have been associated with the consumption of vegetables and vegetable based products, the most notable being the outbreak of listeriosis associated with the consumption of coleslaw in the Maritimes provinces of Canada in 1981. There have been outbreaks connected with the consumption of other vegetables, for example a 1979 outbreak in the Boston area involving 20 cases. The vegetables linked with this outbreak were celery, tomatoes, and lettuce.

Table 1.3: Incidence of *L. monocytogenes* in raw vegetables

Vegetables	% positive samples
Salad ingredients	1.8 a
Mixed salad	19.0 a
Potatoes	21.2 b
Radishes	14.4 b
Cabbage	1.1 b
Cucumber	2.2 b
Mushrooms	0.0 b
Lettuce	0.0 b

a= Adapted from Heisick *et al.*, 1989

b= Adapted from Velani and Roberts, 1991

1.4.4 Incidence of *L. monocytogenes* in dairy products

Table 1.4 shows the incidence of *L. monocytogenes* in milk and dairy products. The review of Farber and Peterkin (1991), does not specify whether the cheeses and other products reported were made from pasteurized or raw milk. Johnston *et al.* (1986) indicate that many of the samples that they examined failed the phosphatase test and that some of the manufacturers used unpasteurized milk. The incidence of the organism is relatively low in the pasteurized milk and cheeses, with the exception of a report (Fernandez Garayzabal *et al.*, 1986) of the incidence in pasteurized milk in Spain. These researchers state that all milk examined passed the phosphatase test and had been high-temperature, short-time (HTST) processed at 78°C for 16 seconds. They found that 6 of 24 samples (21.4%) were positive for *L. monocytogenes*. They hold that the presence of the organism after pasteurization could be due to either contamination in extremely high numbers or to high heat resistance of the organism.

The reported incidence of *L. monocytogenes* in raw milk varies, depending upon the research method, season of study and geographic location. For example, Dominguez Rodriguez *et al.* (1985) reported the incidence of *L. monocytogenes* in raw milk in Spain as 45.3%, yet Lovett *et al.* (1987) reported that the organism was absent from, all raw milk tested in California. The two groups of researchers used very different methods of collecting samples. On the one hand, Dominguez Rodriguez *et al.* (1985) sampled from one bulk tank 95 times over 16 months, and found that 45.3% of their samples contained *L. monocytogenes*. Their sampling method does not indicate whether the organism comes from one farm or several and the authors only sampled one bulk tank in one dairy in Madrid. Dominguez Rodriguez *et al.* (1985) used the cold-enrichment technique to encourage the growth of *Listeria* species in the raw milk, which involved storing the samples of milk at 4°C, and removing specimens of the stored milk after 7 and 15 days. Lovett *et al.* (1987), on the other hand, sampled raw milk from farm bulk tanks in California, Massachusetts and the Tri-State area (Ohio,

Kentucky, and Indiana) over a period of 10 months. They found that California milk had an incidence of 0% *L. monocytogenes* (of 100 samples), Massachusetts had an incidence of 6.5% (of 200 samples), and the Tri-State region had an incidence of 3.4% (of 350 samples). Other researchers sampling raw milk at the farm level in California did find *L. monocytogenes* (Donnelly *et al.*, 1986). The method used by Lovett *et al.* (1987) was the Food and Drug Administration (FDA) procedure involving the mixture of the raw milk sample with an enrichment broth, storage at 30°C, and sampling after 24 and 48 hours. Although the method used by Lovett *et al.* (1987) and most other researchers gives an accurate appraisal of the incidence of *L. monocytogenes* in raw milk at the source (farm), the method of Dominguez Rodriguez *et al.* (1985) gives a more accurate indication of this incidence at the dairy. Doyle and Schoeni (1986) indicate that the cold-enrichment procedure is more sensitive than the FDA method. Most researchers today use a modified FDA procedure with Fraser broth or the United States Department of Agriculture (USDA) procedure with Fraser broth. The modified methods make use of the greater assortment of selective agars available today, and reportedly detect up to 92% of the positive samples (Warburton *et al.*, 1991). Within Canada, the incidence of *L. monocytogenes* at the farm level varies, but ranges between 1.3% (Farber *et al.*, 1988) and 5.4% (Slade *et al.*, 1988).

Raw milk	% positive samples	Location	Reference
	1.3	Switzerland	a
	4.0	U. S. A	a
	4.2	U. S. A	a
	1.6	Canada	a
	5.4	Canada	a
	1.3	Canada	a
	1.9	Canada	b
	2.6	Scotland	a
	45.3	Spain	a
	3.8	Hungary	a
	3.6	United Kingdom	a

Pasteurized Milk	% positive samples	Location	Reference
	0.5	Australia	a
	1.1	United Kingdom	a
	21.4	Spain	c

Cheese	% positive samples	Location	Reference
Soft	10.0	United Kingdom	a
	1.8	Australia	a
	1.6	Italy	a
	0.5	Canada	a
Ripened soft	8.2	United Kingdom	a
Unripened soft	1.1	United Kingdom	a
Semihard	2.0	Switzerland	a
Soft and semihard	0.9	Canada	d
White mold cured	2.7	Switzerland	a
Red smear	9.6	Switzerland	a
"Other cheeses"	5.6	Switzerland	a
Various cheeses	2.0	Hungary	a
Hard	1.5	U. S. A.	a

Other products	% positive samples	Location	Reference
Goat milk	0.8	United Kingdom	a
Ewe milk	1.8	United Kingdom	a
Goat milk cheese	4.6	United Kingdom	a
Ewe milk cheese	0.7	United Kingdom	a
Ice cream	0.3	Canada	a
Ice cream	2.0	United Kingdom	a
Ice cream novelties	1.9	Canada	a

Adapted from: a= Farber and Peterkin, 1991, b= Fedio and Jackson, 1990, c= Fernandez Garayzabal, 1986, d= Johnston *et al.*, 1986

The heat resistance of *L. monocytogenes* is most commonly presented in terms of the D-value and z-value. The D-value is the amount of time, at a specified temperature, required to reduce the cell population by 1 log cycle (90% or 10 fold). The z-value is the increase in temperature required to reduce the D-value 10 fold. These units of measurement will be used throughout the following sections.

1.5.1 Heat resistance of *L. monocytogenes* in meats and meat products

L. monocytogenes can be a contaminant of beef and pork, and beef and pork products. The organism has been found in as high as 92% of raw beef samples tested, and up to 80% of minced pork samples tested (Table 1.1). Thus, the inactivation of the organism is a very important issue.

Within a meat product, several factors will influence the heat resistance of *L. monocytogenes*. Firstly, meat is heated by conduction, not convection (Hallström *et al.*, 1988), and this results in a very large shoulder on the destruction curve of organisms, because heating by convection is much faster than heating by conduction. Secondly, the amount of fat in the product may influence heat resistance, and Mackey and Bratchell (1989) state that *L. monocytogenes* heated in beef with 30% fat shows a D-value at 62.8°C of 1.5 minutes; approximately twice the D-value of *L. monocytogenes* in milk. Finally, curing salts have been shown to increase the heat resistance of *L. monocytogenes*. Mackey *et al.* (1990) demonstrated that the addition of curing salts (200 ppm nitrite, 300 ppm nitrate and 3.5% w/v salt, calculated on the lean fraction) to beef or tryptic soy broth markedly increased heat resistance. Excellent reviews on the heat resistance of *L. monocytogenes* in meat and meat products have been published by Farber (1991), Farber and Peterkin (1991), and Mackey and Bratchell (1989).

of *L. monocytogenes* in meatballs. The meatballs contained beef, eggs, bread, onion, garlic, salt, and spices. These were heated on a grill at 110° to 120°C for 15 minutes, by which time the centre of the meatballs had reached 78° to 85°C. All samples which contained an initial inoculum of 10^4 to 10^5 cells/ gram were positive for *L. monocytogenes* after cooking, whereas 12.5% of samples containing an initial inoculum of 10^2 to 10^3 cells/g were positive for the organism after cooking. Gaze *et al.* (1989) undertook a study to determine the destruction rate of *L. monocytogenes* in beef steak homogenate. They found that the D-values at 60°C for 2 strains of *L. monocytogenes* were 8.32 minutes (strain Scott A) and 6.27 minutes (strain 11994). At 70°C, the D-values were 0.20 minutes (strain Scott A) and 0.14 minutes (strain 11994). Boyle *et al.* (1990) examined the destruction of *L. monocytogenes* in a beef slurry and a beef homogenate. In the beef slurry, the D_{60} value was 2.54 minutes and the D_{70} value was 0.23 minutes. These authors also found that heating ground beef inoculated with strain Scott A that had been stored at 4°C for 48 hours, to internal temperatures of 50°, 60°, and 65°C, resulted in 0.4 to 0.6, 2.1 to 2.3, and 4.6 to 5.5 $\log_{10}$ reduction, respectively, from the initial inoculum level of 7.84 $\log_{10}$ cfu/g. Fain *et al.* (1991) examined the destruction rate of *L. monocytogenes* in lean (2% fat) and fatty (30.5% fat) ground beef. They found that the D-values for *L. monocytogenes* suspended in lean ground beef heated in Thermal Death Time (TDT) tubes at 52°, 57°, and 63°C were 81.3, 2.6, and 0.6 minutes, respectively. In fatty ground beef the D-values were 71.1, 5.8, and 1.2 minutes at 52°, 57°, and 63°C, respectively. These results indicate that beef fat has a protective effect on *L. monocytogenes* at 57° and 63°C, but not at 52°C.

The survival rate of *L. monocytogenes* in chicken meat has also been examined. Gaze *et al.* (1989) found that the z-values of 2 strains of *L. monocytogenes* in chicken were greater than they were in beef steak. The z-values that they reported were 6.72°C

monocytogenes heated in beef and in chicken. The inactivation of *L. monocytogenes* on chicken heated by microwave has also been examined (Lund *et al.*, 1989). *L. monocytogenes* was able to survive the microwave process used and the organism was isolated from the birds which had undergone the holding period in the recommended cooking procedure. The authors attribute the presence of the organism on the cooked chicken to uneven cooking, resulting in "cold spots". The standing step was important to allow the heat to be evenly distributed through the bird, however it appears that the final temperature after standing was not sufficient to inactivate *L. monocytogenes*. Fain *et al.*(1991) examined the destruction rate of *L. monocytogenes* in a turkey emulsion. Despite technical difficulties experienced by the authors, they calculated the D-value at 56.9°C to be 0.35 minutes. *L. monocytogenes* was found to survive a moist heating method in chicken breasts (Harrison and Carpenter, 1989b). Subsequent storage of the vacuum packaged chicken and chicken wrapped in oxygen permeable film at 4° and 10°C resulted in increases in the cell population at both storage temperatures. The final internal temperature of the breasts ranged from 65.6°C (an undercooked chicken) to 82.2°C (overcooked meat).

A comparison of the data is difficult to make, because of variations in experimental protocol, however, from the data, general conclusions about factors affecting the heat resistance of *L. monocytogenes* in meats and meat products can be made. The destruction rate of *L. monocytogenes* in meat depends upon the strain of *L. monocytogenes*, the cooking method, the animal from which the meat came, the amount of fat in the meat, and the temperature of heating. The National Advisory Committee on Microbiological Criteria for Foods (Brown, 1990) recommends a minimum process resulting in a 4D reduction in *L. monocytogenes* for meat and poultry products with an extended refrigerated shelf life, which are ready to eat or are prepared

with little or no additional heat treatment. This recommendation may result in small numbers of organisms being able to survive the heating treatment, particularly in microwave cooked products, and the organism may be able to multiply during storage at refrigeration temperatures.

1.5.2 Heat resistance of *L. monocytogenes* in vegetables

Very little work has been done examining the heat resistance of *L. monocytogenes* in vegetables, possibly because many vegetables are either eaten raw or after severe heat treatments, such as boiling or canning. As well, it appears that very few vegetables are contaminated with *L. monocytogenes*, and that some vegetables produce bacteriostatic compounds (Gaze *et al.*, 1989, Nguyen-the and Lund, 1991). Beuchat *et al.* (1986) examined the effect of pH on the growth and thermal inactivation of *L. monocytogenes* in cabbage and cabbage juice. They found that while the populations of 2 strains of *L. monocytogenes* (Scott A and LCDC 81-861) decreased slightly during storage at 5°C in sterilized cabbage, the population of strain LCDC 81-861 increased with storage in raw cabbage. The rate of inactivation of *L. monocytogenes* in clarified cabbage juice was affected by pH. At pH 5.6, heating the juice for 60 minutes at 50°C resulted in only slight decreases in the cell population, but heating at 58°C for 10 minutes resulted in no viable cells. At pH 4.0, heating the juice for 30 minutes at 50°C resulted in no viable cells, as did heating for 10 minutes at 52°C.

1.5.3 Heat resistance of *L. monocytogenes* in ravioli, crab meat and egg

The ability of *L. monocytogenes* to survive the cooking process for ravioli has been examined (Beuchat and Brackett, 1989). While the organism was found to grow in egg, meat, and cheese ravioli at 5°C, the process of boiling the ravioli for 3, 5, or 7 minutes was sufficient to reduce a population of 3×10^5 organisms/ g to non-detectable

levels. Farber and Peterkin (1991) have reported that *L. monocytogenes* has a D_{60} value in raw whole egg of 1.46 minutes, and a D_{60} value of 2.61 minutes in crab meat.

1.5.4 Heat resistance of *L. monocytogenes* in dairy products

There have been several papers reviewing the heat resistance of *L. monocytogenes* in milk (Doyle, 1988; El-Gazzar and Marth, 1991 Farber, 1989; Farber and Peterkin, 1991; Mackey and Bratchell, 1989;). Most of the data indicate that the organism cannot survive HTST pasteurization (71.7°C for 15 seconds) although there are some reports indicating otherwise. Fernandez Garayzabal *et al.* (1987) found that *L. monocytogenes* survived pasteurization in a plate heat exchanger at 72°C for 15 seconds. For the organism to be detected, a period of resuscitation was necessary, either in an enrichment broth, or using primary enrichment. Doyle *et al.* (1987), also using a plate heat exchanger, found that *L. monocytogenes* survived pasteurization in 6 of 9 trials when pasteurization occurred between 71.7° and 73.9°C for 16.4 seconds. Despite the results found by these authors, pasteurization at 71.7°C for 15 seconds is generally accepted to be adequate to inactivate *L. monocytogenes*, and most authors have found the $D_{71.7}$ value for the organism to be between 0.9 and 2.7 seconds, depending upon the strain examined, when using the sealed tube method. Farber (1991) recommends that further work be undertaken to examine the influence of the heat shock response, growth at elevated temperatures and anaerobic recovery on the survival of *L. monocytogenes* after pasteurization.

Spray drying of milk reduces the population of *L.monocytogenes* in skim milk. Drying with an inlet temperature of 165°C and an outlet temperature of 67°C resulted in $\log_{10}$ cell reductions of 1-1.5 $\log_{10}$ *L. monocytogenes*/ g; and storage at 25°C results in yet further reductions in cell numbers (Doyle, *et al.*, 1985).

Because of the intracellular nature of *L. monocytogenes*, several authors have hypothesized that it may be able to survive pasteurization when it is within leukocytes.

Bunning *et al.* (1986) indicated that *L. monocytogenes* suspended in mouse phagocytes and heated in raw bovine milk had a $D_{71.7}$ value of 1.9 seconds (measured using a slug-flow heat exchanger). Doyle *et al.* (1987), using milk from cows artificially infected with *L. monocytogenes* Scott A found, conversely, that six of nine trials were positive for the organism when heated at 71.7° to 73.9°C for 16.4 seconds. Farber *et al.* (1988) found that high temperature, short time (HTST) pasteurization (above 69°C for 16.2 seconds) resulted in no viable *L. monocytogenes* from naturally or artificially contaminated milk. The initial intracellular populations were similar in both the Doyle *et al.* (1987) and the Farber *et al.* (1988) experiments. Bunning *et al.* (1988) also examined the survival of *L. monocytogenes* suspended within bovine milk phagocytes and found that the organism could not survive vat pasteurization ($D_{62.8}$ = 53.8 seconds), but that HTST pasteurization could result in survivors if initial counts were high ($D_{71.7}$ = 4.1 seconds). Their results indicate that the vat pasteurization method has a 33.4 D margin of safety, while the HTST pasteurization method only has a 3.7 D margin of safety.

1.6 Thermal destruction of bacteria

1.6.1 Introduction

The thermal destruction of bacteria is dependent upon a number of factors. Among the more important are: the type and strain of the organism, the type of heat applied to the system, the growth conditions, the heating menstruum, the stage of growth and density of the organism, and the conditions used to determine survival.

The type and strain of bacteria has an effect upon the resistance of the bacteria to heat. Bacteria have been divided into three groups, referred to as the psychrophiles, mesophiles, and thermophiles, and generally speaking, the thermophiles are more heat resistant than are the mesophiles, which are, in turn, more thermotolerant than the psychrophiles. The strain of a bacterium also has a small effect on the heat resistance.

For example, *L. monocytogenes* Scott A in chicken meat has a D_{60} value of 5.29 minutes. Strain 1194 has a D_{60} value of 5.02 minutes. The z-values were 6.72°C and 7.39°C, respectively (Gaze *et al.*, 1989).

The type of heat applied to bacteria also affects their destruction rate. In general, moist heat is more destructive to microorganisms than is dry heat. Survival of microorganisms after cooking in a microwave may be due to uneven heat distribution. An examination of the destruction of *L. monocytogenes* on chicken breasts determined that the organism was able to survive cooking in a microwave when the internal temperature reached 73.9°C (Harrison and Carpenter, 1989a).

The growth conditions also play a role in thermal inactivation of bacteria. Growth conditions which may affect bacterial heat resistance include the temperature of growth, the water activity (a_w) of the medium, the permeability of solutes to the cell, the percentage of lipid in the growth medium, and the presence of magnesium ions in the medium. As the temperature of growth decreases, the heat resistance of the cell decreases, but, Smith and Marmer (1991) found that stationary phase *L. monocytogenes* that had been grown at low temperatures would acquire the heat resistance of cells grown at 37°C after exposure to 37°C for 5 hours. There is not a direct relationship between a_w and heat resistance; the thermotolerance of the cell depends upon the solute used to decrease the water activity of the system and the bacteria under study. For example, if glucose or salt are used to decrease the water activity of tryptic soy broth, *E. coli* and *P. fluorescens* will show increased heat resistance, but *S. aureus* will not (Calhoun and Frazer, 1966). Magnesium increases the heat resistance of cells. This may be due to the role of magnesium in the stabilization of ribosomes, or its effect on lipopolysaccharide (LPS) and teichoic acid. Thus, growth of bacteria in a medium free from this cation would result in easily destabilized ribosomes and loss of LPS or degraded teichoic acid (Hitchener and Egan, 1977, Hurst, 1977).

The heating menstruum also affects the thermotolerance of the bacterial cell. Components which increase the heat resistance of microorganisms include: magnesium, lipids, and milk solids. The role of magnesium in cellular thermotolerance has been discussed above as a necessity for ribosomal stability and outer membrane and cell wall integrity. Lipids play a protective role during heating, as do milk solids. Components which may increase heat resistance include lowered water activity and pH. The influence of lowered water activity depends upon the permeability of the solute used to lower the a_w into the bacteria under examination. For example, exposure of *Salmonella oranienburg* to 10% salt in egg yolk before pasteurization increases the heat resistance of the organism (Corry, 1973). Many compounds which effectively reduce the heat resistance of microorganisms do so by reducing the pH of the heating medium. pH also affects heat resistance, and generally, when the pH is out of the range of pH 6.0-8.0, the heat resistance of the organism decreases. One exception is the study of Humphrey *et al.* (1991), who found that exposure to, and heating of, *S. typhimurium* in broth adjusted to above pH 9.0 with NaOH resulted in greater heat resistance than exposure and heating in broth of pH 7.0.

The stage of growth and cell density also influences the efficacy of heat treatment on bacterial cells. Cells in the exponential stage of growth are least heat resistant, while cells in the stationary phase display the greatest degree of thermotolerance. Because food research which focuses upon thermal destruction of bacterial cells is concerned with worst-case scenarios, most research in this area is done using stationary phase cells.

1.6.2 Bacterial injury

Injury is structural or metabolic damage to a cell, and is measured by the organism's inability to establish colonies on a selective medium, but not on a non-selective medium. When establishing a protocol for examining thermal injury, the

researcher must ensure that: a) there are large differences in viable counts between the selective and non-selective media after thermal injury, b) the thermal injury menstruum, heating time, and temperature result in minimal death but maximal injury, and c) the selective agar is inhibitory to injured cells but not uninjured cells (Tomlins and Ordal, 1976).

