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UNIVERSITY OF ALBERTA

Studies on the incidence and origins of *Listeria monocytogenes* in raw milk and factors affecting its thermal destruction

by

Willis Martin Fedio

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Animal Health Division

O.S. Longman Building, 6909 - 116 Street, P.O. Box 8070, Edmonton, Alberta, Canada T6H 4P2 Fax 403/422-3438

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Dr. Matt Schoonderwoerd
Head,
Bacteriology Section

Dr. Ralph Shute
Head,
Health Management Branch

University of Alberta
Edmonton
Canada T6G 2P5

Department of Food Science
Faculty of Agriculture and Forestry
2-06 Agriculture-Forestry Centre, Telephone (403) 492-3236
Fax (403) 492-4265

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H. Jackson
Professor, Food Microbiology
Department of Food Science
University of Alberta

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NAME OF AUTHOR

Willis Martin Fedio

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PERMANENT ADDRESS: 12335-92 Street
Edmonton, Alberta
T5G 1B5

Date: 24 Sept, 1991

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **Studies on the incidence and origins of *Listeria monocytogenes* in raw milk and factors affecting its thermal destruction**

submitted by **Willis Martin Fedio**

in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Food Microbiology**

Dr. P. Sporns, Chairman

Dr. H. Jackson, Supervisor

Dr. D. L. Collins-Thompson

Dr. L. Ozimek

Dr. M. M. Palcic

Dr. R. Whitehouse

Date: Sept. 17, 1991

To Sophie and Chloé

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Abstract

The incidence of *Listeria monocytogenes* in 426 bulk tank milk samples collected in Alberta was found to be 1.9%.

Sources of *L. monocytogenes* were examined on four dairy farms with a history of shipping contaminated milk ('Listeria positive'). Feed materials, including silage and silage effluent, hay, grain and beet pellets were positive for listeriae. Silage, silage effluent and beet pellets yielded *L. monocytogenes*. Listeriae and *L. monocytogenes* were also isolated from environmental samples.

Fecal shedding of listeriae was detected in 33% of samples from 'Listeria positive' farms, with 14.5% of the samples containing *L. monocytogenes*. Rectal fecal samples from three farms with no history of shipping contaminated milk ('Listeria negative' farms) showed 24.6% positive for listeriae, and 13.2% positive for *L. monocytogenes*.

Of non-aseptic individual cow milk samples, 8% contained listeriae, and 5% *L. monocytogenes*. Aseptic quarter sampling of *Listeria*-positive cows revealed that *L. monocytogenes* was being shed in the milk by only one quarter of one animal which was subsequently shown to have listerial mastitis. Milk from the infected quarter was found to contain 2,000 to 5,000 organisms/mL. Intramammary therapy with erythromycin and cephaparin failed to resolve the infection.

The high incidence of *L. monocytogenes* in the feed, fecal and environmental samples compared with aseptic quarter samples indicates the farm environment and not the udder is the most likely source of the organism in raw milk.

L. monocytogenes tempered at 48°C for 1 h in broth before heating at 60°C showed a marked increase in heat resistance compared with untreated controls. The increase in thermotolerance was retained even after storage at 1°C for 24 h. Acquired thermotolerance was also observed when the preheating and heating menstruum were UHT milk (2% milk fat). The composition of the preheating menstruum affected the development of acquired thermotolerance. Cells treated with chloramphenicol, rifamycin and 2,4-dinitrophenol during tempering showed reduced enhancement of heat resistance following tempering, compared to untreated controls. However, the heat resistance was still greater than that of untempered controls, indicating that protein synthesis, RNA synthesis and energy yielding mechanisms are involved in the tempering effect.

Tempering at 48°C for 1 h stabilized the ribosomes of *L. monocytogenes* to subsequent heating, compared with untempered controls. The significance of this finding in relation to thermotolerance is discussed.

Table of Contents

List of Tables

List of Figures

1. Introduction

1.1 Cause for concern in the food industry

Listeria monocytogenes was recognized as a pathogen of man and other animals in the early years of this century, but its role as a food-borne pathogen was not established until the 1980's. Two large outbreaks, in 1983 and 1985, involving 62 deaths were linked to the consumption of pasteurized dairy products (Fleming *et al.*, 1985; Linnan *et al.*, 1988). One effect of these outbreaks was to stimulate research on the causative organism, *L. monocytogenes*. In particular, attention was focused on the incidence and origins of the organism in the raw milk supply and its heat resistance relative to normal pasteurization treatments.