During heating, there are several effects on the microbial cell. Components of the cell which become damaged during heating include the cell barrier (cell wall or outer membrane and cytoplasmic membrane), ribosomal RNA (r-RNA), ribosomes, heat-labile proteins, and, to a small extent, DNA. Determining which, if any, of these components is the primary site of injury in the cell has proven difficult. Damage to one site in a cell may quickly lead to damage in another.

The cell wall and cytoplasmic membrane become damaged during heating. In the cell wall, teichoic acid is degraded during heating. This degradation releases D-alanine, which sequesters magnesium ions from the medium. Magnesium is necessary for ribosomal integrity. The cytoplasmic membrane is also damaged by heat, in both Gram-positive and Gram-negative organisms. Electron micrographs show that heating causes the formation of holes in the cytoplasmic membrane. In *E. coli*, LPS is lost from the outer membrane, and this loss is an indication of outer membrane damage, as is the loss of lipids from *S. typhimurium* (Hitchener and Egan, 1977). Reconstruction of the cytoplasmic membrane is vital to cell recovery, so that cells can rebuild intracellular pools of lost components.

Ribosomal RNA proportions change dramatically during heating. The 16-S r-RNA is totally destroyed, while the 23-S r-RNA undergoes minor alterations. It is believed that the 5-S r-RNA is not affected by mild heating (Genthner and Martin, 1990, Hurst, 1977, Hurst and Hughes, 1978, Tomlins and Ordal, 1976).

The degradation of the 16-S r-RNA markedly affects ribosomes, as it functions as the core of the 30-S subunit. The 50-S subunit of the ribosome undergoes a minor

alteration to a 47-S subunit. Some proteins which are part of the ribosome are extremely heat labile. Loss of these proteins has a great effect on cell recovery, because without them, the 50-S subunit cannot mature, and a 40-S particle accumulates.

Proteins other than ribosomal proteins are degraded during heating. Several enzymes are heat labile, many of which are dehydrogenases. Enzyme inactivation, however, is not seen as a major site of injury. During injury, the cell synthesizes the "heat shock proteins", transient proteins which are only synthesized at high temperatures or other conditions of stress. The roles of these heat shock proteins are still unknown. There may be no relationship between the heat shock proteins and increased thermotolerance, and extended production of heat shock proteins is lethal to the cell (Neidhardt and VanBogelen, 1987).

DNA is also affected by heat in some cells. For example, *Yersinia enterocolitica* DNA undergoes no change. Conversely, *E. coli* DNA, stored *in vivo* at 57°C, undergoes single strand breakage similar to those seen when the cell has been treated with gamma radiation. DNA damage due to heat is believed to be through the heat activation of nucleases in the cell, explaining why DNA *in vitro* is unaffected by mild heating (Hurst, 1984).

Injured cells have a longer lag phase during recovery than do non-injured cells. Many cell types have greater nutritional requirements for recovery, and thus require a complex recovery medium, however some types of cells, notably injured *Salmonella* species, recover better on minimal medium. Improved recovery on minimal medium may be best for repair of DNA single strand breaks (Tomlins and Ordal, 1976). The first site of repair must be the cytoplasmic membrane, so that the cell can re-establish intracellular pools of components leaked during injury. Ribosomal RNA is also re-synthesized during the initial period of recovery. Ribosomal RNA synthesis is not the rate determining step of recovery, because recovery is not completed by the time r-RNA synthesis stops. Later in the recovery period, ribosomes are re-synthesized or are

synthesized *de novo*. Use of already present ribosomal particles by *S. typhimurium* will occur if the recovery medium is lacking in magnesium ions. Protein synthesis, including r-protein synthesis, occurs in the last stages of recovery, if protein synthesis is required for cell recovery. For example, Hurst (1984) reports that *Y. enterocolitica* requires only RNA synthesis for repair; no protein, DNA, or cell wall synthesis is required for recovery. *S. typhimurium* and *E. coli* both require protein synthesis for complete recovery.

1.6.3 Tempering

One possible explanation for the survival of *L. monocytogenes* in pasteurized milk, which has begun to be explored is the possibility that *L. monocytogenes* can survive pasteurization temperatures if first exposed to a sub-lethal heat treatment. The first workers to show that this phenomenon could occur in a bacterial cell were Tsuchido, Takano and Shibasaki (1974). They exposed *Escherichia coli* to various temperatures (ranging from 0° to 45°C) before challenging the cells at 50°C for 20 minutes, and found that the higher the temperature of the sublethal heat treatment, the greater the number of survivors at 50°C. They also heated the *E. coli* to 50°C at different rates, and found that the slower the rate of heating, the greater the number of survivors. Not much more work was done on this topic until Mackey and Derrick (1986) reported that exposure of *Salmonella typhimurium* to a sublethal temperature (48°C) for 30 minutes resulted in increased survivors after the heating challenge at 50° to 59°C. Later, Mackey and Derrick (1987a) reported that the rate of heating also affects the number of survivors to the heat challenge at 50° to 59°C. A slower heating rate (0.6°C/ minute) resulted in greater numbers of survivors than a faster heating rate (10°C/ minute). Mackey and Derrick (1987b) also determined that *Salmonella thompson* displays increased thermotolerance in broth as well as food systems. The food systems chosen for study were: 10% and 40% reconstituted skim milk, whole

egg, and beef mince. Increased thermotolerance occurred after exposure to 48°C for 30 minutes in all samples, the increase in thermotolerance ranging from 2 to 10 fold, depending upon the menstruum. The authors did not propose any explanation for these findings.

To this point, the bacterial species examined for increased thermotolerance were all Gram-negative. Then, the Italian researchers, Quintavalla *et al.* (1988) determined that the rate of heating to 65°C of *Streptococcus faecium*, a Gram-positive organism, markedly influenced the D-value. The D_{65} value found for cells heated instantaneously was 5.4 minutes. Cells heated at 0.48°C/ minute had a D_{65} of 27.8 minutes, while cells heated at 0.13°C/ minute had a D_{65} of 42.9 minutes, 5 and 8 times greater, respectively, than the instantaneously heated cells. Of interest is their finding that lower initial cell concentrations (10^4 and 10^2 cells/ mL) result in even greater D-values when heated at 0.13°C/ minute, compared with both the higher cell concentration and the instantaneously heated cells (D_{65}=134.2 minutes for 10^2 cells/ mL, D_{65}=1225.7 minutes for 10^4 cells/ mL).

Listeria has also been examined to determine whether or not it can develop transient increased thermotolerance. Quintavalla and Barbuti (1989), as well as Fedio and Jackson (1989), determined that *Listeria* species will acquire increased thermotolerance when exposed first to a sublethal heat treatment. Fedio and Jackson (1989) also established that *L. monocytogenes* acquires increased thermotolerance in 2% UHT milk. At this time, Fedio and Jackson (1989) suggested the term "temper" be used to denote the phenomenon of heat-induced elevation of thermotolerance. Farber and Brown (1990) determined that *L. monocytogenes* will not only become tempered in sausage meat, but will also remain tempered after storage at 4°C for 24 hours. Bunning *et al.* (1990) repeated the work of Mackey and Derrick (1986) and Fedio and Jackson (1989) and confirmed that tempering increases thermotolerance in *L. monocytogenes* and *S. typhimurium*. Linton *et al.* (1990) examined the acquisition of

thermotolerance by logarithmic phase *L. monocytogenes*. The cells were exposed to 40°, 44°, or 48°C for up to 20 minutes, followed by a heat challenge at 55°C for 50 minutes. The authors found that, for logarithmic phase cells, the optimum condition for tempering was 48°C for 20 minutes. Linton *et al.* (1990) concluded that logarithmic phase *L. monocytogenes* become tempered, and they recommended that future experiments should examine the effect of heating rate upon increased thermotolerance. Soon after, a paper was published on the effect of heating rate and menstruum on heat resistance in *L. monocytogenes* (Quintavalla and Campanini, 1991). *L. monocytogenes* survived better after challenging if heated in meat than if heated in broth, and the heating rate again affected thermotolerance. A slow rate of heating resulted in more survivors after challenging than did a fast heating rate.

Smith and Marmer (1991) examined the effect of growth temperature on heat tolerance of *L. monocytogenes*. The lower the temperature of growth, the less thermotolerant the cells were. Protein synthesis appeared to be involved in thermotolerance, although the role of the synthesized proteins was not determined. The temperatures used in the experiment were all within the normal range of growth for the organism (10°, 19°, 28°, and 37°C). The authors found that stationary phase cells grown at the lower temperatures (10° or 19°C), when held at 37°C for 5 hours, acquired the same level of thermotolerance as cells grown at 37°C. Cells grown at 28°C were slightly less thermotolerant than cells that were grown at 28°C then held at 37°C before challenging at 52°C.

Recently, Whitaker and Batt (1991) added to the known genera of bacteria which display increased thermotolerance, demonstrating the phenomenon in *Lactococcus lactis* subsp. *lactis*. In this study, 13 proteins were found to be synthesized in greater quantities at 42°C than at 30°C, determined by two-dimensional polyacrylamide gel electrophoresis. The authors hypothesized that the proteins are related to increased thermotolerance, but the roles of the proteins are unknown.

1.7 Statement of objectives

The objective of this study was to examine some factors which may influence the acquisition and retention of increased thermotolerance in *L. monocytogenes*. The results are presented in 3 chapters, and a statement of conclusions.

1.8 References

Beuchat, L. R. and R. E. Brackett. 1989. Observations on survival and thermal inactivation of *Listeria monocytogenes* in ravioli. *Letters in Applied Microbiology* **8**: 173- 175

Beuchat, L. R., R. E. Brackett, D. Y.-Y. Hao and D. E. Conner. 1986. Growth and thermal inactivation of *Listeria monocytogenes* in cabbage and cabbage juice. *Canadian Journal of Microbiology* **32**: 791- 795

Boyle, D. L., J. N. Sofos and G. R. Schmidt. 1990. Thermal destruction of *Listeria monocytogenes* in a meat slurry and in ground beef. *Journal of Food Science* **55**: 327- 329

Brown, W. L. 1990. Guidelines for *Listeria monoctogenes* thermal inactivation studies for extended shelf-life pre-cooked refrigerated foods. Poster presented at the Institute of Food Technologists Annual Meeting, Anaheim

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. *Applied and Environmental Microbiology* **52**: 1398- 1402

Bunning, V. K., R. G. Crawford, J. T. Tierney and J. T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella typhimurium* after sublethal heat shock. *Applied and Environmental Microbiology* **56**: 3216- 3219

Bunning, V. K., C. W. Donnelly, J. T. Peeler, E. H. Briggs, J. G. Bradshaw, R. G. Crawford, C. M. Beliveau and J. T. Tierney. 1988. Thermal inactivation of *Listeria monocytogenes* within bovine milk phagocytes. *Applied and Environmental Microbiology* **54**: 364- 370

Calhoun, C. L. and W. C. Frazier. 1966. Effect of available water on thermal resistance of three nonsporeforming species of bacteria. *Applied Microbiology* **14**: 416- 420

Corry, J. E. L. 1973. The water relations and heat resistance of microorganisms. **In:** D. J. D. Hockenhull (ed.), Progress in industrial microbiology, Vol. 12 Churchill Livingstone, London

Dominguez Rodriguez, L., J. F. Fernandez Harayzabal, J. A. Vazquez Boland, E. Rodriguez Ferri and G. Suarez Fernandez. 1985. Isolation de micro-organismes du genre *Listeria* á partir de lait cru destiné a la consommation humaine. *Canadian Journal of Microbiology* **31**: 938- 941

Donnelly, C. W., E. H. Briggs and G. J. Baigent. 1986. Analysis of raw milk for the epidemic serotype of *Listeria monocytogenes* linked to an outbreak of listeriosis in California. *Jounal of Food Protection* **49**: 847

Doyle, M. P. 1988. Effect of environmental and processing conditions on *Listeria monocytogenes*. *Food Technology* April, 169- 171

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. *Applied and Environmental Microbiology* **53**: 1433- 1438

Doyle, M. P., L. M. Meske, and E. H. Marth. 1985. Survival of *Listeria monocytogenes* during the manufacture and storage of nonfat dry milk. *Journal of Food Protection* **48**: 740- 742

Doyle, M. P. and J. L. Schoeni. 1986. Comparison of methods for detecting *Listeria monocytogenes* in cheese. *Journal of Food Protection* **49**: 849

El-Gazzar, F. E. and E. H. Marth. 1991. *Listeria monocytogenes* and listeriosis related to milk, milk products and dairy ingredients: a review I. *Listeria monocytogenes*, listeriosis and responses of the pathogen to environmental conditions. *Milchwissenschaft* **46**: 14- 19

Fain, A. R. Jr., J. E. Line, A. B. Moran, L. M. Martin, R. V. Lechowich, J. M. Carosella and W. L. Brown. 1991. Lethality of heat to *Listeria monocytogenes* Scott A: D-value and z-value determinations in ground beef and turkey. *Journal of Food Protection* **54**: 756- 761

Farber, J. M. 1989. Thermal resistance of *Listeria monocytogenes* in foods. *International Journal of Food Microbiology* **8**: 285- 291

Farber, J. M. and B. E. Brown. 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. *Applied and Environmental Microbiology* **56**: 1584- 1587

Farber, J. M. and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews* **55**: 476- 511

Farber, J. M., G. W. Sander and S. A. Malcolm. 1988. The presence of *Listeria* species in raw milk in Ontario. *Canadian Journal of Microbiology* **34**: 95- 100

Farber, J. M. 1991. *Listeria monocytogenes. Journal of the Association of Official Analytical Chemists* **74**: 701- 704

Fedio, W. M. and H. Jackson. 1989. Effect of tempering on the heat resistance of *Listeria monocytogenes. Letters in Applied Microbiology* **9**: 157- 160

Fenlon, D. R. 1985. Wild birds and silage as reservoirs of *Listeria* in the agricultural environment. *Journal of Applied Bacteriology* **59**: 537- 543

Fernandez Garayzabal, J. F., L. Dominguez Rodriguez, J. A. Vazquez Boland, J. L. Blanco Cancelo and G. Suarez Fernandez. 1986. *Listeria monocytogenes* dans le lait pasteurisé. *Canadian Journal of Microbiology* **32**: 149- 150

Fernandez Garayzabal, J. F., L. Dominguez Rodriguez, J. A. Vazquez Boland, E. F. Rodriguez Ferri, V. Briones Dieste, J. L. Blanco Cancelo and G. Suarez Fernandez. 1987. Survival of *Listeria monocytogenes* in raw milk treated in a pilot plant size pasteurizer. *Journal of Applied Bacteriology* **63**: 533- 537

Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audrier, C. V. Broome and A. L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *New England Journal of Medicine* **312**: 404- 407

Gaze, J. E., G. D. Brown, D. E. Gaskell and J. G. Banks. 1989. Heat resistance of *Listeria monocytogenes* in homogenates of chicken, beef steak and carrot. *Food Microbiology* **6**: 251- 259

Genthner, F. J. and S. E. Martin. 1990. Ribosome assembly in *Salmonella typhimurium* 7136 during recovery from heat shock. *Food Microbiology* **7**: 53- 67

Hallström, B., C. Skjöldebrand and C. Trägardh. 1988. Thermal processing equipment. **In:** Heat transfer and food products. Elsevier Applied Science Publishers Ltd New York, NY

Harrison, M. A. and S. L. Carpenter. 1989a. Survival of *Listeria monocytogenes* on microwave cooked poultry. *Food Microbiology* **6**: 153- 157

Harrison, M. A. and S. L. Carpenter. 1989b. Survival of large populations of *Listeria monocytogenes* on chicken breasts processed using moist heat. *Journal of Food Protection* **52**: 376- 378

Heisick, J. E., D. E. Wagner, M. L. Nierman and J. T. Peeler. 1989. *Listeria* spp. found on fresh market produce. *Applied and Environmental Microbiology* **55**: 1925- 1927

Hitchener, B. J. and A. F. Egan. 1977. Outer-membrane damage in sublethally heated *Escherichia coli* K-12. *Canadian Journal of Microbiology* **23**: 311- 318

Humphrey, T. J., N. P. Richardson, A. H. L. Gawler and M. J. Allen. 1991. Heat resistance of *Salmonella enteritidis* PT4: the influence of prior exposure to alkaline conditions. *Letters in Applied Microbiology* **12**: 258- 260

Hurst, A. 1977. Bacterial injury: a review. *Canadian Journal of Microbiology* **14**: 416- 420

Hurst, A. 1984. Revival of vegetative bacteria after sublethal heating. **In:** M. H. E. Andrew and A. D. Russell (ed.) The revival of injured microbes. Academic Press, Toronto

Hurst, A. and A. Hughes. 1978. Stability of ribosomes of *Staphylococcus aureus* S6 sublethally heated in different buffers. *Journal of Bacteriology* **133**: 564- 568

Johnson, J., M. P. Doyle and R. G. Cassens. 1986. Survival of *Listeria monocytogenes* in ground beef. *Journal of Food Protection* **49**: 847

Johnston, M. A., A. Loit, U. Purvis and J. Farber. 1986. Surveillance of soft and semi-soft cheese for *Listeria*. *Journal of Food Protection* **49**: 847

Karaioannoglou, P. G. and G. C. Xenos. 1980. Survival of *Listeria monocytogenes* in meatballs. *Hellenic Veterinary Medicine* **23**: 111- 117

Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, A. Audrier, B. D. Plikaytis, S. L. Fannin, A. Kleks and C. V. Broome. 1988. Epidemic listeriosis associated with Mexican-style cheese. *New England Journal of Medicine* **319**: 823- 828

Linton, R. H., M. D. Pierson and J. R. Bishop. 1990. Increase in heat resistance of *Listeria monocytogenes* Scott A by sublethal heat shock. *Journal of Food Protection* **53**: 924- 927

Lovett, J., D. W. Francis and J. M. Hunt. 1987. *Listeria monocytogenes* in raw milk: detection, incidence, and pathogenicity. *Journal of Food Protection* **50**: 188- 192

Lund, B. M., M. R. Knox and M. B. Cole. 1989. Destruction of *Listeria monocytogenes* during microwave cooking. *Lancet* January 28, 1989, pg. 218

Mackey, B. M. and N. Bratchell. 1989. The heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology* **9**: 89- 94

Mackey, B. M. and C. M. Derrick. 1986. Elevation of the heat resistance of *Salmonella typhimurium* by sublethal heat shock. *Journal of Applied Bacteriology* **61**: 389- 393

Mackey, B. M. and C. M. Derrick. 1987a. Changes in the heat resistance of *Salmonella typhimurium* during heating at rising temperatures. *Letters in Applied Microbiology* **4**: 13- 16

Mackey, B. M. and C. M. Derrick. 1987b. The effect of prior heat shock on the thermoresistance of *Salmonella thompson* in foods. *Letters in Applied Microbiology* **5**: 115- 118

Mackey, B. M., C. Pritchet, A. Norris and G. C. Mead. 1990. Heat resistance of *Listeria*: strain differences and effects of meat type and curing salts. *Letters in Applied Microbiology* **10**: 251- 255

Neidhardt, F.C. and R. A. VanBogelen. 1987. Heat shock response. p. 1334-1345 **In**: F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger (ed.) *Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology. American Society for Microbiology, Washington

Nguyen-the, C. and B. M. Lund. 1991. The lethal effect of carrot on *Listeria* species. *Journal of Applied Bacteriology* **70**: 479- 488

Northolt, M. D., H. J. Beckers, U. Vecht, L. Toepoel, P. S. S. Soentoro and H. J. Visselink. 1988. *Listeria monocytogenes*: heat resistance and behavior during storage of milk and whey and making of Dutch types of cheese. *Netherlands Milk Dairy Journal* **42**: 207- 219

Quintavalla, S. and S. Barbuti. 1989. Caractérisation microbiologique et résistance thermique de *Listeria* isolée de produits de viande. *Sciences des Aliments* **9**: 125- 128

Quintavalla, S. and M. Campanini. 1991. Effect of rising temperature on the heat resistance of *Listeria monocytogenes* in meat emulsion. *Letters in Applied Microbiology* **12**: 184- 187

Quintavalla, S., M. Campanini and L. Miglioli. 1988. Effetto della velocita di riscaldamento sulla resistenza termica de *Streptococcus faecium*. *Industria Conserve* **63**: 252

Ryser, E. T., E. H. Marth and M. P. Doyle. 1985. Survival of *Listeria monocytogenes* during manufacture and storage of cottage cheese. *Journal of Food Protection* **48**: 746- 750

Seeliger, H. P. R. and D. Jones. 1986. Genus *Listeria* Pirie. p. 1235- 1245 **In:** P. H. A. Sneath, N. S. Mair and E. Sharp (ed.) Bergey's manual of systematic bacteriology, 7th edition, volume 2. The Williams & Wilkins Co., Baltimore

Slade, P. J., D. L. Collins-Thompson and F. Fletcher. 1988. Incidence of *Listeria* species in Ontario raw milk. *Canadian Institute of Food Science and Technology Journal* **21**: 425- 429

Smith, J. L. and B. S. Marmer. 1991. Temperature shift effects on injury and death in *Listeria monocytogenes* Scott A. *Journal of Food Safety* **11**: 73- 80

Tomlins, R. I. and Z. J. Ordal. 1976. Thermal injury in bacteria. **In:** F. A. Skinner and W. B. Hugo (ed.) Inhibition and inactivation of vegetative microbes. Academic Press, London

Tsuchido, T., M. Takano and I. Shibasaki. 1974. Effect of temperature-elevating process on the subsequent isothermal death of *Esherichia coli* K-12. *Journal of Fermentation Technology* **52**: 788- 792

Velani, S. and D. Roberts. 1991. *Listeria monocytogenes* and other *Listeria* spp. in prepackaged mixed salads and individual salad ingredients. *PHLS Microbiol. Digest* **8**: 21- 22

Warburton, D. W., J. M. Farber, A. Armstrong, R. Caldeira, N. P. Tiwari, T. Babiuk, P. Lacasse and S. Read. 1991. A Canadian comparative study of modified versions of the "FDA" and "USDA" methods for the detection of *Listeria monocytogenes*. *Journal of Food Protection* **54**: 669-676

Whitaker, R. D. and C. A. Batt. 1991. Characterization of the heat shock response in *Lactococcus lactis* subsp. *lactis*. *Applied and Environmental Microbiology* **57**: 1408- 1412

2. Stability of the tempered state in *Listeria monocytogenes* stored at restrictive temperatures, 0° and 4°C, and incubated at a non-restrictive temperature, 37°C[1]

2.1 Introduction

Following several outbreaks of listeriosis in North America and Europe associated with the consumption of milk and milk products, research scientists began to take a new look at the causative organism, *Listeria monocytogenes*, a Gram-positive, non-sporulating rod. Of particular concern was the apparent ability of the organism to survive the pasteurization process. For example, in the Massachusetts outbreak (Fleming *et al.*, 1983), in which 49 persons were affected, pasteurized milk was suspected to be the vehicle for infection, although pasteurization procedures appeared to be adequate. This led some researchers to speculate that *L. monocytogenes* could survive pasteurization if it was contained within bovine leukocytes as opposed to being freely suspended in milk. Bunning *et al.* (1988), however, found that *L. monocytogenes* suspended within bovine phagocytes was not significantly more heat resistant than freely suspended cells. One other possibility that could account for the presence of *L. monocytogenes* in pasteurized milk, apart from post pasteurization contamination, is the phenomenon of tempering.

The term "temper" was first proposed by Fedio and Jackson in 1989, and describes increased thermotolerance of bacterial cells resulting from a mild heat treatment prior to a severe heat challenge. Tsuchido, Takano and Shibasaki (1974) first examined the phenomenon of increased thermotolerance in *Escherichia coli*, and found that as the temperature of the pre-treatment of the cells increased, so did the length of time that the cells were able to withstand an even higher temperature. They found that a

---

[1] A version of this chapter was presented in poster format at the 23rd International Dairy Congress, Montréal, Canada, 1990: Black, E. K. and H. Jackson, Stability of the tempering effect in *Listeria monocytogenes*.

heat treatment at 45°C for 10 minutes resulted in cells which were more heat resistant to a challenge of 50°C for 20 minutes than untreated contols. They also found that heating the cell suspension to 50°C at a rate of 1.0°C/ min. from 0°C resulted in more survivors than heating to 50°C at rates of 2.1°, 5.0°, 2.7°, or 750°C/ min (instantaneously). More than a decade later, Mackey and Derrick (1986, 1987a) showed that the same phenomenon occurred in *Salmonella typhimurium,* and later, *S. thompson.* They tempered *S. typhimurium* at temperatures ranging between 42° and 48°C for 30 minutes, then challenged the cells at 55°C for 25 minutes. They found that tempering at 48°C for 30 minutes resulted in the greatest number of survivors after the heating challenge. *S. thompson* was examined in several test systems: Tryptic Soy Broth (TSB), whole egg, 10% milk solids, 40% milk solids, and minced beef. The cells were tempered for 30 minutes between the temperatures of 42° and 48°C, and challenged at 54° or 60°C. Mackey and Derrick (1986, 1987a) found that the heat resistance of the tempered cells was similar in TSB, liquid whole egg, 10% reconstituted skim milk, and minced beef, while the heat resistance in 40% reconstituted skim milk was much greater. In all cases, the tempered cells were more heat resistant than the untempered cells. For example, untempered *S. thompson* had a D_{60} value of 0.46 minutes in minced beef, while the tempered cells had a D_{60} value of 1.26 minutes. Mackey and Derrick (1987b) also showed that a slow heating rate to the heat challenge temperature resulted in greater numbers of survivors than did a faster heating rate. Cells were heated at rates between 0.6° and 38°C/ min. to 52°, 55°, or 59°C then held at that temperature for 25 minutes. Cells which were heated at a rate of 5°C/ min. or greater showed little change in heat resistance relative to cells heated instantaneously, whereas cells heated at the slowest rate (0.6°C/ min.) required a challenge that was 2 to 3 times longer than instantaneously heated cells to decrease the population by 99%. Quintavalla et *al.* (1988) showed that the heating rate also affects the thermotolerance of a Gram-positive organism, *Streptococcus faecium.* The D_{65}

values for cells heated at 0.13° and 0.48°C/ min. were 42.9 and 27.8 minutes, respectively. Instantaneously heated cells had a D_{65} value of 5.4 minutes.

The first studies on tempering in *L. monocytogenes* were reported in 1989 (Fedio and Jackson, 1989; Quintavalla and Barbuti, 1989). Fedio and Jackson (1989) tempered *L. monocytogenes* in TSB plus 0.6% Yeast Extract (TSBYE) or 2% UHT treated milk at 48°C for 1 hour. Tempered cells survived a challenge of 60°C better than did untempered cells. Quintavalla and Barbuti (1989) conducted similar experiments using both *L. innocua* and *L. monocytogenes*. They tempered the organisms at 45°C for 30 minutes, then examined the time required to reduce the cell population by 7 log cycles at 65°C. A 7 log cycle reduction in *L. innocua* required 14.3 minutes, and *L. monocytogenes* required 10.5 minutes. Untempered cells of *L. innocua* and *L. monocytogenes* had a 7 log reduction after 3.64 minutes. Following the work of these authors, other workers began to examine the effect of the tempering and challenging menstrua upon the destruction rate of tempered *L. monocytogenes*. Quintavalla and Campanini (1990) and Farber and Brown (1990) found that *L. monocytogenes* was capable of acquiring increased heat resistance in a meat product.

Questions arose about the stability of the tempering effect, *i.e.*, how long does the increased thermotolerance persist and how is the persistence affected by storage conditions? Smith and Marmer (1991) found that *L. monocytogenes* grown at 10°, 19°, or 28°C were less heat resistant compared to cells grown at 37°C, when challenged at 52°C. They found that even a short exposure to 37°C resulted in the cells exhibiting thermotolerance similar to cells grown at 37°C. Cells grown at 19°C which were exposed to 37°C for one hour were significantly more thermotolerant than cells grown at 28°C but not exposed to 37°C before challenging. Humphrey (1990) found that storage at 4° or 8°C of *Salmoneila enteritidis* phage type 4 grown at 37°C resulted in decreased heat resistance. The decrease was observed within one hour of storage at 4°C, and the longer the cells were kept at this temperature, the lower the D-value at

55°C. Farber and Brown (1990) examined sausage meat in which *L. monocytogenes* had been tempered for 2 hours at 48°C. The inoculated and tempered meat was stored at 4°C for 24 hours before challenging the cells at 64°C for up to 8 minutes. After storage at the refrigeration temperature for 24 hours, tempered *L. monocytogenes* retained its increased thermotolerance.

The experiments presented here were undertaken to determine if *L. monocytogenes* would remain tempered when stored at restrictive temperatures of 0 and 4°C, and whether the effect was reversible at a non-restrictive temperature of 37°C. The studies were carried out in three liquid systems: TSBYE, pH adjusted TSBYE, and 2% UHT milk.

2.2 Materials and methods

2.2.1 Culture preparation

L. monocytogenes Scott A was used throughout the experiments. Stock cultures were stored on slants of Tryptic Soy Agar plus 0.6% Yeast Extract (TSAYE) (Becton Dickinson, Cockeysville, MD). Experimental cultures were produced by placing a loopful of the stock culture in 5 mL of Tryptic Soy Broth plus 0.6% Yeast Extract (TSBYE) (Difco Laboratories, Detroit MI, Becton Dickinson, Cockeysville, MD). The cell suspension was incubated at 37°C for 24 hours and shaken at 125 revolutions per minute (rpm) in a controlled environment incubator shaker (New Brunswick Scientific, NJ), after which time a 1 mL suspension was removed and pipetted into 225 mL of TSBYE in a 500 mL Erlenmeyer flask. The flask was incubated at 37°C for 24 hours and shaken at 125 rpm. After incubation, 1 mL of this suspension was removed and pipetted into 225 mL of TSBYE in a 500 mL Erlenmeyer flask. Again, the flask was incubated at 37°C for 24 hours at 125 rpm. Following this final incubation, the suspension was centrifuged at 6000 rpm for 15 minutes in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Inc., Missisauga, Ont.),

resuspended in the test broth, and re-centrifuged. The test broths were: TSBYE, 2% UHT milk (a commercially available brand) or pH 6.7 TSBYE adjusted to the desired pH with 12 N HCl. The cells were finally resuspended in 225 mL of test broth to give a final concentration of either 10^7 or 10^9 organisms/ mL. Up to 50 mL of this suspension was placed in 250 mL Erlenmeyer flasks. Suspensions of 10^7 organisms/ mL were only used in incubation studies at 37°C.

2.2.2 Tempering and storage at 0° and 4°C

Erlenmeyer flasks containing cell suspension were placed at 48°C for 1 hour, shaking at 125 rpm in a Metabolyte water bath shaker (New Brunswick Scientific, NJ). After tempering, the flasks were placed in a Hotpack incubator (Andor Scientific Services Ltd., Alberta) at 0° or 4°C and removed for challenging at appropriate intervals. The untempered cells were immediately placed at 0° or 4°C after dispensing into the Erlenmeyer flasks.

2.2.3 Challenging

Flasks of the stored cells were placed for 10 minutes into a controlled environment incubator shaker (New Brunswick Scientific, NJ) at 37°C. Twenty-five mL of the suspension was added to 225 mL of TSBYE preheated to 60°C. Samples were removed at intervals of 0, 4, 8, 12, 16, and 20 minutes of challenging, and diluted as appropriate in 0.1% peptone water (Difco Laboratories, Detroit, MI). The diluted samples were plated onto TSAYE and incubated at 37°C for 48 hours before enumerating the colony forming units.

2.2.4 Tempering and incubation at 37°C

The flasks with cell suspensions containing 10^7 or 10^9 organisms/ mL, were tempered for one hour at 48°C and shaken at 125 rpm in a Metabolyte water bath shaker. They were then placed in a controlled environment incubator shaker at 37°C,

shaken at 125 rpm, for up to 2 hours, before challenging at 60°C, as for the suspensions stored at 0° and 4°C. Samples were removed after 0, 10, and 20 minutes of exposure to 60°C, for the suspensions containing 10^9 organisms/ mL, and after 0, 6, and 12 minutes of exposure to 60°C, for the suspensions containing 10^7 organisms/ mL, and diluted in 0.1% peptone water. The dilutions were plated onto TSAYE. Plates were incubated at 37°C for 48 hours before enumerating the colony forming units.

2.2.5 Repetitions

All experiments were repeated a minimum of 2 times. The figures presented represent typical results. Duncan's Multiple Range Test was performed to determine statistical differences between treatments.

2.3 Results

The tempered cells stored at 0°C for one week in either broth or milk, remained more heat resistant when challenged at 60°C than the stored, untempered cells. Figures 2.1 and 2.2 show that as the storage period continued, the thermotolerance of all cells decreased, and after 7 days of storage in TSBYE and 2% UHT milk, the tempered cells had lost much of their increased heat resistance, but were still slightly more heat resistant than the original untempered culture.

When stored at 4°C, the cells remained tempered for a long period of time, irrespective of the tempering and storage menstruum. The cells stored in TSBYE (figure 2.3) decreased in thermotolerance gradually, until after day 11 of storage, they displayed the same heat resistance as the untempered control cells. In 2% UHT milk, even after 21 days of storage, the tempered cells remained more heat resistant than the untempered control cells (figure 2.4). In all cell suspensions, a gradual decline in initial cell populations was noted. The stored, untempered cells also decreased in

thermotolerance, and stored, tempered cells were always more thermotolerant than cells that were stored for the same length of time but not tempered.

One possible reason why the tempered cells stored in milk retained their increased thermotolerance longer than the cells stored in TSBYE may be the difference in pH between the two systems. Milk has a pH of 6.7, and TSBYE has a pH of 7.2. To examine this possibility, TSBYE was adjusted to pH 6.7 with 12 N HCl. Cells tempered and stored in TSBYE that was adjusted to pH 6.7 with hydrochloric acid (figure 2.5) showed a similar loss of tolerance to the cells stored in pH 7.2 TSBYE (figure 2.3). Initial decreases in cell populations were noted with storage.

The results of the experiment on incubation at 37°C for 30 to 120 minutes, using a suspension of 10^9 organisms/ mL (figures 2.6 and 2.7), show that the heat resistance of the cells tempered and incubated in either medium (TSBYE or 2% UHT milk) is highly significantly different after 20 minutes at 60°C from that of the tempered cells which were not incubated at 37°C. At the concentration of 10^7 organisms/ mL, it can be seen (figures 2.8 and 2.9) that the heat resistance of the tempered cells is significantly greater after 12 minutes than that of cells incubated at 37°C, and this was confirmed by statistical analysis. Although it would appear that incubation at 37°C leads to a progressive decline in heat resistance, this observation was not supported by statistical analysis.

2.4 Discussion

Irrespective of the storage medium, tempered *L. monocytogenes* retains enhanced heat resistance, in relation to untempered cells, for several days when stored at 0° or 4°C. Although tempered cells stored in TSBYE eventually lost increased thermotolerance, this process required at least one week. The stability of the tempering effect may be attributable to the slow metabolic activity of *L. monocytogenes* at the lower temperatures, which are close to minimum growth temperature, and also the high

initial cell population. The pH adjusted TSBYE still showed decreasing thermotolerance over the storage period, albeit a longer period of storage was required for the decline to be seen.

The heat resistance of the suspension of 10^9 organisms/ mL tempered and incubated at 37°C was highly significantly less after 20 minutes at 60°C than that of the tempered cells. The heat resistance of the tempered cell suspension of 10^7 organisms/ mL also was highly significantly different than that of the tempered and incubated cell suspension after challenging at 60°C. Bunning *et al.* (1990), found that *L. monocytogenes* heated to 42°C for 1 hour lost increased thermotolerance within one hour of being shifted back to 35°C, but retained increased heat resistance for up to 4 hours when held at 42°C. The results of this study are similar to those found by Bunning *et al.* (1990).

The results show that *L. monocytogenes* Scott A is able to become tempered in milk, broth, and pH adjusted broth, and that storage of the tempered cells at 0° and 4°C may enable the cells to remain tempered for a long period of time.

The reversibility of the tempering effect observed at 37°C is consistent with the view and the evidence (Fedio, 1991, Mackey and Derrick, 1990) that tempering in itself involves, at least in part, metabolic events. Fedio (1991) examined the effects of chloramphenicol, rifamycin, and 2,4-dinitrophenol on the acquisition of thermotolerance by *L. monocytogenes*. He found that, while the presence of any of these inhibitors partially restricted the acquisition of increased thermotolerance, the tempered cells in the presence of the inhibitors showed greater thermotolerance than untempered control cells. Inhibition by chloramphenicol indicates that *de novo* protein synthesis is required for the acquisition of thermotolerance, inhibition by rifamycin suggests that RNA synthesis is necessary to increase thermotolerance, and 2,4-dinitrophenol is an uncoupling agent, so its presence in the cells releases electron transport from respiratory control, indicating that energy yielding mechanisms are

required for increased thermotolerance. Mackey and Derrick (1990) found that chloramphenicol did not completely inhibit the acquisition of thermotolerance by *S. typhimurium*, and the addition of the inhibitor before a shift from 48°C to 37°C did not prevent the loss of resistance by the cells, indicating that protein synthesis is not required for the return of the cells to the pre-tempering state. In other words, tempered cells, modified in heat resistance by exposure to 48°C, will revert to their original heat resistance when shifted back to a permissive temperature of 37°C.

The practical significance of the results presented in this paper is difficult to evaluate in absolute terms. Clearly, tempering of *L. monocytogenes* leads to increased heat resistance, and this is maintained during extended periods of refrigerated storage. In addition, the circumstances for organisms to become "tempered" do exist in dairy processing plants, during such operations as product mixing, formulation and standardization. These two points together lead to the obvious question: "Could inadvertent tempering in dairy operations lead to the survival of *L. monocytogenes* in finished dairy products?" This question needs to be answered in terms of both minimal pasteurization treatments and sub-pasteurization heat treatments. It would appear improbable that the enhanced heat resistance derived from tempering, even under optimal conditions, would allow the organism to survive minimum regulatory pasteurization at 72°C for 15 seconds, based upon the observed D-values. In addition, it should be noted that recent observations in Alberta (K. Walden, Dairy Division, Alberta Agriculture, personal communication) show that most dairy processors use pasteurization time and temperature conditions considerably in excess of the minimum.

The ability of tempered *L. monocytogenes* to survive sub-pasteurization treatments, such as thermization as commonly used in cheesemaking is more problematical. The data presented here would indicate that it would be prudent to re-evaluate the efficacy of such processes in destroying *L. monocytogenes*, especially if the opportunity for tempering exists prior to heat treatment.