1.2 Objectives

At the onset of this study, the primary objectives were to determine the incidence of *L. monocytogenes* in raw milk in Alberta and to identify the origins of the organism at the farm level.

A parallel study was initiated later to investigate factors affecting the heat resistance of the organism. This part of the study was prompted by the work of Mackey and Derrick (1986, 1987) who showed that the heat resistance of vegetative bacterial cells could be increased by mild preheating.

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2. Literature Review

2.1 Historical survey

The Gram-positive bacillus now known as *Listeria monocytogenes* was first described in detail by Murray *et al.* in 1926. They described a fatal disease of rabbits which was characterized by a large mononuclear monocytosis and named the causative organism *Bacterium monocytogenes*. In 1927, Pirie reported a bacterial disease in wild gerbils, the so-called "Tiger River Disease", caused by an apparently identical microorganism which he called *Listerella hepatolytica* (Pirie, 1927). Pirie reported that infection could be produced in gerbils by subcutaneous inoculation or by feeding, and that the chief lesions produced by the organism were intestinal ulceration (when infection was by feeding), necrosis of the liver and spleen and bacteremia. Inoculation of gerbils subcutaneously with Murray's isolate produced effects indistinguishable from those caused by the Tiger River bacillus in similar doses, suggesting differences in host response to the same pathogenic organism (Pirie, 1927). After the identity with *Bacterium monocytogenes* had been recognized, the organism was named *Listerella monocytogenes*.

There is little doubt that the bacterium described by Murray *et al.* had been isolated before by other workers. As early as 1891, Hayem, in France, and in 1893, Henle, in Germany, observed Gram-positive rods in tissue sections from patients who had died of disease that in retrospect was almost certainly listeric infection (Gray and Killinger, 1966). In 1911, the Swedish worker Hülphers described a short Gram-positive rod, isolated from a rabbit with miliary necrosis of the liver, which he named *Bacterium hepatis* (Errebo Larsen, 1969). Although the original isolates were not preserved, photographs of the lesions and of surface and stab cultures are compatible with listeriosis and growth of *Listeria* (Seeliger, 1958). In 1921, Dumont and Catoni, in France, isolated a Gram-positive rod shaped bacterium from a case of meningitis in man (Gray and Killinger, 1966). The culture was isolated and preserved at the Pasteur Institute in Paris and later identified as *Listeria monocytogenes* in 1942 (Seeliger, 1958).

In 1929, Nyfeldt reported the first isolation of *L. monocytogenes* from a human, a boy with infectious mononucleosis (Barza, 1985). Nyfeldt thought that he had found the causative organism of infectious mononucleosis and three years later described similar isolates from 3 of 10 patients with infectious mononucleosis and

referred to the organism as *Listerella monocytogenes hominis* (Gray and Killinger, 1966).

The first report of listeric infection in domestic farm animals was that of Gill who reported a disease in sheep where the affected animals were described as having "circling disease" (Gill, 1931). Gill was able to isolate the Gram-positive rod shaped bacterium from the brain of diseased sheep and draw an association between it and ovine encephalitis (Gill, 1933). Gill (1937) named the organism *Listerella ovis*.

In 1951, the first confirmed case of human listeriosis in Canada occurred in Ontario where a 24 year old pregnant woman was found to be infected with *L. monocytogenes* serotype 4b (Stoot, 1954).

2.2 Taxonomy

The name *Listerella* had already been given to a Mycetozoan (slime mold) by Jahn in 1906, therefore the generic name *Listeria* was proposed (Pirie, 1940). *Listeria* was adopted in the sixth edition of Bergey's Manual of Determinative Bacteriology, approved by the Judicial Commission on Bacterial Nomenclature and Taxonomy in 1954 and became the official generic name (Gray and Killinger, 1966). The species name *monocytogenes*, as suggested by Murray *et al.* (1926), was conserved, making the official name *Listeria monocytogenes*, Pirie (Gray and Killinger, 1966).