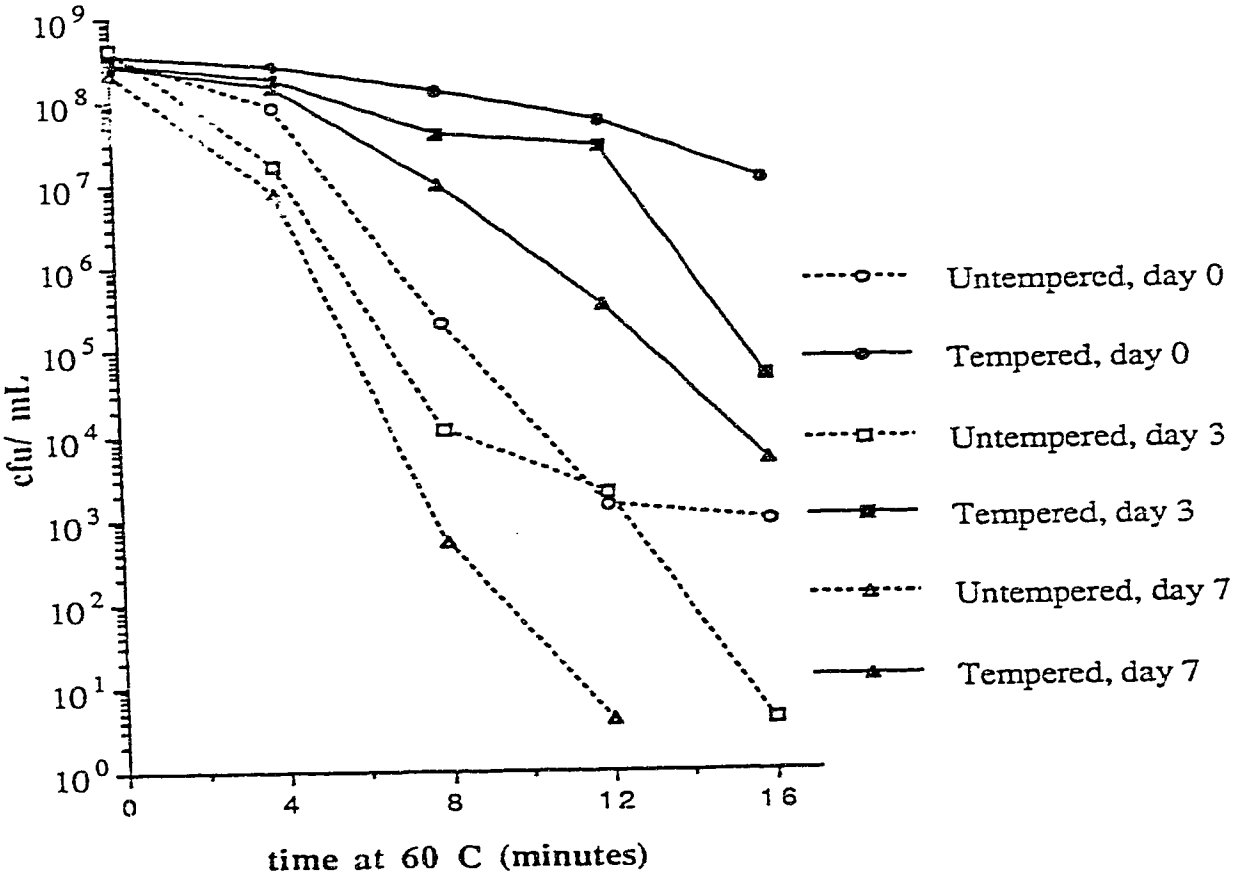

Figure 2.1: Effect of storage at 0°C in TSBYE on tempered and untempered *Listeria monocytogenes*

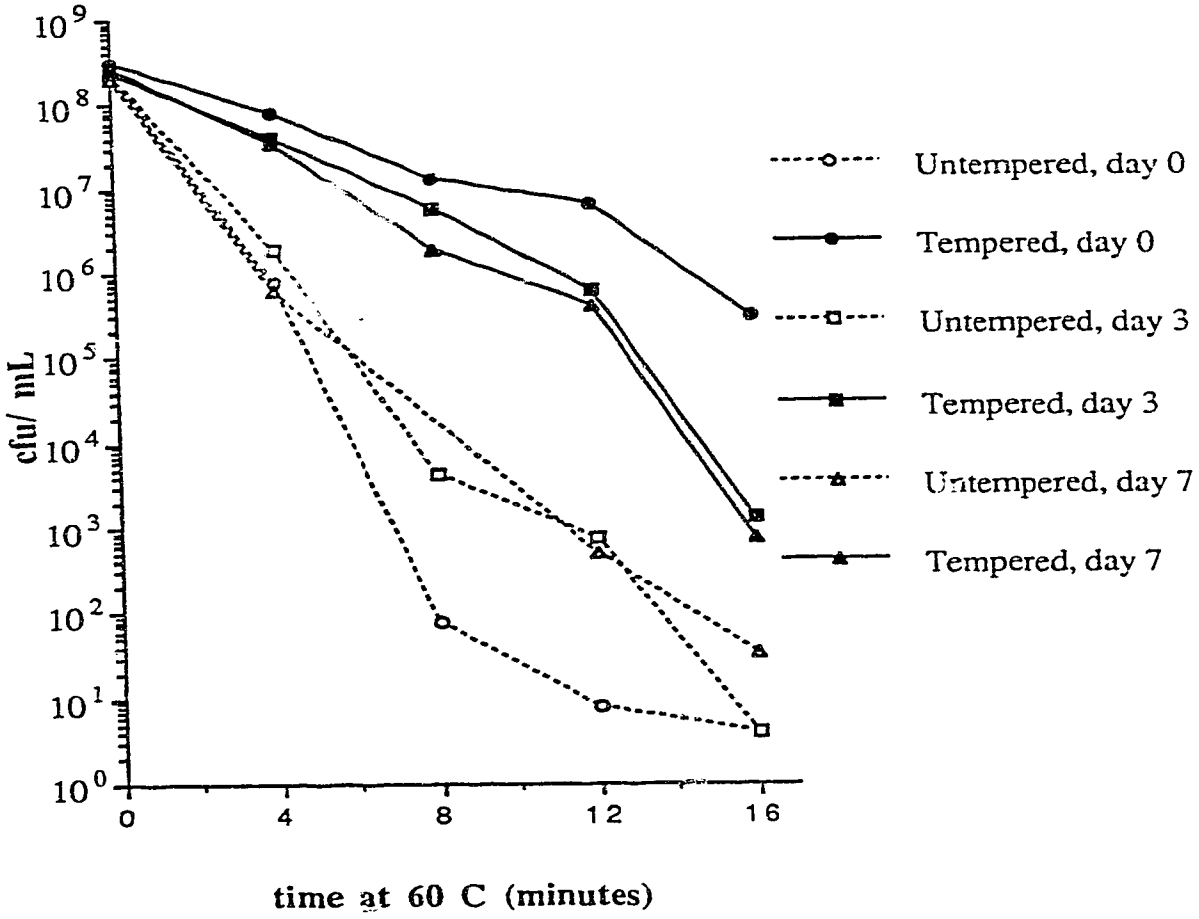

Figure 2.2: Effect of storage at 0°C in 2% UHT milk on tempered and untempered *Listeria monocytogenes*

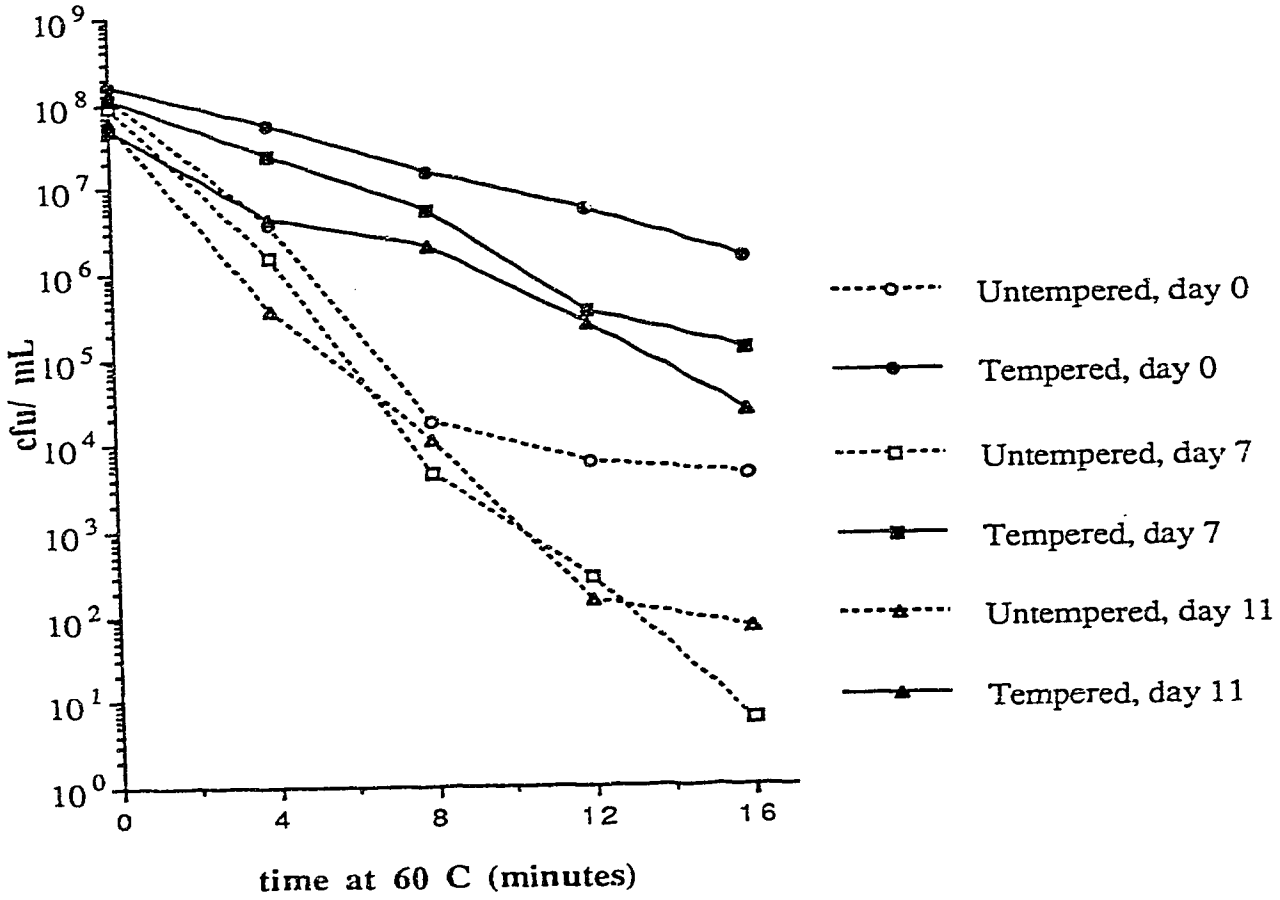

Figure 2.3: Effect of storage at 4°C in TSBYE on tempered and untempered *Listeria monocytogenes*

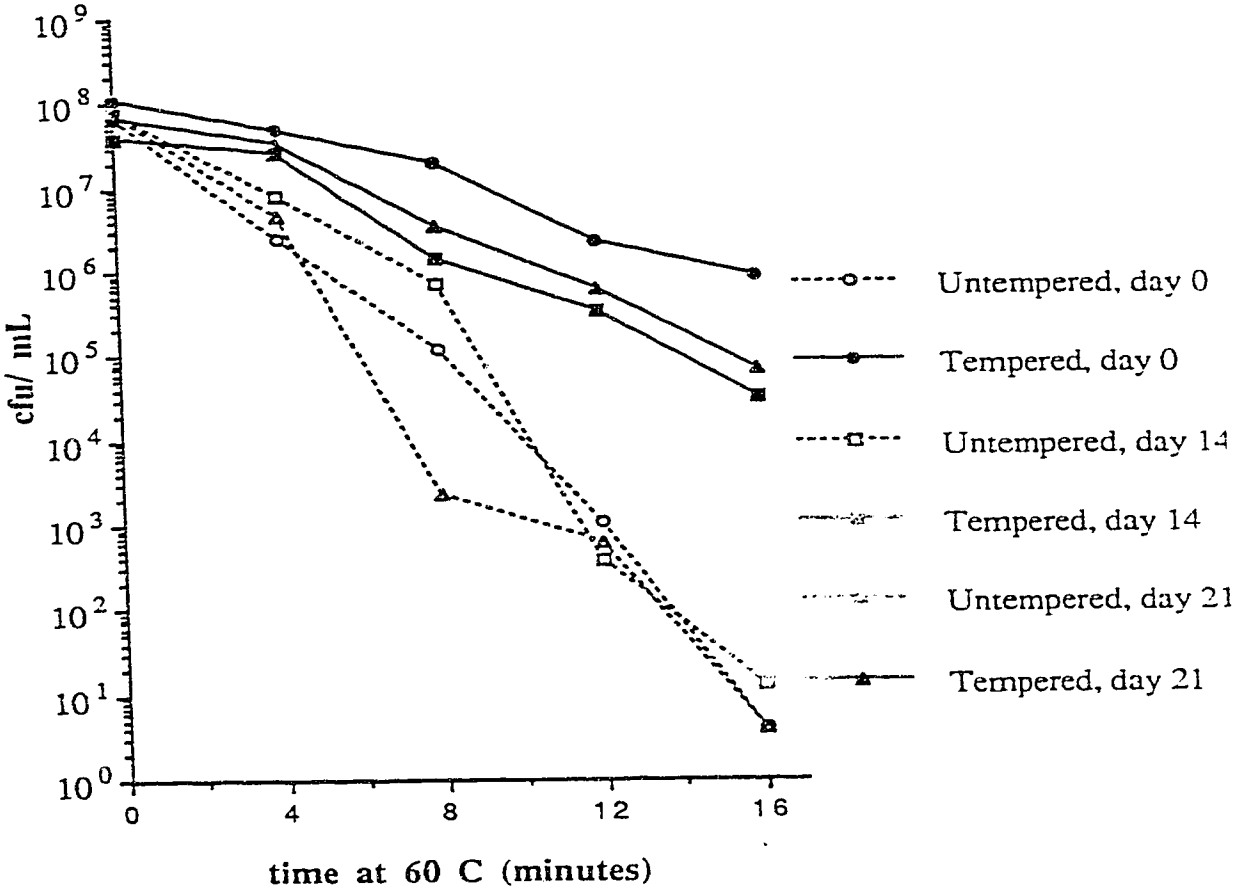

Figure 2.4: Effect of storage at 4°C in 2% UHT milk on tempered and untempered *Listeria monocytogenes*

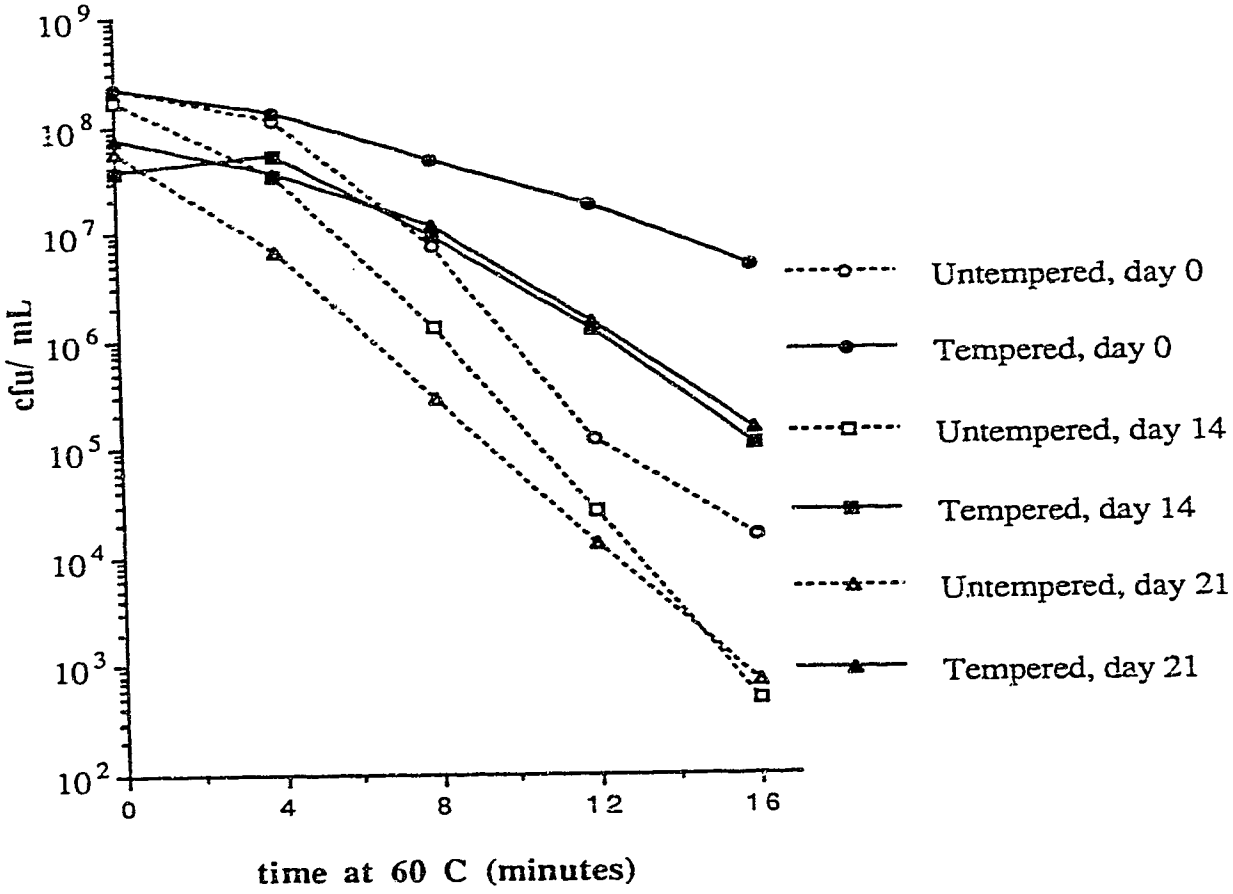

Figure 2.5: Effect of storage at 4°C in TSBYE (pH 6.7) on tempered and untempered *Listeria monocytogenes*

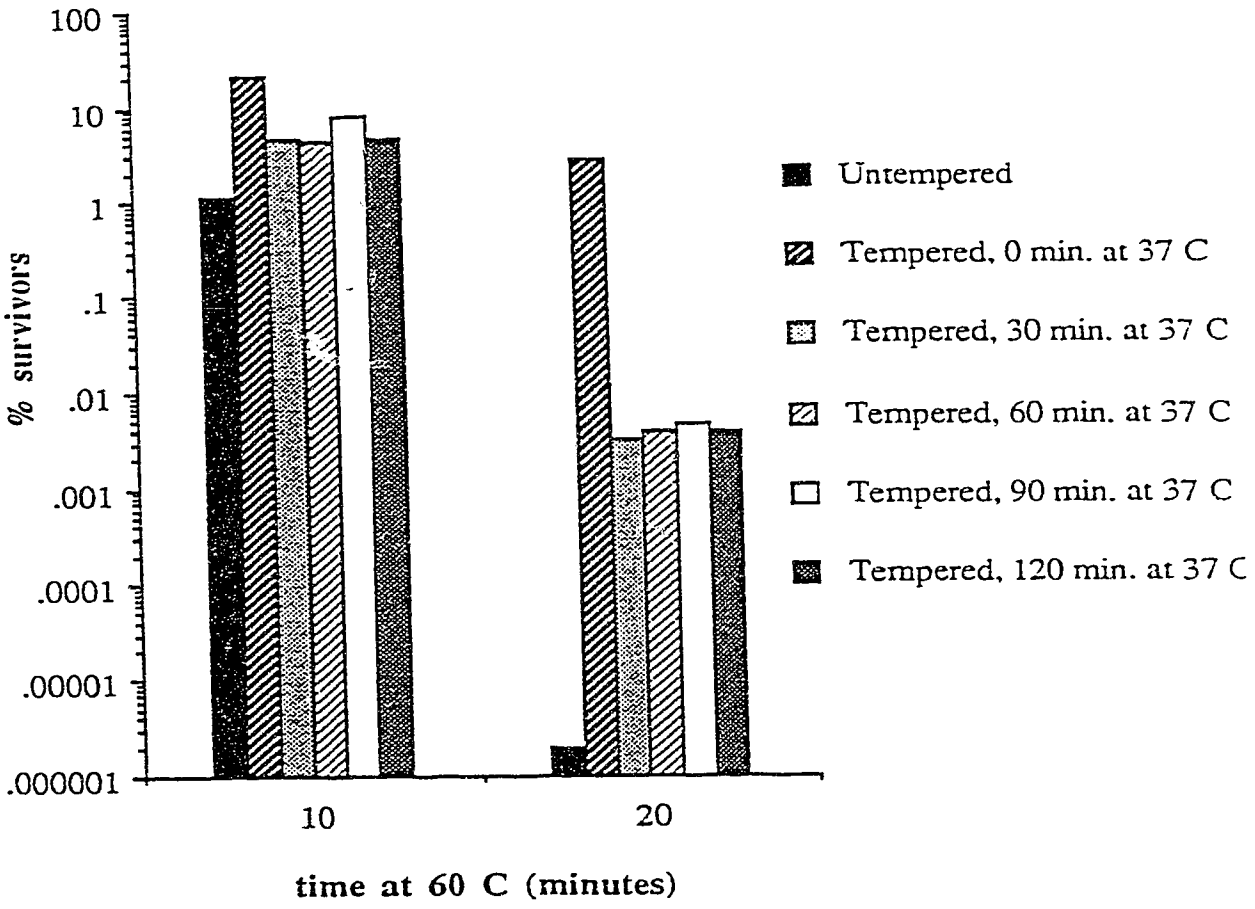

Figure 2.6: Effect of incubation at 37°C in TSBYE on 10^9 organisms/ mL of tempered *Listeria monocytogenes*

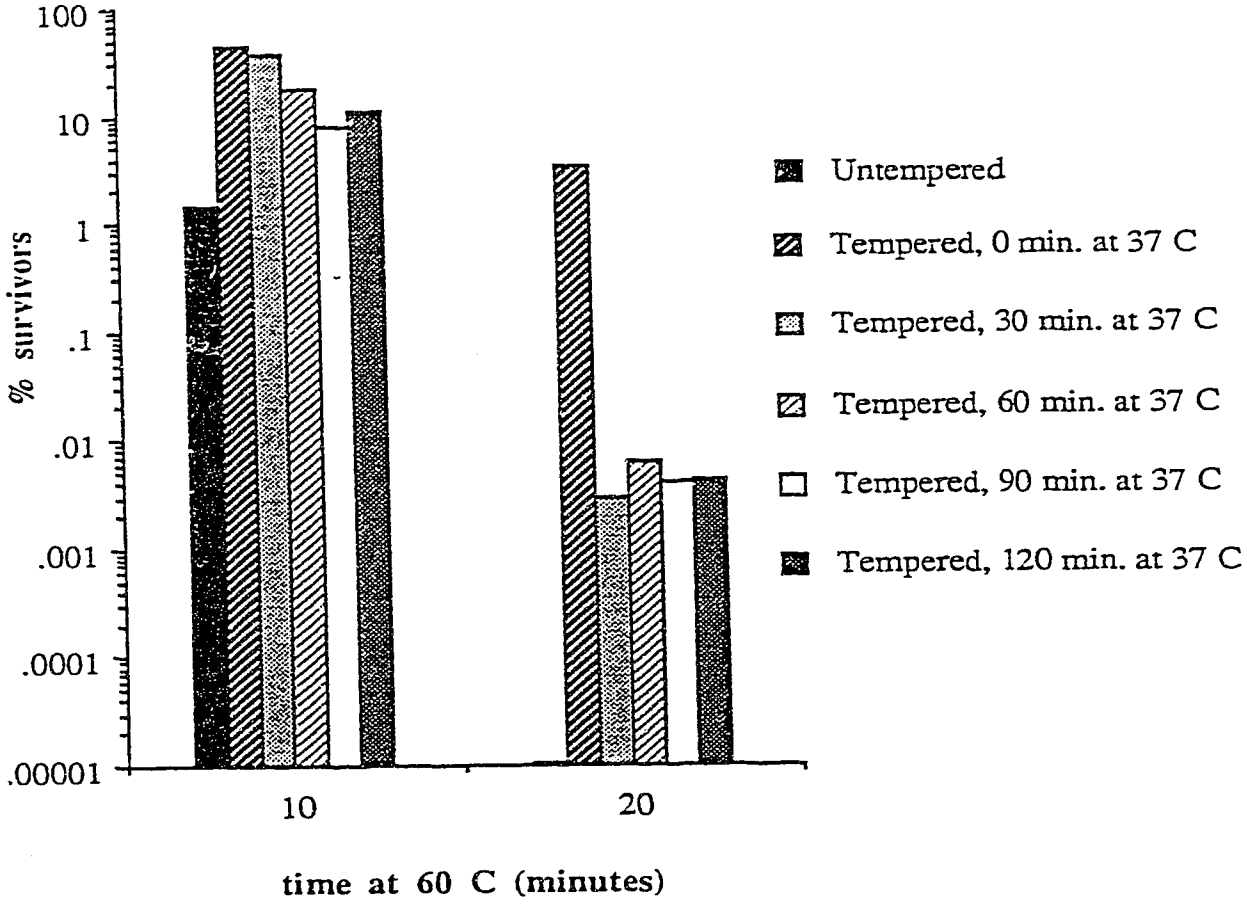

Figure 2.7: Effect of incubation at 37°C in 2% UHT milk on 10^9 organisms/ mL of tempered *Listeria monocytogenes*

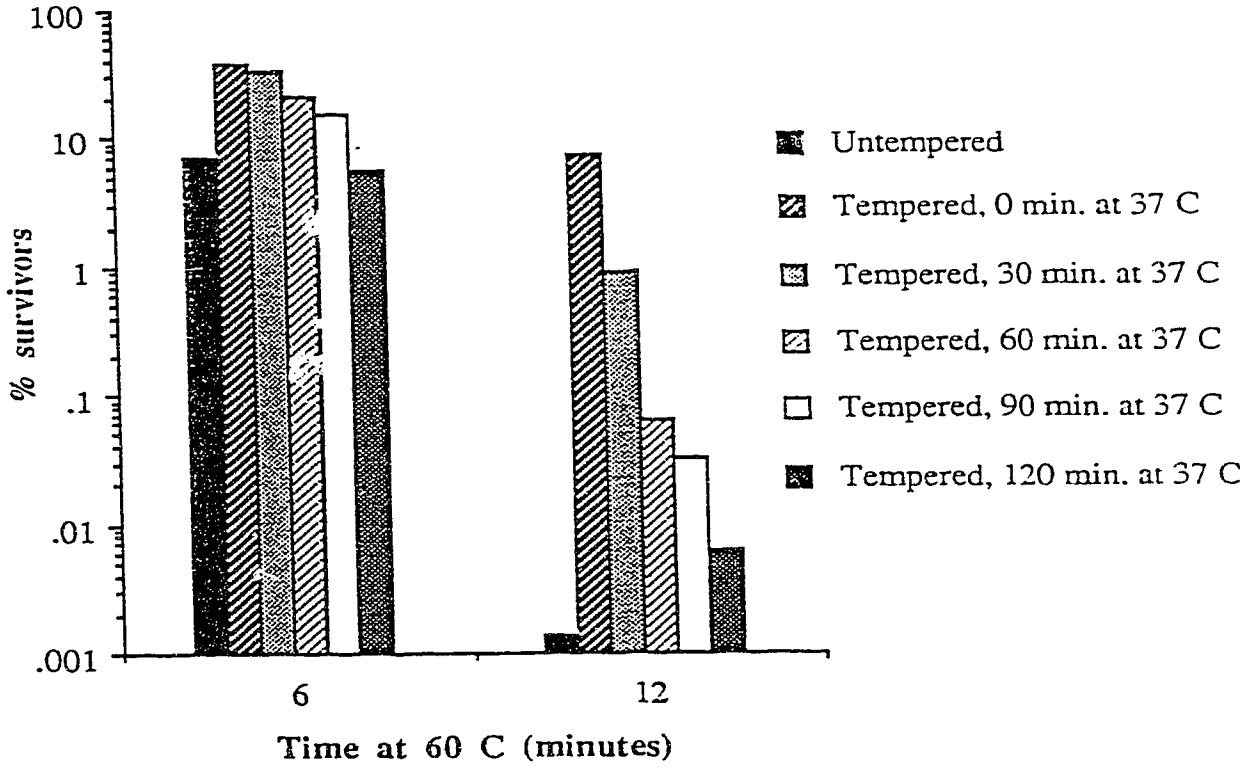

Figure 2.8: Effect of incubation at 37°C in TSBYE on 10^7 organisms/ mL of tempered *Listeria monocytogenes*

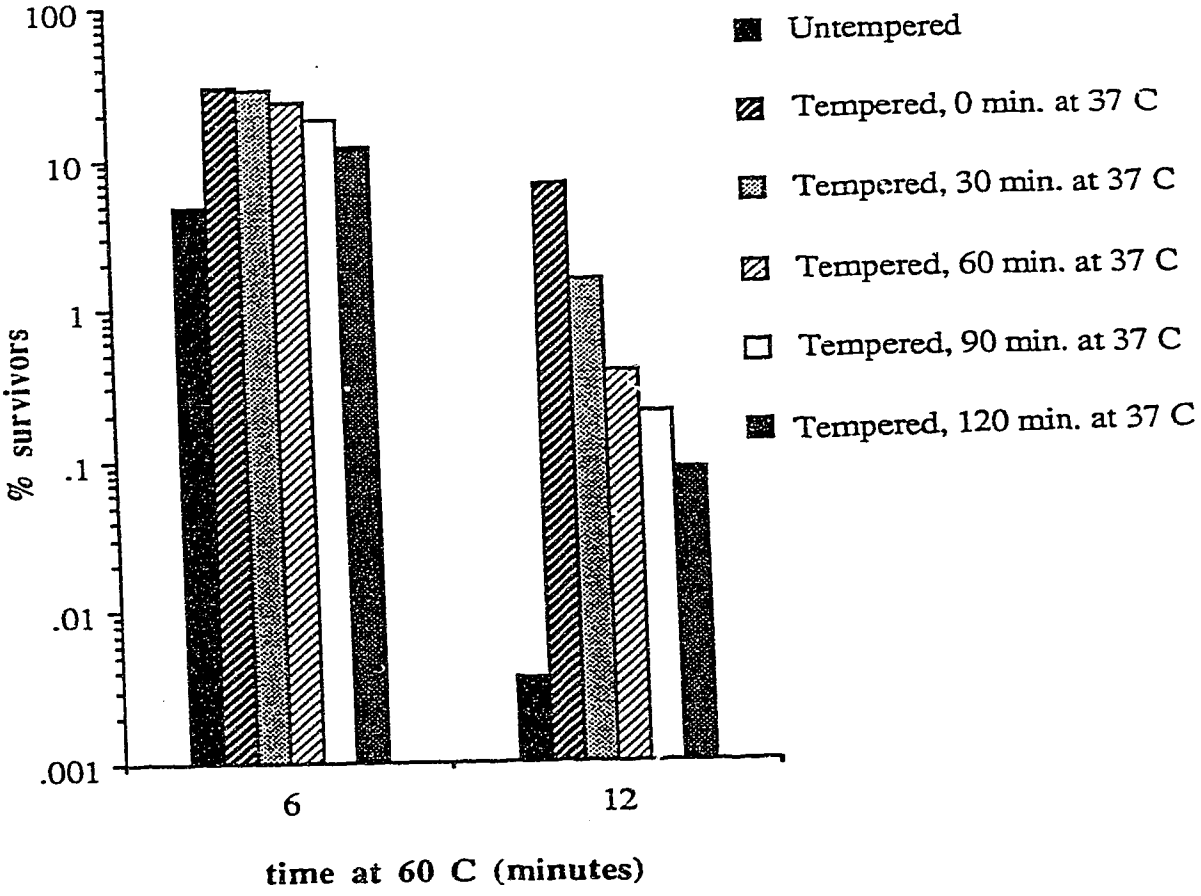

Figure 2.9: Effect of incubation at 37°C in 2% UHT milk on 10^7 organisms/ mL of tempered *Listeria monocytogenes*

2.5 References

Bunning, V. K., C. W. Donnelly, J. T. Peeler, E. H. Briggs, J. G. Bradshaw, R. G. Crawford, C. M. Beliveau and J. T. Tierney. 1988. Thermal inactivation of *Listeria monocytogenes* within bovine milk phagocytes. *Applied and Environmental Microbiology* **54**: 364- 370

Bunning, V. K., R. G. Crawford, J. T. Tierney and J. T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella typhimurium* after sublethal heat shock. *Applied and Environmental Microbiology* **56**: 3216- 3219

Farber, J. M. and B. E. Brown. 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. *Applied and Environmental Microbiology* **56**: 1584- 1587

Fedio, W. M. 1991. Effects of tempering menstrua and the ribosomal response to tempering. pp. 89- 109 **In:** Studies on the Incidence and Origins of *Listeria monocytogenes* in Raw Milk and Factors Affecting its Thermal Destruction. Ph. D. thesis, University of Alberta, Edmonton

Fedio, W. M. and H. Jackson. 1989. Effect of tempering on the heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology* **9**: 157- 160

Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audrier, C. V. Broome and A. L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *New England Journal of Medicine* **312**: 404- 407

Humphrey, T. J. 1990. Heat resistance in *Salmonella enteritidis* phage type 4: the influence of storage temperatures before heating. *Journal of Applied Bacteriology* **69**: 493- 497

Mackey, B. M. and C. M. Derrick. 1986. Elevation of the heat resistance of *Salmonella typhimurium* by sublethal heat shock. *Journal of Applied Bacteriology* **61**: 389- 393

Mackey, B. M. and C. M. Derrick. 1987a. The effect of prior heat shock on the thermoresistance of *Salmonella thompson* in foods. *Letters in Applied Microbiology* **5**: 115- 118

Mackey, B. M. and C. M. Derrick. 1987b. Changes in the heat resistance of *Salmonella typhimurium* during heating at rising temperatures. *Letters in Applied Microbiology* **4**: 13-16

Mackey, B. M. and C. M. Derrick. 1990. Heat shock protein synthesis and thermotolerance in *Salmonella typhimurium. Journal of Applied Bacteriology* **69**: 373- 383

Quintavalla, S. and S. Barbuti. 1989. Charactérisation microbiologique et résistance thermique de *Listeria* isolée de produits de viande. *Sciences des Aliments* **9**: 125- 128

Quintavalla, S., M.Campanini and L. Miglioli. 1988. Effetto della velocitá de riscaldamento sulla resistenza termica de *Streptococcus faecium. Industria Conserve* **63**: 252

Quintavalla, S. and M. Campanini. 1990. Effect of rising temperature on the heat resistance of *Listeria monocytogenes* in meat emulsion. *Applied and Environmental Microbiology* **12**: 184- 187

Smith, J. L. and B. S. Marmer. 1991. Temperature shift effects on injury and death in *Listeria monocytogenes* Scott A. *Journal of Food Safety* **11**: 73- 80

Sokolovic, Z. and W. Goebel. 1989. Synthesis of listeriolysin in *Listeria monocytogenes* under heat shock conditions. *Infection and Immunity* **57**: 295- 298

Tsuchido, T., M. Takano and I. Shibasaki. 1974. Effect of temperature-elevating process on the subsequent isothermal death of *Escherichia coli* K-12. *Journal of Fermentation Technology* **52**: 788- 792

3. Effect of pH on the tempering effect in *Listeria monocytogenes*

3.1 Introduction

Tsuchido *et al.* (1974) reported that exposure of *Escherichia coli* to sublethal temperatures before treatment at a lethal temperature resulted in greater numbers of survivors. For example, cells held at 40°C for 10 minutes were more resistant to a challenging treatment of 50°C for 20 minutes than were cells incubated at 30°C for 10 minutes before the same challenge. More than a decade later, Mackey and Derrick (1986) showed that the same phenomenon occurs in *Salmonella*. *S. typhimurium* held at 48°C for 30 minutes before challenging at 50°, 52°, 55°, 57° or 59°C was more heat resistant than the cells that were not treated. *S. thompson*, when held at 48°C for 30 minutes, then challenged at 54° to 60°C, was more thermotolerant than the cells held at 37°C then challenged (Mackey and Derrick, 1987). They also reported that the phenomenon was observed in several other serovars of *Salmonella*, including: *S. infantis, S. montevideo, S. stanley* and *S. cubana.*

The examination of *Listeria* species for the phenomenon of increased heat resistance due to exposure to a sublethal heat treatment began in 1989. At that time, Fedio and Jackson coined the term "temper" to describe this phenomenon. They tempered *L. monocytogenes* at 48°C for 1 hour before exposing the cells to 60°C. They found , in Tryptic Soy Broth plus 0.6% Yeast Extract (TSBYE) and 2% UHT milk, that the tempered cells were more heat resistant than the untempered cells. Also that year, Quintavalla and Barbuti (1989) examined the heat resistance of tempered and untempered *L. monocytogenes* and *L. innocua*. Their results confirmed those of Fedio and Jackson (1989). *L. monocytogenes* and *L. innocua* tempered at 48°C for 30 minutes had a D_{65} value of 10.5 and 14.3 minutes, respectively. Untempered *L. monocytogenes* and *L. innocua* had a D_{65} value of 0.52 minutes. Farber and Brown (1990) examined the heat resistance of tempered and untempered *L. monocytogenes* in

a sausage mixture. These authors found that the organism required 2 hours at 48°C for a significant difference to be found in the D_{64} value. They also stored tempered *L. monocytogenes* in the meat for 24 hours at 4°C prior to challenging and concluded that the cells retained increased thermotolerance during storage. Linton *et al.* (1990) found that *L. monocytogenes* in the logarithmic phase of growth was capable of becoming tempered. Recently, Quintavalla and Campanini (1991) established that the rate of heating of *L. monocytogenes* in sausage meat affects the thermotolerance of the organism. Cells which were heated at a rate of 0.5°C/ minute to the challenge temperatures of 60°, 63°, or 66°C had greater D-values than the cells heated instantaneously.

Few studies examining the heat resistance of *L. monocytogenes* have taken into consideration the effect of pH. Beuchat *et al.* (1986) examined the effect of pH of cabbage juice on the thermotolerance of *L. monocytogenes* Scott A. Cabbage juice at pH 5.6 was less destructive to the organism when held at 50°, 52°, 54°, or 56°C for up to one hour, than it was at pH 4.6. At pH 5.6, only a slight decrease in cell numbers was observed after one hour at 50°C, while at pH 4.6 the population was reduced by approximately 2 log cycles in the same amount of time. El-Kest and Marth (1988), while examining the effect of 1 ppm chlorine upon the inactivation of *L. monocytogenes* at 5°, 25°, and 35°C, also examined the effect of pH. They found that as the pH of the chlorine solution increased, the inactivation of the organism decreased, even as the temperature increased. El-Kest and Marth (1988) attribute the decrease in effectiveness of chlorine at high pH levels to the formation of OCl^-, which is 80 to 100 times less effective than HOCl at inactivating bacteria.

The effect of pH on tempering has only been examined indirectly. Various authors have tempered *L. monocytogenes* in several systems, such as Tryptic Soy Broth plus 0.6% Yeast Extract (TSBYE) (pH 7.2) (Fedio and Jackson, 1989, Linton *et al.*, 1990, and Bunning *et al.*, 1990), 2% UHT milk (pH 6.7) (Fedio and Jackson,

1989), and sausage meat (approximately pH 6.5) (Farber and Brown, 1990). These systems have many other differences than just pH, such as protein, sugar, and lipids, in their compositions and proportions, therefore a direct comparison of the effect of pH cannot be made. As well, the food and broth systems examined to date only cover a narrow range of pH, while *L. monocytogenes* is capable of growth over a much broader range of pH 4.3-9.0 (Seeliger and Jones, 1986). At a neutral pH, the organism will grow between 1° and 43°C (Seeliger and Jones, 1986), however, the minimum pH allowing growth is variable, depending upon temperature, strain, and acidulant. For example, pH adjusted Brain Heart Infusion (BHI) broth at pH 5.1 and 4°C will support the growth of *L. monocytogenes* LCDC 81-861 when the pH is adjusted with lactic acid. Under the same conditions of temperature and acidulant, *L. monocytogenes* Scott A will not grow below pH 5.5. At 30°C, using the same acidulant, the strain LCDC 81-861 will grow at a minimum pH of 4.9, while strain Scott A will grow at a minimum pH of 5.1. In BHI broth adjusted with acetic acid, *L. monocytogenes* strains LCDC 81-861 and Scott A will not grow below pH 5.7 at 4°C (Farber *et al.*, 1989).

Thus, the purpose of this study was to determine the effect of pH on the acquisition of thermotolerance, and to examine the heat resistance of *L. monocytogenes* over a broad range of pH.

3.2 Materials and methods

3.2.1 Culture preparation

L. monocytogenes Scott A was maintained on slants of Tryptic Soy Agar plus 0.6% Yeast Extract (TSAYE) (Becton Dickinson, Cockeysville, MD) refrigerated at 4°C. A colony was picked from this pure culture and placed in 5 mL of TSBYE (Difco Laboratories, Detroit, MI, Becton Dickinson, Cockeysville, MD). The inoculated broth was incubated at 37°C in a shaking incubator, set at 125 rpm, for 24 hours. One mL of

this culture was placed in 225 mL of TSBYE, which was then incubated at 37°C, 125 rpm, in a shaking incubator. Twenty-four hours later, one mL of cells was taken and inoculated into 225 mL of TSBYE, and was incubated at 37°C for 24 hours at 125 rpm. This third culture was used for all experiments. The suspension was centrifuged in a Sorvall RC-5B refrigerated centrifuge (Du Pont Inc., Mississauga, Ont.) for 15 minutes at 6000 rpm. The supernatant was discarded, and the cell pellet was resuspended in 225 mL of pH adjusted TSBYE. The suspension was centrifuged again for 15 minutes at 6000 rpm. The supernatant was discarded, and the pellet was again resuspended in pH adjusted TSBYE. Up to 50 mL of this resuspension was placed into each of 2 X 250 mL sterile Erlenmeyer flasks.

3.2.2 The pH adjusted broths

The pH of the TSBYE was adjusted with either 12 N HCl or 10 N NaOH to a final pH of 5, 6, 7, 8, or 9, and then autoclaved. After autoclaving at 121°C for 25 minutes and cooling to 23°C, the pH of the broths was again measured.

3.2.3 Tempering and challenging

The tempering parameters chosen were 48°C for 60 minutes, shaking in a Metabolyte water bath shaker (New Brunswick Scientific, NJ) at 125 rpm.

Twenty-five mL of the cell suspension was removed and placed in 225 mL of pH adjusted TSBYE in a Erlenmeyer flask at 60°C, and 125 rpm in a Metabolyte water bath shaker (New Brunswick Scientific, NJ). Samples of this cell suspension were taken after 0, 4, 8, 12, 16, and 20 minutes at 60°C. The appropriate dilutions were made in 0.1% peptone water (Difco Inc., Detroit, MI), and portions were plated onto TSAYE. After one hour at 48°C, 25 mL of the tempered cells were placed in 225 mL of pH adjusted TSBYE in an Erlenmeyer flask at 60°C, shaking at 125 rpm in a Metabolyte water bath shaker (New Brunswick Scientific, NJ), then sampled, diluted,

and plated as for the untempered cells. Plates were incubated at 37°C for 48 hours before counting.