In the eighth edition of Bergey's Manual of Determinative Bacteriology, *Listeria* was placed in a section headed "Gram-positive, asporogenous, rod shaped bacteria". In addition to *L. monocytogenes*, three other species of *Listeria* were described: *L. murrayi, L. grayi and L. denitrificans* (Seeliger and Welshimer,1974). *L. monocytogenes* was the only species associated with diseases of man and other animals; however, *L. monocytogenes* was recognized as being heterogeneous, containing both pathogenic and non-pathogenic isolates (Seeliger and Welshimer, 1974). The specific designation of the non-pathogenic strains of *L. monocytogenes* was considered misleading by Groves and Welshimer (1977) who felt that it could result in erroneous interpretation of the role of *L. monocytogenes* in human and veterinary medicine.

Results of a numerical taxonomic study in 1977 of *Listeria* and related bacteria showed that the genus *Listeria* contained three subgroups corresponding to: 1) *Listeria monocytogenes*, 2) *Listeria grayi* and 3) non-hemolytic listeria strains (Wilkinson and Jones, 1977). *L. murrayi* was not sufficiently distinct from *L. grayi* to warrant separate species status and both were considered as part of the same subgroup (Wilkinson and Jones, 1977).

By examining virulent and non virulent strains of *L. monocytogenes* in mouse virulence tests, it became apparent that non-hemolytic strains were eliminated from the test animals, whereas hemolytic strains multiplied rapidly in the spleens and livers (Sceliger, 1984). The non-hemolytic, apathogenic listeria were given the name *Listeria innocua* (Seeliger, 1981).

Strongly hemolytic *Listeria* associated with sheep abortions in Bulgaria were reported by Ivanov in 1962. Isolates designated *L. monocytogenes* serotype 5 were considered to be sufficiently different from other strains of *L. monocytogenes* to be given special species status, and the name *Listeria bulgarica* was suggested (Ivanov, 1975).

Rocourt *et al.* (1982) characterized 66 strains of *L. monocytogenes* (as defined by Seeliger and Welshimer (1974)) by DNA relatedness and found 5 distinct groups: 1) *Listeria monocytogenes sensu stricto*, 2) *L. bulgarica* (*L. monocytogenes* serovar 5), 3) *L. innocua* and two unnamed but phenotypically distinct groups (groups 4 and 5). All five groups were clearly distinct from *L. murrayi* and *L grayi* (Rocourt *et al.*, 1982). Rocourt and Grimont (1983) proposed the names *Listeria welshimeri* and *Listeria seeligeri* for the the isolates falling into genomic groups 4 and 5, pointing out that the separation of both of the new species was not only supported by the results of DNA relatedness, but also by determinations of biochemical characteristics and studies of pathogenicity for adult mice.

L. monocytogenes serovar 5 was named *L. bulgarica* by Ivanov in 1975, however, no type strain was designated and the name lost standing in bacterial nomenclature when it was omitted from the 'Approved lists of bacterial names' (Skerman *et al.*, 1980). Although Seeliger *et al.* (1982) recommended that serovar 5 isolates be classified as either a subspecies "*L. monocytogenes* subsp. *perhaemolytica*" or as a distinct species named either "*L. perhaemolytica*" or "*L. ivanovii*", it was not in the form of a proposal for recognition of a new species and the name was not changed until 1984 when Seeliger *et al.* (1984) formally named the organism *Listeria ivanovii*.

An organism isolated by Sohier *et al.* in 1948 was placed within the genus *Listeria* without species status and was later named *L. denitrificans* by Prevot in 1961 (Rocourt *et al.*, 1987). *L. denitrificans* has been shown by numerical taxonomic, morphological, biochemical, serological, and nucleic acid studies to be sufficiently different from the other *Listeria* species to justify its removal from the genus *Listeria* (Welshimer and Meridith, 1971; Stuart and Pease, 1972; Stuart and Welshimer, 1973, 1974; Jones, 1975; Rocourt *et al.*, 1982; Collins *et al.*, 1983). In Bergey's Manual of

Systematic Bacteriology, vol. 2, it remained within the genus *Listeria* as a misclassified organism (Seeliger and Jones, 1986). Rocourt *et al.* (1987) examined the 16S ribosomal ribonucleic acid of the organism and found oligonucleotide sequences that were actinomycete-specific, as compared with *L. monocytogenes* which is a member of the *Bacillus-Clostridium* subdivision of Gram-positive bacteria. The low similarity coefficients found between *L. denitrificans* and the genera comprising the *Arthrobacter* subgroup of the actinomycetes subdivision, together with previously described chemotaxonomic properties indicated that it should be transferred to a new genus, *Jonesia* gen. nov., as *Jonesia denitrificans* comb. nov.(Rocourt *et al.*, 1987).