3.2.4 Repetitions

All experiments were repeated twice. The figures represent typical results.

3.3 Results

The effect of pH on tempering and thermotolerance is shown in figures 3.1 to 3.10. These findings are consolidated in Figure 3.11. This figure shows that the pH of a broth system dramatically affects the thermotolerance and the ability to become tempered of *L. monocytogenes*. In the acidic range of pH examined (pH 4.8 to pH 6.9), it is evident that as the pH of the medium approaches neutral pH, the thermotolerance of the untempered and tempered cells increases. The difference in D_{60} values between the tempered and untempered cells increases exponentially towards neutral pH. The maximum D_{60} value for both the tempered and untempered cells was at pH 7.5. After the peak in heat resistance found at pH 7.5, the thermotolerance of the tempered and untempered cells decreased. Acidic conditions (pH 4.8) in the medium were more lethal to the untempered and tempered cells than were basic conditions (pH 9.1). At the lower end of the pH range examined, there was virtually no difference seen between the tempered and untempered cells. At the higher end of the pH range examined, although the cells did not survive heat treatment as well as at neutral pH, the tempered cells were slightly more themotolerant than their untempered counterparts. Untempered cells challenged at neutral pH were more thermotolerant than tempered cells at low pH (below pH 5.5). For the tempered cells, the basic samples were more heat resistant than were the acidic samples, however, neither the cells in the basic nor the acidic media showed as much increased heat resistance as the cells in the neutral medium.

A decrease in cell numbers was noted after tempering in the acidic media as a result of the combination of the tempering temperature (48°C) and the low pH. This phenomenon was not observed at the basic pHs examined.

3.4 Discussion

Humphrey *et al.* (1991) observed that *Salmonella enteritidis* PT4 had greater thermotolerance when exposed to a medium of pH 9.2 ± 0.2 for 5 minutes relative to the organism exposed to pH 7.0 ± 0.2. The results presented here indicate that this type of increased heat resistance due to exposure to high pH does not occur in *L. monocytogenes*. The untempered cells at pH 9.1 were less thermotolerant than the untempered cells at pH 7, as shown in figure 3.1. Thus, it may be concluded that the induction of increased thermotolerance by exposure to high pH in *S. enteritidis* is not a universal phenomenon and more experiments of this nature should be undertaken with this and other Gram-negative organisms.

From our results, we may conclude that *L. monocytogenes* Scott A is able to become more thermotolerant within the range of neutral to basic pH. Tempering was evident within a wide range of pH as shown in figure 3.1. It should not, however, be assumed that this experimental system in any way is representative of food systems. For example, our results show that tempered *L. monocytogenes* should have a D_{60} value of approximately 5 to 5.5 minutes at pH 6.5, and a D_{60} value of 1.5 minutes for untempered cells. Farber and Brown (1990) found that, for sausage meat, 60 minutes at 48°C only resulted in a slight increase in heat resistance relative to the untempered sausage meat. Tempering at 48°C for 120 minutes resulted in a much greater increase in heat resistance to a challenge at 64°C. Comparing 2 liquid systems, Fedio and Jackson (1989) reported that tempering *L. monocytogenes* in 2% UHT milk at 48°C for 60 minutes was slightly less effective than tempering the organism in TSBYE for the same length of time and temperature.

Unlike *S. enteritidis*, exposure to high pH does not increase the thermotolerance of *L. monocytogenes*, although basic pH levels result in more survivors after challenging than do acidic pHs when the cells are exposed to either a mild or a severe heat treatment. Further studies should be undertaken to determine if weak acids have a greater effect upon the ability of *L. monocytogenes* to withstand high temperatures, and the range of pH which will permit tempering.

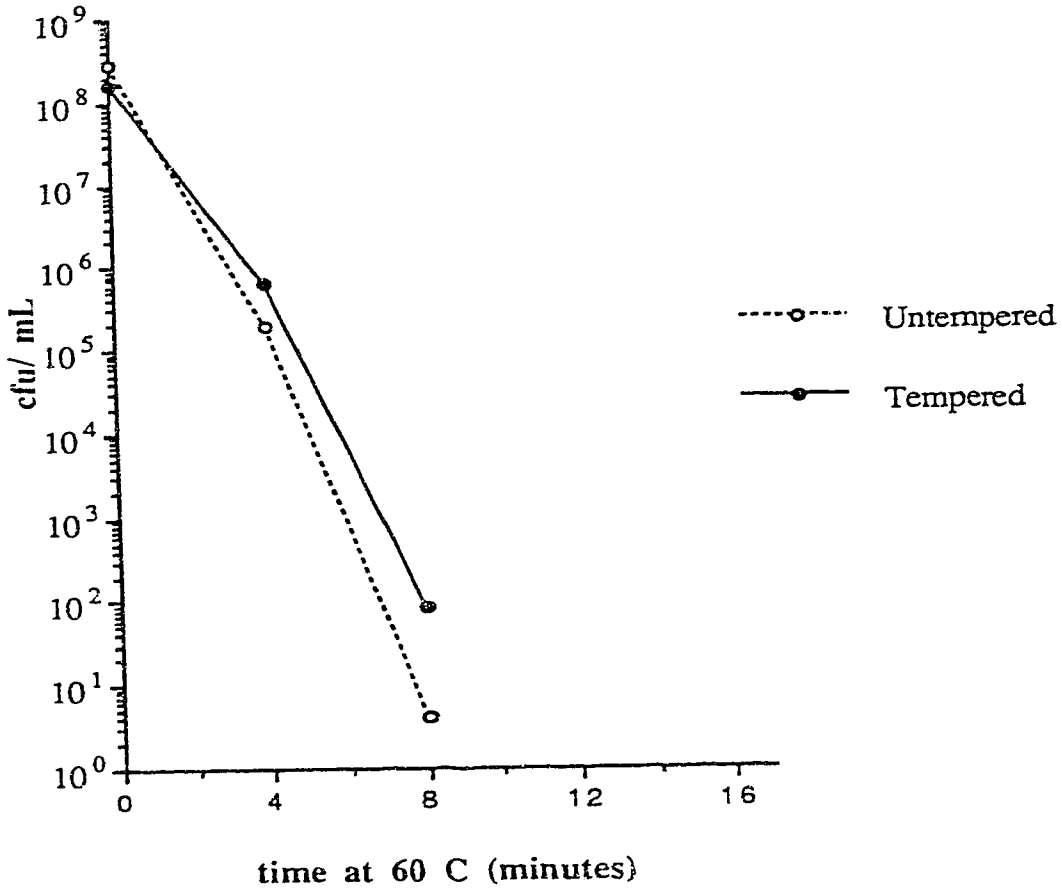

Figure 3.1: Effect of tempering and challenging in pH 4.8 TSBYE on *Listeria monocytogenes*

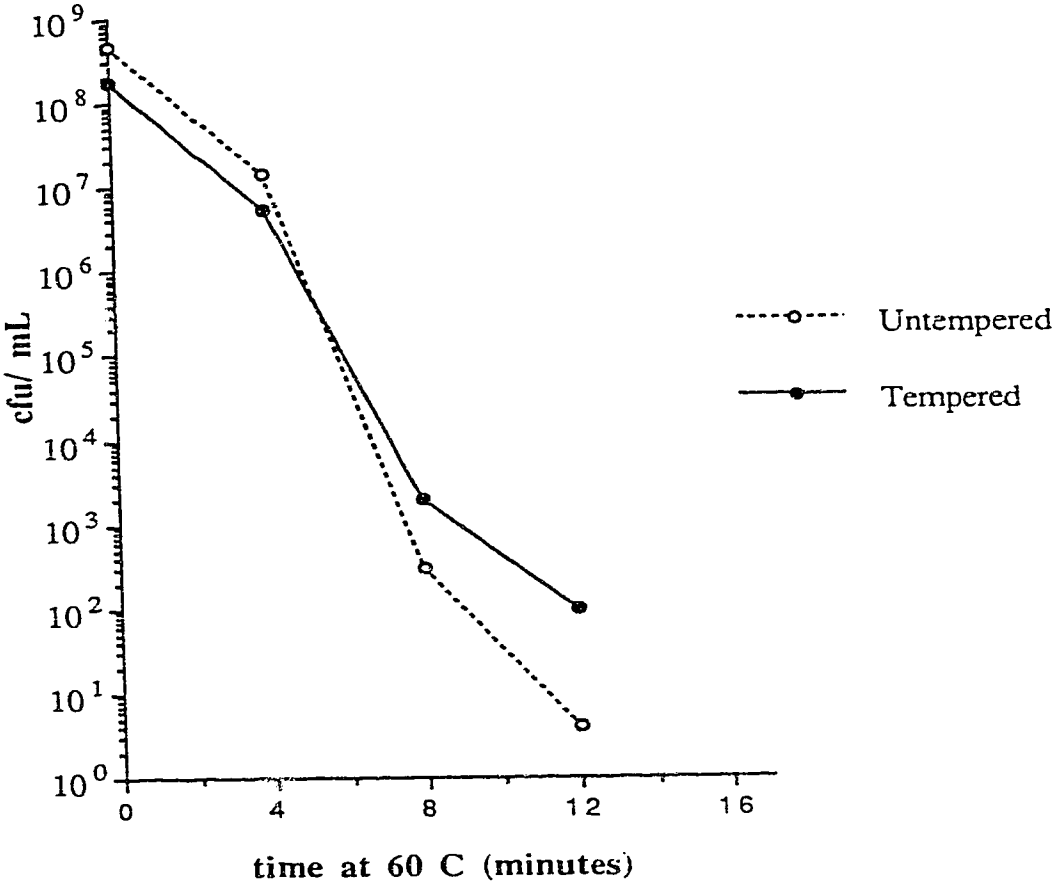

Figure 3.2: Effect of tempering and challenging in pH 4.9 TSBYE on *Listeria monocytogenes*

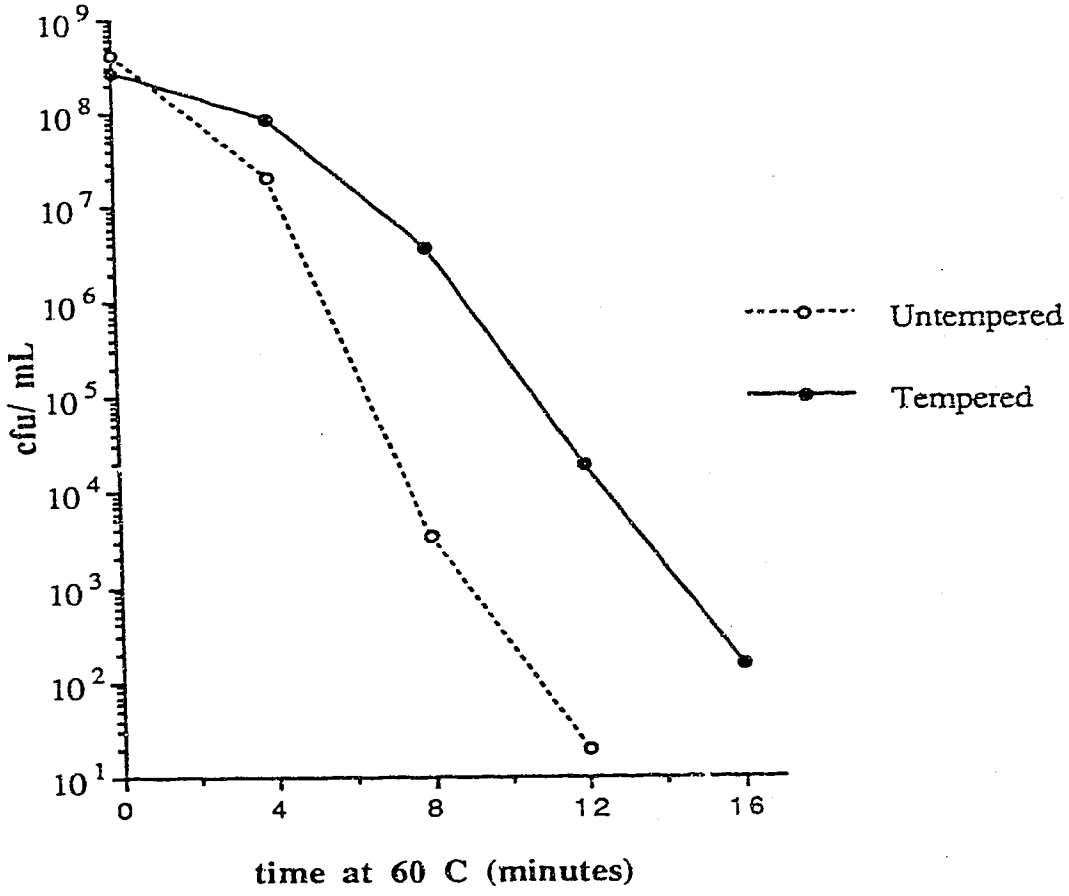

Figure 3.3: Effect of tempering and challenging in pH 6.1 TSBYE on *Listeria monocytogenes*

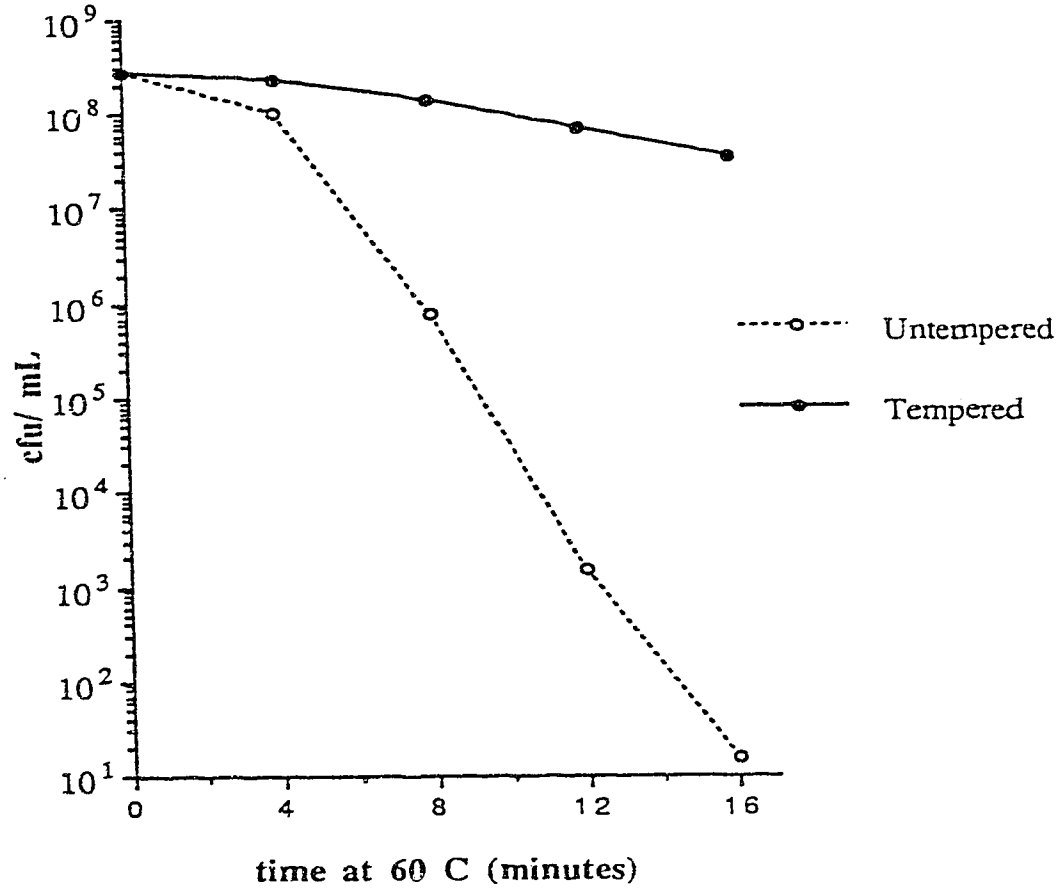

Figure 3.4: Effect of tempering and challenging in pH 6.8 TSBYE on *Listeria monocytogenes*

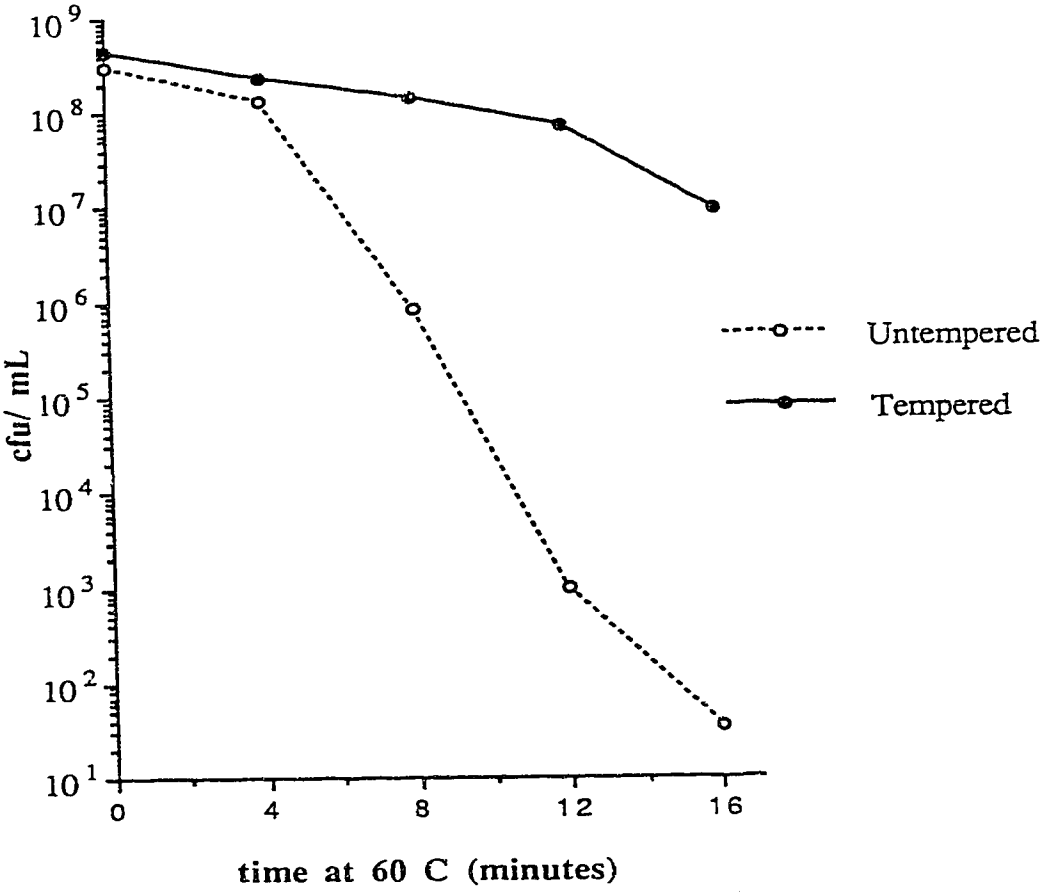

Figure 3.5: Effect of tempering and challenging in pH 6.9 TSBYE on *Listeria monocytogenes*

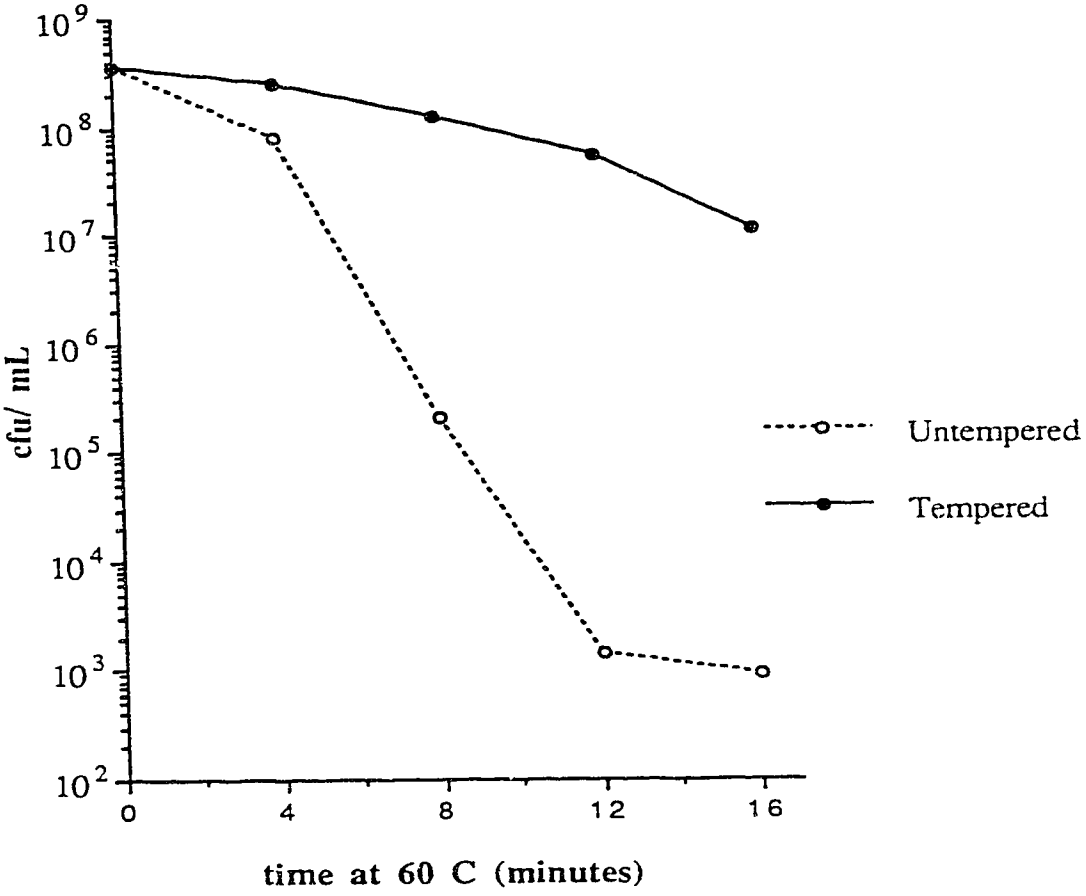

Figure 3.6: Effect of tempering and challenging in pH 7.2 TSBYE on *Listeria monocytogenes*

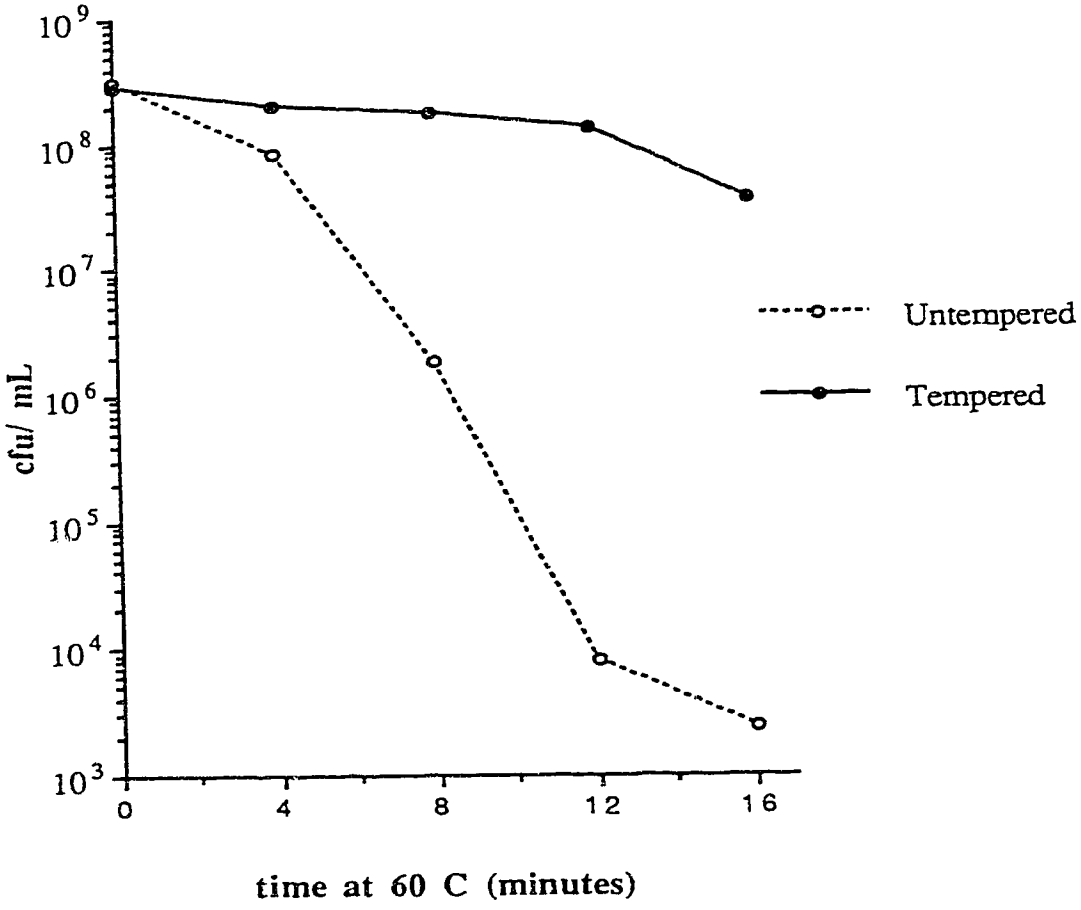

Figure 3.7: Effect of tempering and challenging in pH 7.5 TSBYE on *Listeria monocytogenes*

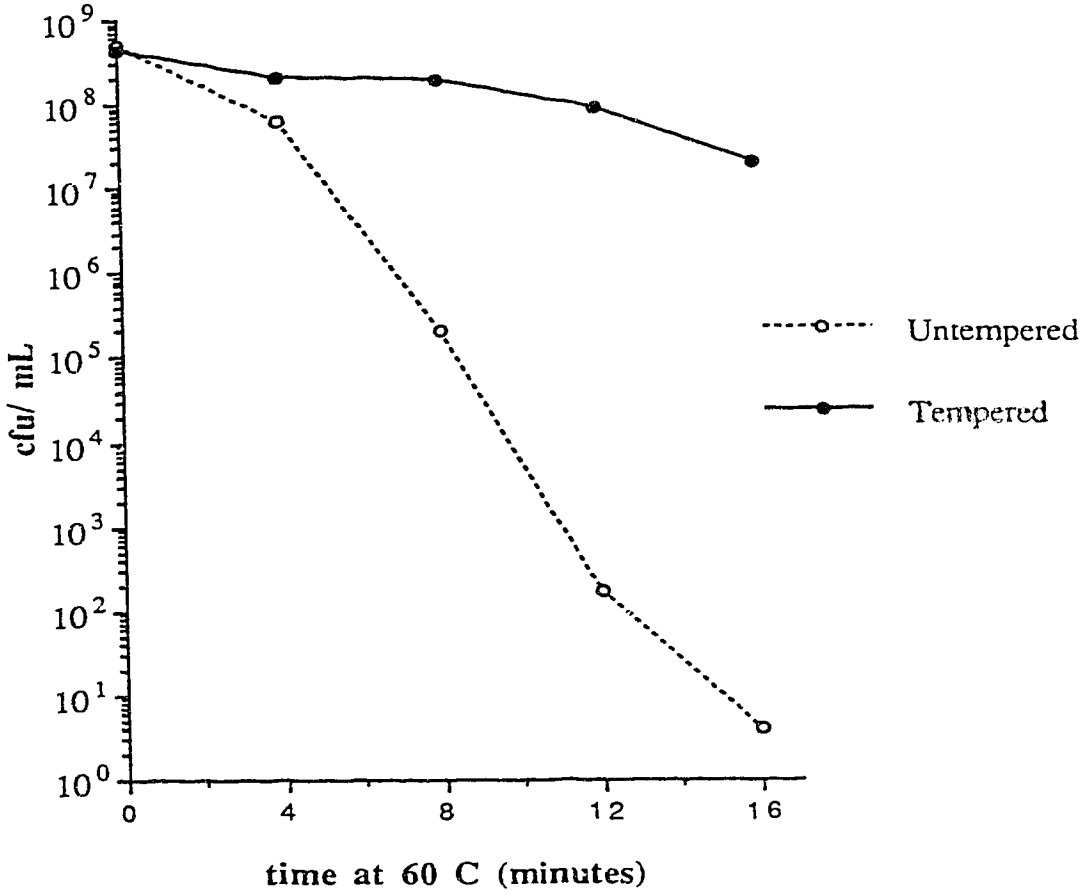

Figure 3.8: Effect of tempering and challenging in pH 7.8 TSBYE on *Listeria monocytogenes*

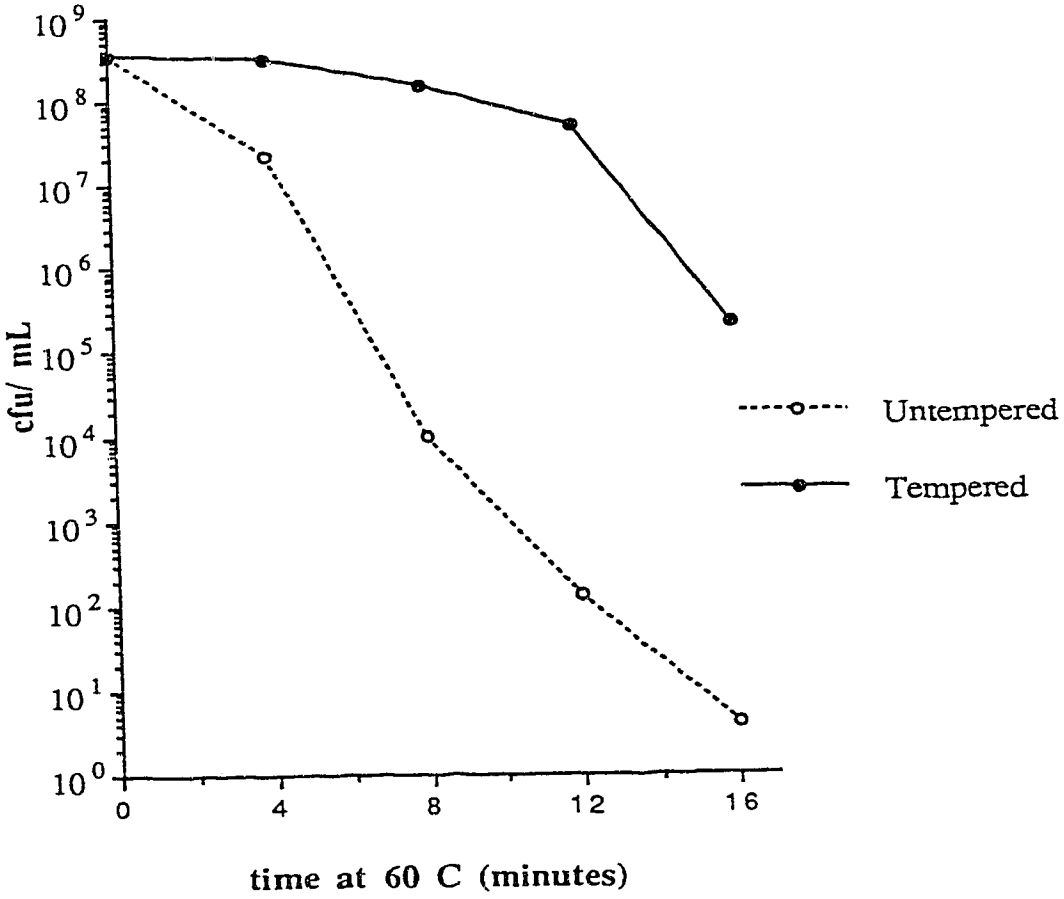

Figure 3.9: Effect of tempering and challenging in pH 8.4 TSBYE on *Listeria monocytogenes*

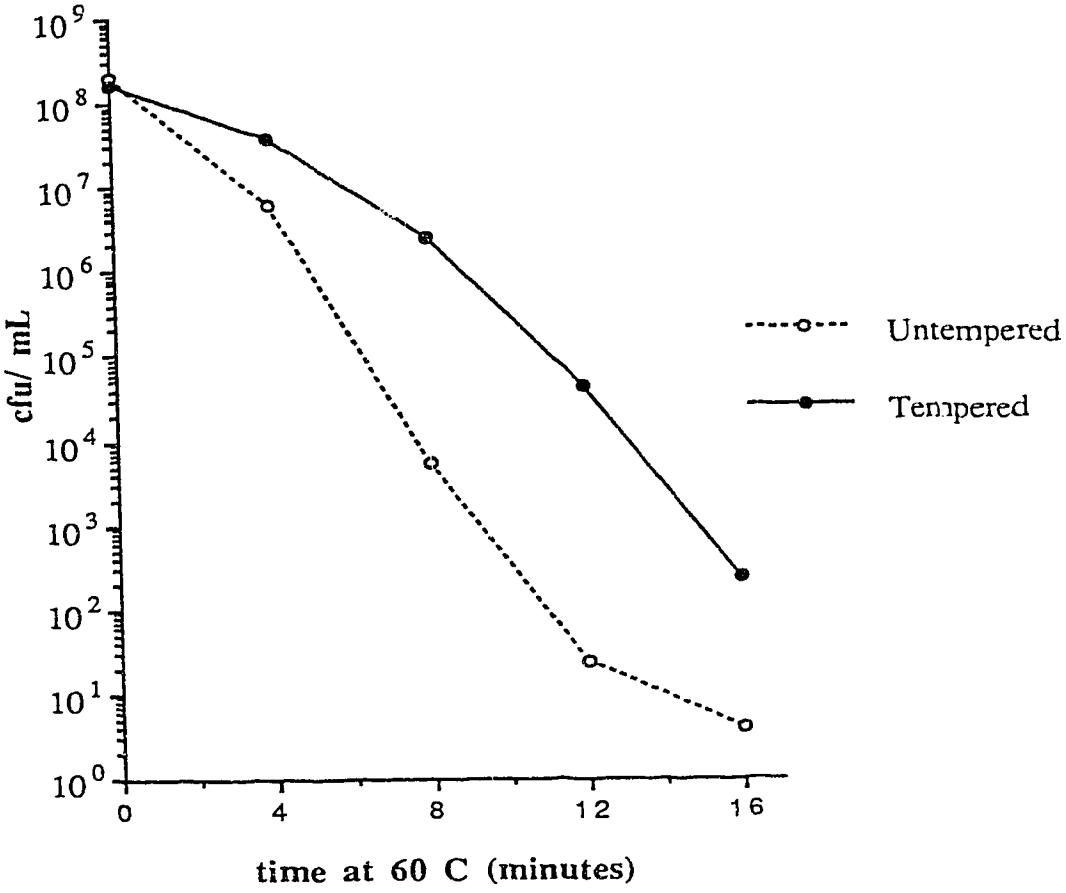

Figure 3.10: Effect of tempering and challenging in pH 9.1 TSBYE on *Listeria monocytogenes*

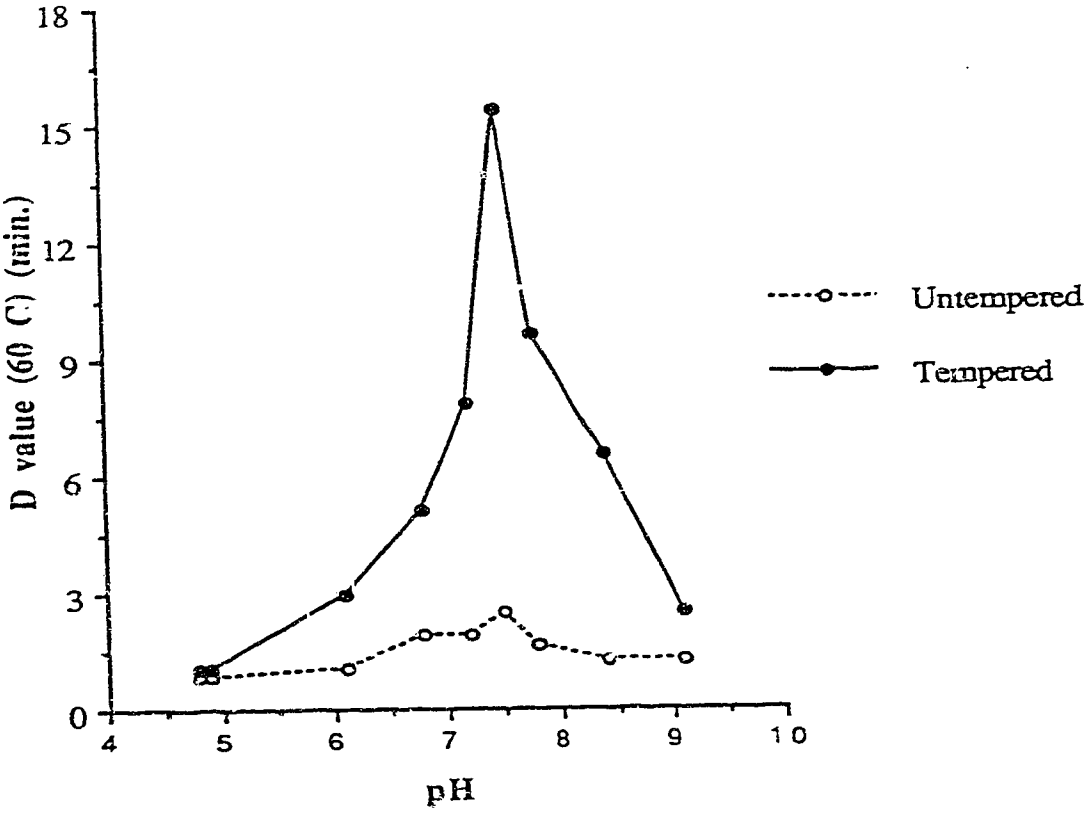

Figure 3.11: Effect of the pH of TSBYE on the heat resistance of tempered and untempered *Listeria monocytogenes*

3.5 References

Beuchat, L.R., R.E. Brackett, D.Y-Y. Hao and D.E. Conner. 1986. Growth and thermal inactivation of *Listeria monocytogenes* in cabbage and cabbage juice. *Canadian Journal of Microbiology* **32**: 791- 795

Bunning, V.K., R.G. Crowford, J.T. Tierney, and J.T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella typhimurium* after sublethal heat shock. *Applied and Environmental Microbiology* **56**: 3216- 3219

El-Kest, S.E., and E.H. Marth. 1988. Temperature, pH and strain of pathogen as factors affection inactivation of *Listeria monocytogenes* by chlorine. *Journal of Food Protection* **51**: 622- 625

Farber, J.M., and B.E. Brown. 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. *Applied and Environmental Microbiology* **56**: 1584- 1587

Farber, J.M., G.W. Sanders, S. Dunfield, and R. Prescott. 1989. The effect of various acidulants on the growth of *Listeria monocytogenes*. *Letters in Applied Microbiology* **9**: 181- 183

Fedio. W.M., and H. Jackson. 1989. Effect of tempering on the heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology* **9**: 157- 160

Humphrey, T.J., N.P. Richardson, A.H.L. Gawler, and M.J. Allen. 1991. Heat resistance of *Salmonella enteritidis* PT4: the influence of prior exposure to alkaline condition. . *Letters in Applied Microbiology* **12**: 258- 260

Linton, R.H., M.D. Pierson, and J.R. Bishop. 1990. Increase in heat resistance of *Listeria monocytogenes* Scott A by subletnal heat shock. *Journal of Food Protection* **53**: 924- 927

Seeliger, H.P.R. and D. Jones. 1986. Genus *Listeria* Pirie, p. 1235- 1245 **In:** P.H.A. Sneath, N.S. Mair and E. Sharp (ed.), Bergey's manual of systematic bacteriology, 7th ed., Vol. 2. The Williams & Wilkins Co., Baltimore

4. Effects of the tempering and challenging media on the thermotolerance of *L. monocytogenes*

4.1 Introduction

Within the last decade, several incidents of listeriosis have been associated with the consumption of dairy products. In 1983, in the Massachusetts area, 49 people contracted listeriosis after consuming either 2% milk or whole milk, which appeared to have been properly processed (Fleming *et al.*, 1985). The mortality rate was 29%. In California in 1985, 142 people became ill after eating cheese which had been made from milk contaminated with *L. monocytogenes* (Linnan *et al.*, 1988). The mortality rate was 39%. A case of listeriosis was associated with ice cream and fresh cream consumption in 1989 (Farber and Peterkin, 1991).

Because of the Massachusetts outbreak, questions were raised about the thermotolerance of *L. monocytogenes* and its apparent ability to survive pasteurization. Subsequently, several papers were published reporting on two possibilities to explain why *L. monocytogenes* may have been able to survive pasteurization: the protective influence of bovine phagocytes, and tempering, *i.e.* increased thermotolerance due to a sublethal exposure to heat.