L. murrayi and *L. grayi* are considered as *species insertae sedis* in Bergey's Manual of Systematic Bacteriology vol.2 (Seeliger and Jones, 1986). Numerical taxonomic, serological and nucleic acid studies have shown that *L. grayi* and *L murrayi* are very closely related and that both species are more distantly related to the other listeria (Stuart and Welshimer, 1973, 1974; Wilkinson and Jones, 1975, 1977; Rocourt *et al.*, 1982). Stuart and Welshimer (1974) proposed that *Listeria grayi* and *Listeria murrayi* be transferred to a new genus, *Murraya*, as *M. grayi*, and *M. grayi* subsp *murray*, respectively and proposed the formation of a new family, *Listeriaceae* for the genera *Listeria* and *Murraya*. These designations were not in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980), but were repeated in Bergey's Manual of Systematic Bacteriology, vol.2 (Seeliger and Jones, 1986).

Eight species of *Listeria* are described in Bergey's Manual of Systematic Bacteriology, vol.2 :

Listeria monocytogenes
Listeria innocua
Listeria welshimeri
Listeria seeligeri
Listeria ivanovii
*Listeria grayi**
*Listeria murrayi**
*Listeria denitrificans**

**species incertae sedis*

2.3 Nature of the organism

2.3.1 Morphology and cultural characteristics

Listeria monocytogenes is a small Gram-positive, nonsporeforming, short rod shaped bacterium, 0.4-0.5 μm in diameter and 0.5-2 μm in length that occurs singly, in short chains or arranged at an angle to each other to give V forms or occurs in groups lying parallel to each other along the long axis (Seeliger and Jones, 1986). It is motile by means of a few peritrichous flagella and exhibits characteristic "tumbling motility" when cultured at 20-25°C, but may be non-motile when cultured at 37°C (Gray and Killinger, 1966). Stab cultures into semi-solid motility medium produce a typical, inverted "pine tree" effect (Gray and Killinger, 1966). This temperature dependent motility is commonly referred to as "umbrella motility".

L. monocytogenes grows on nutrient agar to give colonies after 24-48 h that range from 0.5-1.5 mm in diameter, depending on colony density. Colonies are round, translucent, low convex with a finely textured surface, entire margin and a blue-gray coloration (Seeliger and Jones, 1986). When cultures grown on a translucent medium are viewed under a binocular scanning microscope using obliquely transmitted light as described by Henry (1933), the colonies have a finely textured surface, almost like ground glass, and a distinctive blue-green color (Gray *et al.*, 1948; Gray, 1957). This coloration is so characteristic that colonies of *L. monocytogenes* can be identified on suitable agars containing high numbers of contaminants. Cultures grown on agars containing 0.1-1% w/v glucose have a characteristic sour, buttermilk-like odor (Seeliger and Jones, 1986). When grown on blood agar at 37°C for 48 h, colonies are 0.2-1.5 mm in diameter and translucent, becoming grayish-white to opaque with age (Lovett, 1989). The organism produces a narrow zone of β-hemolysis on blood agar which frequently does not extend beyond the edge of the colony, and may be detected only by removing the colony (Seeliger and Jones, 1986), or by stabbing the organism into the agar.