Fedio and Jackson (1989) first examined tempering of *L. monocytogenes* in tryptic soy broth plus yeast extract and in 2% UHT milk. They found that the organism became tempered in milk, and that the subsequent rate of destruction of the organism at the heating challenge temperature of 60°C was less than the rate found for untempered organisms. Other authors have since examined various food and experimental systems and confirmed the increased thermotolerance of tempered *L. monocytogenes*. Farber and Brown (1990) examined sausage meat, Quintavalla and Barbuti (1989) investigated a broth system, Quintavalla and Campanini (1991) tempered *L. monocytogenes* in a meat system, and Bunning *et al.* (1990), also looked at a broth system.

To date, however, no information has been published on the effect of systematic changes in the composition of the suspending medium on the tempering effect. Fat content was chosen as the variable to be studied in view of the known effect of lipids in increasing thermotolerance of micro-organisms and because of the different levels found in heat processed fluid dairy products. The results presented here are the data from a series of experiments in which tempering and heat resistance were determined in reconstituted skim milk, 2% Ultra-High Temperature (UHT) milk, pasteurized cereal cream (10% butterfat), and pasteurized whipping cream (35% butterfat). A minimal defined medium and a complex medium were also examined.

4.2 Materials and methods

4.2.1 Culture preparation

L. monocytogenes Scott A was maintained on slants of Tryptic Soy Agar plus 0.6% Yeast Extract (TSAYE) (Becton Dickinson, Cockeysville, MD) refrigerated at 4°C. A colony was picked from this pure culture and placed in 5 mL of Tryptic Soy Broth plus 0.6% Yeast Extract (TSBYE) (Difco Laboratories, Detroit, MI, Becton Dickinson, Cockeysville, MD). The inoculated broth was incubated at 37°C in a shaking incubator, set at 125 rpm, for 24 hours. One mL of this culture was placed in 225 mL of TSBYE, which was then incubated at 37°C, 125 rpm, in a shaking incubator. Twenty four hours later, one mL of culture was taken and inoculated into 225 mL of TSBYE, and incubated at 37°C for 24 hours at 125 rpm. This third culture was centrifuged in a Sorvall RC-5B refrigerated centrifuge (Du Pont Inc., Mississauga, Ont.) for 15 minutes at 6000 rpm. The supernatant was discarded, and the cell pellet was resuspended in 225 mL of the test medium. The suspension was centrifuged again for 15 minutes at 6000 rpm. The supernatant was discarded, and the pellet was again resuspended in the test medium. The test media used were reconstituted skim milk, 2%

UHT milk, 10% butterfat cereal cream, 35% butterfat whipping cream, Modified Ralovich's Minimal (MRM) medium, and TSBYE.

4.2.2 Media preparation

Minimal medium was a modification of Ralovich's minimal medium (Ralovich, 1984) (complete formulation given in the appendix), substituting distilled water for agar. The pH of the final medium was 7.0.

Skim milk was made from 10% spray-dried skim milk powder, sterilized after reconstitution by autoclaving at 100°C for 30 minutes, on 2 consecutive days.

Two percent Ultra-High Temperature (UHT) treated milk was a commercially available brand which was aseptically dispensed in appropriate amounts for the experiments.

Pasteurized cereal cream (10% butterfat) and pasteurized whipping cream (35% butterfat) were purchased and then heat treated by holding at 80°C for 10 minutes for 3 consecutive days in 500 mL portions before being aseptically transferred as appropriate. The effectiveness of these heat treatments in destroying initial contaminants in these products was evidenced by absence of atypical colonies.

4.2.3 Tempering

Up to 50 mL of the cells resuspended in the different media were placed in a 250 mL Erlenmeyer flask. The flask was then placed in a Metabolyte water bath shaker (New Brunswick Scientific, NJ) at 48°C for 1 hour at 125 rpm.

4.2.4 Challenging

Twenty-five mL of the untempered cell suspension was removed and placed in 225 mL of the test medium in a 500 mL Erlenmeyer flask at 60°C and 125 rpm in a Metabolyte water bath shaker. Samples of this cell suspension were taken after 0, 4, 8, 12, 16, and 20 minutes at 60°C. The appropriate dilutions were made in 0.1% peptone

water (Difco Laboratories, Detroit, MI), and portions were plated onto TSAYE. After one hour at 48°C, 25 mL of the tempered cells were placed in 225 mL of test medium in a 500 mL Erlenmeyer flask at 60°C, shaking at 125 rpm, and sampled, diluted, and plated as for the untempered cells. Plates were incubated at 37°C for 48 hours before enumerating the colony forming units.

4.2.5 Repetitions

All experiments were repeated a minimum of 2 times. D-values were determined from best fit graphs of survivors. Duncan's Multiple Range Test was performed to determine statistical differences between treatments.

4.3 Results

The results on the effects of fat content and tempering are shown in table 4.1. It can be seen that *L. monocytogenes* became tempered in all media examined and that the D-values of tempered cells were all significantly different from those of untempered controls in the same media. There was no significant difference between the D_{60} values of the cells tempered and challenged in skim milk, 2% UHT milk, 10% cereal cream, or 35% whipping cream. Cells which were challenged in 35% whipping cream had a highly significantly different heat resistance from that of the cells challenged in skim milk, 2% UHT milk, and 10% cereal cream. No difference was found between the thermotolerances of challenged cells in skim milk, 2% UHT milk, or 10% cereal cream.

Cells which were tempered in MRM medium were not expected to show an increase in thermotolerance due to tempering, because earlier tempering experiments in phosphate buffer, phosphate buffer plus casamino acids, and phosphate buffer plus glucose, were unsuccessful (Fedio, 1991). It can be seen, however, from figure 4.1 that tempering did occur in this medium. The characteristic plateau displayed by *L. monocytogenes* when heated in TSBYE (figure 4.2) was not seen when the MRM medium was the tempering and the challenging menstruum. Further attempts to

determine which components of the minimal medium were responsible for tempering determined that tempering did not occur in suspensions of the four required amino acids (cysteine, isoleucine, leucine and valine) suspended in phosphate buffer, in phosphate buffer plus glucose, or in phosphate buffer plus glucose, amino acids and magnesium chloride.

4.4 Discussion

L. monocytogenes became tempered in several milk products varying in fat content from 0.1% to 35%, in a complex broth, and in a minimal medium. The heat resistance of the untempered cells challenged in 35% butterfat whipping cream was highly significantly different than the heat resistance of the cells challenged in skim milk, 2% UHT milk, or 10% butterfat cereal cream. There was no significant difference between the thermotolerance of cells tempered and challenged in skim milk, 2% UHT milk, 10% butterfat cereal cream, and 35% butterfat whipping cream. The protective role of butterfat is well documented, and most dairies increase the pasteurization time for high butterfat and high viscosity products, such as 35% cream and ice cream mix. Our results indicate that tempering occurs in dairy products with differing levels of butterfat, however, that the level of butterfat has no significant effect on the D_{60} value of the tempered cells. The D_{60} values of the untempered and challenged cells, though, are influenced by the level of butterfat present in the challenging medium. Cells challenged in 35% butterfat whipping cream had a highly significantly different D_{60} value when compared with cells challenged in skim milk, 2% UHT milk, and 10% cereal cream.

Tempering was observed in a complete minimal medium, but experiments using an incomplete medium were not successful. This implies that even if *L. monocytogenes* is grown in a complex medium such as TSBYE, the cells may still become tempered in a medium which is not as rich as the medium in which they were

grown. It is evident that *L. monocytogenes* requires many nutrients to produce a tempered state when exposed to a sublethal heat treatment; the presence of amino acids, dextrose and magnesium chloride in a phosphate buffer alone did not permit tempering. Fedio (1991) found that *L. monocytogenes* became tempered in phosphate buffer plus yeast extract. He recommended that more work be done to determine which nutrients are necessary to permit tempering.

In conclusion, the medium in which *L. monocytogenes* Scott A is suspended for tempering and challenging affects the heat resistance of the cells. Untempered cells display greatest heat resistance in 35% butterfat whipping cream (significantly different at 99%). No significant difference was found between the D_{60} values of the cells tempered and challenged in skim milk, 2% UHT milk, 10% cereal cream, or 35% whipping cream. *L. monocytogenes* Scott A was able to become tempered in both a rich medium (TSBYE) and a minimal one (MRM medium), but not in an incomplete medium consisting of phosphate buffer, essential amino acids, dextrose and magnesium chloride. Further experimentation is needed to determine which components of the MRM medium are necessary for tempering to occur in *L. monocytogenes*.

Table 4.1: Mean D_{60} values of *L. monocytogenes* challenged in various butterfat products

% butterfat	Untempered		Tempered*	
0.1 (skim milk)	1.5	a**	4.4	c
2.0 (UHT milk)	1.6	a	8.7	c
10 (cereal cream)	1.4	a	5.4	c
35 (whipping cream)	4.0	b	7.0	c

* = at 48°C for 60 minutes in the test medium before challenging at 60°C
** = no significant difference using Duncan's Multiple Range Test between treatments with the same letters

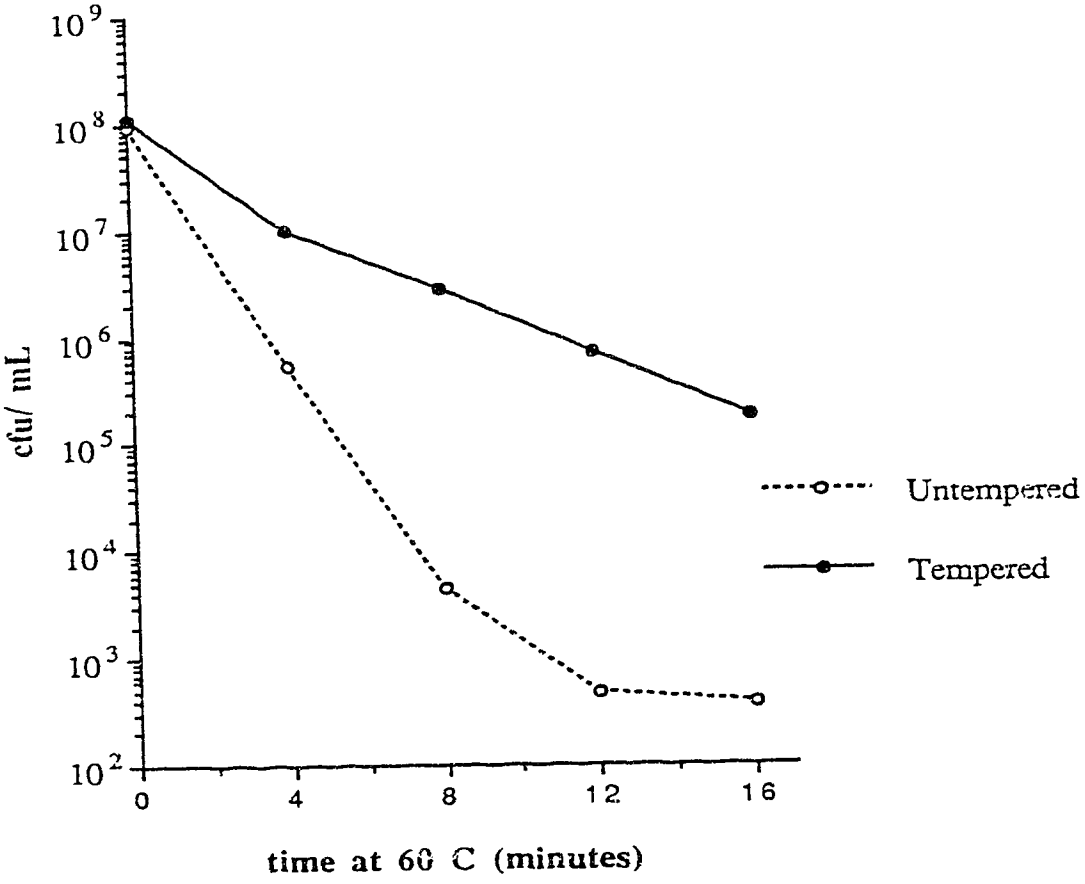

Figure 4.1: Effect of tempering and challenging in modified Ralovich's minimal medium on the heat resistance of *L. monocytogenes*

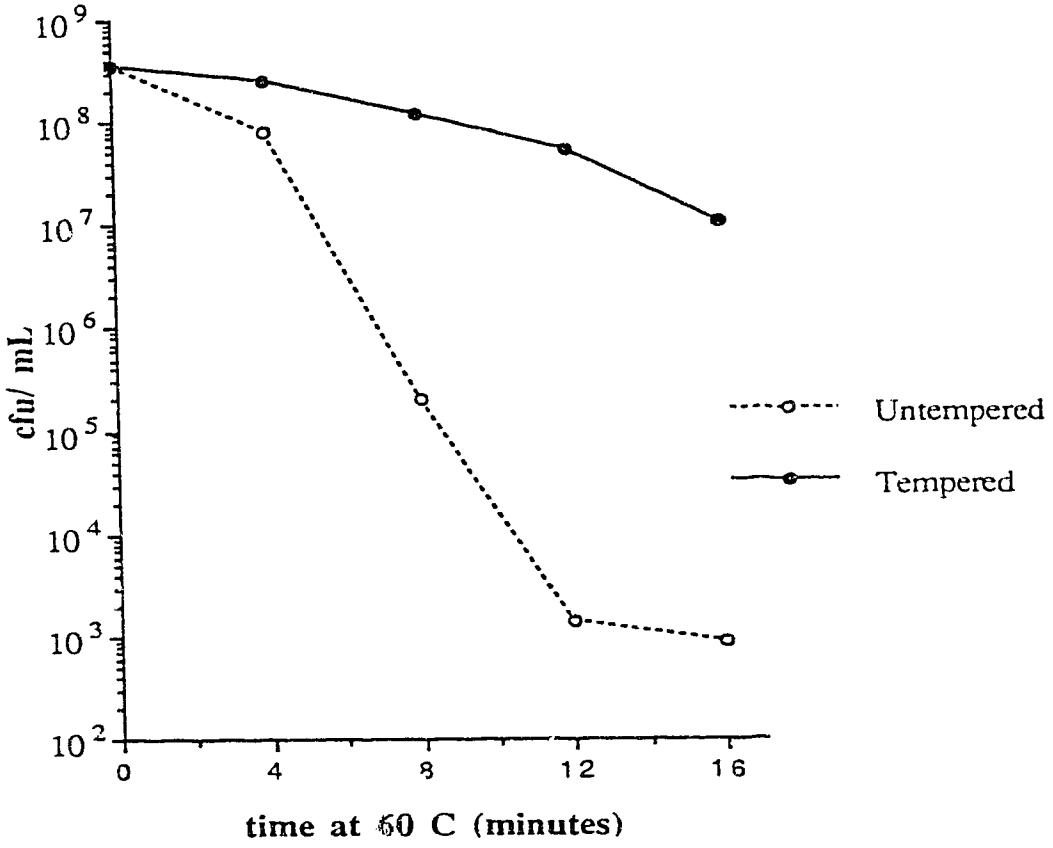

Figure 4.2: Effect of tempering and challenging in TSBYE on the heat resistance of *L. monocytogenes*

4.5 References

Bunning, V. K., R. G. Crawford, J. T. Tierney and J. T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella typhimurium* after sublethal heat shock. *Applied and Environmental Microbiology* **56**: 3216- 3219

Farber, J. M. and B. E. Brown. 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. *Applied and Environmental Microbiology* **56**: 1584- 1587

Farber, J. M. and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews* **55**: 476- 511

Fedio, W. M. 1991. Effects of tempering menstrua and the ribosomal response to tempering. pp. 89- 109 **In:** Studies on the Incidence and Origins of *Listeria monocytogenes* in Raw Milk and Factors Affecting its Thermal Destruction. Ph. D. thesis, University of Alberta, Edmonton

Fedio, W. M. and H. Jackson. 1989. Effect of tempering on the heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology* **9**: 157- 160

Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audrier, C. V. Broome and A. L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *New England Journal of Medicine* **312**: 404- 407

Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, A. Audrier, B. D. Plikaytis, S. L. Fannin, A. Kleks and C. V. Broome. 1988. Epidemic listeriosis associated with Mexican- style cheese. *New England Journal of Medicine* **319**: 823- 828

Quintavalla, S. and S. Barbuti. 1989. Caractérisation microbiologique et résistance thermique de *Listeria* isolée de produits de viande. *Science des Aliments* **9**: 125- 128

Quintavalla, S. and M. Campanini. 1991. Effect of rising temperature on the heat resistance of *Listeria monocytogenes* in meat emulsion. *Letters in Applied Microbiology* **12**: 184- 187

Ralovich, B. 1984. Nutritional requirements of *Listeria*. pp. 15- 16 **In**: Listeriosis Research Current Situation and Perspective. Akadémiai Kiadó, Budapest

5. Conclusions

Factors which affect the acquisition and retention of the tempered state in *L. monocytogenes* include: the temperature and medium of storage after tempering, the pH of the tempering medium, the butterfat content of the challenging media, and the nutritional composition of the tempering menstruum.

- When stored at 0° or 4°C, tempered *L. monocytogenes* retained its thermotolerance for an extended period of time in milk, TSBYE, and pH 6.7 TSBYE.
- *L. monocytogenes* tempered in TSBYE and stored at 4°C, decreased in thermotolerance gradually over a period of 11 days of storage, while cells tempered and stored in 2% UHT milk decreased in thermotolerance over a period of 21 days.
- Cells tempered in TSBYE adjusted to the pH of 2% UHT milk (pH 6.7) and stored at 4°C showed less thermotolerance after 14 days of storage than did cells tempered in milk, but greater heat resistance than the cells tempered in TSBYE (pH 7.2) and stored at 4°C.
- Storage at 0°C in both TSBYE and 2% UHT milk results in the retention of the tempered state for at least 7 days.
- Tempered cells of *L. monocytogenes* incubated at 37°C for periods from 30 to 120 minutes showed a statistically significant decrease in heat resistance, indicating that the acquisition of thermotolerance is partially reversible.
- Cells become tempered in TSBYE over a wide range of pH, however the effect is optimal at pH 7.5 and drops off considerably at pH values above and below this figure.
- Exposure to very high pH (pH 9.1) did not result in increased heat resistance of untempered cells, when compared to the heat resistance of untempered cells at neutral pH.

- The butterfat content of the tempering and challenging media affects the heat resistance of *L. monocytogenes*. There was no difference in heat resistance of untempered cells challenged at 60°C in 0.1, 2, and 10% butterfat, but at 35%, the difference was statistically significant.
- When cells were tempered and challenged at 60°C in 0.1, 2, 10, and 35% butterfat, the heat resistance of the tempered cells was greater than that of untempered cells heated at the same butterfat level and the differences were statistically significant.
- *L. monocytogenes* became tempered in modified Ralovich's medium, a minimal defined medium, but not in selected components of the medium consisting of essential amino acids, dextrose and magnesium chloride in a phosphate buffer. The plateau observed when *L. monocytogenes* is heated in TSBYE was not observed in the minimal medium.

Further studies should be undertaken to evaluate pasteurization treatments of products which are exposed to high temperatures during formulation, or inadvertently, even when the product is stored before pasteurization. The pH plays a role in determining the degree of increased thermotolerance shown by *L. monocytogenes*, and more intensive studies will better define this role of pH. As well, more research should be done to determine the factors in the medium responsible for the tempering effect.

Although increased thermotolerance occurs under a wide variety of conditions, the results of the present study, together with those of other workers, would indicate that it is improbable that tempered cells of *L. monocytogenes* could survive normal pasteurization treatments.

Appendix I. Modified Ralovich's minimal medium

Components	Stock solutions (mg/ 100 mL distilled water)	Quantity of stock solution in the medium (mL/ 100 mL medium)
KH_2PO_4 and K_2HPO_4	4500 and 10 500	10
Na_3 citrate•2 H_2O and $(NH_4)_2SO_4$	5000 and 10 000	1.0
* dextrose	25 000	4.0
Na thioglycollate	11 000	1.0
$FeSO_4$•2 H_2O	66	1.0
$MgSO_4$•7 H_2O	20 000	0.1
$CaCl_2$•6 H_2O	1 330	0.1
DL- isoleucine	2 000	1.0
L- leucine	1 000	1.0
DL- valine	2 000	1.0
‡ L- cysteine- HCl	4 000	1.0
* ‡ riboflavin	20	2.0
* ‡ D- biotin	20	2.0
* ‡ thiamine- HCl	100	1.0
distilled H_2O		80.0

*= sterilized by filtration

‡= stored in dark glass bottles

Adapted from: Ralovich, B. 1984 Nutritional requirements of *Listeria*. pp. 15-16 In: Listeriosis Research Present Situation and Perspective. Adadémiai Kiadó, Budapest