L. monocytogenes can grow in a wide pH range (pH 6 to pH 9) (Seeliger and Jones, 1986). Optimal growth is observed in neutral to slightly alkaline media, but the organism will grow over a range from as high as pH 9.6 to as low as pH 5.6 (Gray and Killinger 1966). Cabbage juice (pH 5.6) was found to support the growth of *L. monocytogenes* at 30°C but cabbage juice adjusted to pH 5.0 with lactic acid still supported growth at 30°C but not at 5°C (Conner *et al.*, 1986). In cabbage juice adjusted to pH 4.6 and 4.4 with lactic acid the organism was inactivated at 30°C

(Conner *et al.*, 1986). Parish and Higgins (1989) examined the growth of *L. monocytogenes* in pH adjusted tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) and found that after incubation at 30°C, all four strains examined grew at pH 4.5. In pH adjusted TSBYE, Conner *et al.* (1990) found that the minimum pH at which growth did not occur depended upon the acidulant used. These authors found that the organism would not grow below a test pH of 5.0 using propionic acid, 4.5 using acetic and lactic acids and 4.0 using citric and hydrochloric acids.

Growth of *L. monocytogenes* was observed in tryptic soy broth which was adjusted to a_w of 0.93 by the addition of 37.5% sucrose to the broth but not in broth adjusted to a_w of 0.92 by the addition of 39.4% sucrose (Peltran and Zottola, 1989).

L. monocytogenes has been reported to be quite halotolerant and exhibits growth in 10% NaCl (Seeliger, 1961). However Shahamat *et al.* (1980) found that 10.5% NaCl caused inactivation of *L. monocytogenes* with a 5 log decrease in numbers over a two week period at 37°C. Shahamat *et al.* (1980) found that the survival time for *L. monocytogenes* in trypticase soy broth containing 25.5% salt was greatly influenced by temperature with survival time being 5 d at 37°C, 32 d at 22°C and more than 132 d at 4°C. Growth of *L. monocytogenes* was observed in unclarified cabbage juice containing 2.0% NaCl, but not 5.0% NaCl (Conner *et al.*, 1986).

Refrigeration is used to maintain the temperature of perishable foods at 4 to 5°C in order to prevent the growth of pathogenic microorganisms (Pearson and Marth, 1990). However, refrigeration provides only limited protection from *L. monocytogenes* due to the organism's ability to grow at those temperatures. *L. monocytogenes* can grow from 1°C to about 45°C, with optimum growth between 30 and 37°C (Gray and Killinger, 1966). *L. monocytogenes* in naturally contaminated milk was found to grow at 4°C after a lag period of between 3 to 5 d (Farber *et al.*, 1990). The average generation time was 25.3 h with a final population density of 2 x 10^7 cfu/mL (Farber *et al.*, 1990). Skim, whole and chocolate milk and whipping cream were found to support the growth of *L. monocytogenes* at 4°C (Rosenow and Marth, 1987). After an initial lag of between 5 and 10 d, doubling times of about 36-48h were observed and the final populations were in excess of 10^7cfu/mL for all products and strains examined (Rosenow and Marth, 1987). Minimum growth temperatures ranging from - 0.1 to - 0.4°C were reported for three strains of *L. monocytogenes* grown in both chicken broth and UHT milk, using a rocking temperature gradient incubator (Walker *et al.*, 1990).

2.3.1.1 Heat resistance

Since Fleming *et al.* (1985) linked the consumption of pasteurized milk to cases of human listeriosis, there have been many studies on the heat resistance of the organism. In a survey of pasteurized milk, two percent of more than 700 pasteurized milk samples tested in the U.S. were found to be culture positive for *L. monocytogenes* (CDC, 1988). In Spain, Fernandez-Garayzabal *et al.* (1986) were able to isolate *L. monocytogenes* from 6 of 28 (21.4%) pasteurized whole milk samples that had been given a high temperature, short time (HTST) pasteurization treatment (78°C for 15 s). Although these findings may represent post-pasteurization contamination, the efficacy of pasteurization for killing *L. monocytogenes* was questioned.

Fleming *et al.* (1985) suggested that the intracellular position of *L. monocytogenes* in naturally infected cows milk could protect the organism from heat inactivation. The authors cited a study by Bearns and Girard (1958) which demonstrated that with 'holder' type pasteurization at 61.7°C for 35 min, organisms could survive heating in sterilized skim milk if inoculated at a level of $5x10^4$ cfu/mL or more. Bearns and Girard (1958) observed D-values at 61.7°C of 9.5 to 10.8 min. depending on the strain used. Donnelly *et al.* (1987) found that the test tube method, used by Bearns and Girard (1958) for the determination of heat resistance resulted in extensive tailing of survivor curves, which would give an inaccurate and inflated indication of the heat resistance of the organism.

Using sealed glass tubes containing *L. monocytogenes* suspended in raw whole milk, Bradshaw *et al.* (1985) observed much lower D-values (4.8 min at 57.8°C and 0.3 min at 63.3°C) than those reported by Bearns and Girard (1958). Donnelly *et al.* (1987) found $D_{62°C}$ values ranging from 0.1-0.4 min. Several studies have shown that when 10^5 to 10^6 cfu/mL of *L. monocytogenes* were freely suspended in whole milk, the organism would not survive vat pasteurization (62.8°C for 30 min) or HTST pasteurization (71.7°C for 15 s) (Bradshaw *et al.*, 1985; Donnelly and Briggs, 1986; Donnelly *et al.*, 1987).

Thermal resistance can also be determined using a continuous two phase (air-liquid), slug flow, tubular heat exchanger. Air added to the tubing compartmentalizes the liquid in the tubing and allows the precise measurement of the residence time of the fastest flowing particles in the heating, holding and cooling portions of the instrument (Stroup *et al.*, 1969). The slug flow heat exchanger method is designed to give exposure times and temperatures in the range of HTST pasteurization (Stroup *et*

al., 1969). Bunning *et al.* (1988) used both the sealed tube method and slug flow heat exchanger method to show that the intracellular location of *L. monocytogenes* within bovine phagocytes did not significantly increase their heat resistance. However, the authors concluded that the $D_{71.7°C}$ value of 4.1 s indicated a potentially unsafe 3.7D margin of safety for the HTST pasteurization minimum (71.7°C for 15 s).

Mackey and Bratchell (1989) reviewed heat resistance data for *Listeria monocytogenes* and found, for milk, that heating in sealed tubes and heating using the slug flow heat exchanger yielded different estimates of lethality. Thermal inactivation studies using the sealed tube method indicate that vat pasteurization (62.8°C for 30 min) would result in a 39D reduction in numbers, while high temperature short time (HTST) pasteurization (71.7°C for 15 s) would cause a 9.3D reduction. According to Mackey and Bratchell (1989) the slug flow model predicts a 5.2D reduction in numbers for the HTST treatment and suggested the organism might survive pasteurization if the initial counts were high.

Fernandez-Garayzabal *et al.* (1987) were able to isolate *L. monocytogenes* from raw milk which had been inoculated with the organism at levels of $3x10^6$, $1x10^7$ and $2x10^8$ cfu/mL following pasteurization of the milk at 69°C for 15 s and at 72°C for 15 s but not at 73°C for 15 s. However, *L. monocytogenes* was only isolated from heated samples after cold enrichment for 1 or 3 weeks at 4°C indicating that only a low number of cells survived the heating. The high initial cell numbers of the inoculated milk used in this study demonstrate pasteurizer overload and not a failure of pasteurization under simulated natural conditions (Lovett *et al.*, 1990).

Doyle *et al.* (1987) reported that milk from cows experimentally infected with *L. monocytogenes* was not always free of the organism after HTST pasteurization at 71.7°-73.9°C for 16.4 s but was after 76.4°-77.8°C for 15.4 s. Survivors were detected only following enrichment culture of the heated milk. Since the milk used in this study was collected only from cows that were shedding *L. monocytogenes* in their milk, the concentration of the pathogen (10^3 to 10^4 cfu/mL) is also likely to be higher than what would be normally be found in mixed milk at a processing plant (Lovett *et al.*, 1990).

Milk naturally contaminated with *L. monocytogenes* serotype 1 at levels of about 10^4 cfu/mL was subjected to HTST pasteurization treatments for 16.2 s at temperatures ranging from 60 to 78°C (Farber *et al.*, 1988). In the naturally contaminated milk, *L. monocytogenes* survived one of three trials at 66°C, but no survivors were detected at 67, 69, 72, 75 or 78°C (Farber *et al.*, 1988). When raw whole milk was inoculated with 10^5 cfu/mL *L. monocytogenes* survival was observed

at temperatures up to 67.5°C, indicating that HTST pasteurization was effective in killing *L. monocytogenes* in naturally contaminated milk (Farber *et al.*, 1988).

Lovett *et al.* (1990) found that HTST pasteurization (71.7°C for 15 s) was effective in inactivating *L. monocytogenes* that was freely suspended ($2.6x10^5$ cfu/mL), internalized in bovine phagocytes *in vitro* (5×10^4 cfu/mL) and in milk that was from cows experimentally infected with *L. monocytogenes in vivo* (3×10^3 cfu/mL). The authors used four assay procedures for survivors after the heat treatments, however, no survivors were detected.

The results on heat resistance of *L. monocytogenes* confirm the WHO position that pasteurization is a safe process which reduces the number of *L. monocytogenes* occurring in raw milk to levels that do not pose a threat to human health (WHO Working Group, 1988).

2.3.2 Biochemical characteristics

Listeria monocytogenes is facultatively anaerobic, catalase positive and ferments glucose to produce acid, but no gas. Both aerobically and anaerobically, the catabolism of glucose proceeds by the Embden-Meyerhof pathway (Seeliger and Jones, 1986). Anaerobically, the end product is mainly lactic acid; while the end products of aerobic metabolism include pyruvate, acetoin, lactic acid and other products (Seeliger and Jones, 1986). Acid production from L-rhamnose, alpha-methyl-D-mannoside and D-xylose are characteristics used to differentiate *Listeria* spp. (Table 2.1).

The CAMP test (Christie *et al.*, 1944), can be used to confirm the B-hemolytic reaction of *Listeria* species (Brzin and Seeliger, 1975). Both *L. monocytogenes* and *L. seeligeri* demonstrate enhanced hemolysis within the lytic zone of a B-toxin producing *Staphylococcus aureus* strain. A modified CAMP test using *Rhodococcus equi* was used to identify *Listeria ivanovii* (Ivanov, 1975). Enhanced hemolysis by *L. ivanovii* can be seen within the lytic zone of *R. equi* but not *S. aureus* (Ivanov, 1975).

2.3.3 Serology

The currently accepted serological scheme for *Listeria* is based on that described by Paterson (1940). Agglutination reactions of *Listeria* with highly absorbed rabbit antisera allowed the identification of a number of flagellar (H) and somatic (O) antigens. Paterson (1939) demonstrated the presence of four H antigens in *L. monocytogenes*. For the flagellar antigens he found a common factor, B, and three other factors, A, C, and D. Paterson (1940) recognized five O antigens which

Table 2.1 Biochemical characteristics of *Listeria* spp.

	Listeria				
CHARACTERS	*monocytogenes*	*innocua*	*seeligeri*	*welshimeri*	*ivanovii*
β–hemolytic	+	-	+	-	++
CAMP test (*S. aureus*)	+	-	+	-	-
CAMP test (*R. equi*)	-	-	-	-	+
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
H_2S on TSI	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
Vogues-Proskauer	+	+	+	+	+
Methyl Red	+	+	+	+	+
Acid from:					
Mannitol	-	-	-	-	-
L-Rhamnose	+	V	+	+	+
D-Xylose	-	-	+	+	+
α-methyl-D-mannoside	+	+	-	+	+
Hydrolysis of:					
Esculin	+	+	+	+	V
Hippurate	+	+	+	+	+

(Adapted from Seeliger and Jones, 1986)

\+ 90% or more of strains are positive

– 90% or more of strains are negative

V strain instability

allowed him to classify 54 strains of *L. monocytogenes* into three somatic groups: (1) a group with factors I, II and (III); the latter being a factor common to all listeriae, (2) a group with factors II, IV and (III), and (3) a group with factors V and (III). Based upon agglutination test studies, with both H and O antisera, Paterson (1940) defined four serological types (Table 2.2).

Table 2.2 Paterson scheme for serological identification of *Listeria monocytogenes*.

Type	Flagellar antigens	Somatic antigens
1	A B	I II (III)
2	B D	I II (III)
3	A B	II (III) IV
4	A B C	(III) V

(Adapted from Paterson, 1940)

The demonstration of additional somatic antigenic factors by Seeliger and by Donker-Voet led to an expansion and refinement of Patterson's scheme (Donker-Voet, 1959, 1972; Seeliger, 1961, 1975, 1976).

The Seeliger–Donker-Voet antigenic scheme which divides the genus *Listeria* into 16 serovars is shown in Table 2.3.(Seeliger and Jones, 1986). Additional combinations of antigens have been reported (Ralovich, 1984). *Listeria monocytogenes* strains exhibit the antigenic composition of serovars 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and serovar 7 (Seeliger and Jones, 1986). The antigenic composition of the five *Listeria* spp. are shown in Table 2.4. (Seeliger and Jones, 1986). Serological differentiation aids in the identification of suspected cultures; however it has been of limited use in the elucidation of the epidemiology of listeriosis as most isolates from pathological sources belong to relatively few serovars (1/2a, 1/2b and 1/2c, 3a, 3b, and 3c, 4b and 5) (Seeliger and Höhne, 1979). Recent statistics from Britain, the United States and Canada of the serotype distribution in cases of human listeriosis (Table 2.5), underline the need for additional typing systems in order to elucidate the epidemiology of listeriosis.

Table 2.3 Serovars of *Listeria* spp.

Designation		O–Antigens	H–Antigens
Patterson	Seeliger – Donker-Voet		
1	1/2a*	I II (III)	AB
	1/2b*	I II (III)	ABC
2	1/2c*	I II (III)	B D
3	3a*	II (III) IV	AB
	3b*	II (III) IV (XII) (XIII)	ABC
	3c*	II (III) IV (XII) (XIII)	B D
4	4a*	(III) (V) VII IX	ABC
	4ab*	(III) V VII IX X	ABC
	4b*	(III) V VI	ABC
	4c*	(III) V VII	ABC
	4d*	(III) V VI VIII	ABC
	4e*	(III) V VI (VIII) (IX)	ABC
	5	(III) (V) VI (VIII) X	ABC
	7*	(XII) (XIII)	ABC
	6a (4f)	(III) V (VI) (VII) (IX) XV	ABC
	6b (4g)	(III) (V) (VI) (VII) IX X XI	ABC

**L. monocytogenes*

(Adapted from Seeliger and Jones, 1986)

Table 2.4 Distribution of serovars in *Listeria* spp.

Species	Serotype
Listeria monocytogenes	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c,4d, 4e, 7
Listeria ivanovii	5
Listeria innocua	6a, 6υ, 4ab, undesignated
Listeria welshimeri	6a, 6b
Listeria seeligeri	1/2b, 4c, 4d, 6b, undesignated

(Adapted from Seeliger and Jones, 1986)

Table 2.5 Serovar distribution of *L. monocytogenes* involved in cases of human listeriosis.

Serovar	Country		
	U.K.	U.S.A.	Canada
1/2a	130 (18%)	48 (30%)	4 (12.9%)
1/2b	99 (14%)	52 (32%)	13 (41.9%)
1/2c	29 (4%)	-	-
3	11 (1%)	-	3 (9.8%)
4b	423(59%)	53 (33%)	9 (29%)
other 4	29 (4%)	-	2 (6.5%)
3a,3b,1/2c	-	9 (5%)	
non-typable	1	-	-
total	722	162	31

(McLauchlin, 1987; Gellin and Broome, 1989; Varughese and Carter, 1989)

2.3.4 Bacteriophage typing

Bacteriophages have been isolated from *L. monocytogenes*, *L. innocua*, *L. seeligeri* and *L. ivanovii* (Sword and Pickett, 1961; Audurier *et al.*, 1977, 1979; Rocourt *et al.*, 1982). Isolates of *L. monocytogenes* were divided into eight groups based upon the lytic properties of four bacteriophages (Sword and Pickett, 1961). Audurier *et al.* (1977) established a typing scheme for *L. monocytogenes* using a set of 20 phages and were able to type 645/823 (78.4%) of the strains examined. The authors found that 88% of serotype 4 and 57% of serotype 1 strains could be typed and that the typable strains could be subdivided into 8 principal phage types: 3 types within serotype 1 and 5 phage types within serotype 4. A standardized phage typing scheme was established using 29 bacteriophages by Rocourt *et al.* (1985) which allowed 54% of serogroup 1/2 and 77% of serogroup 4 strains to be typed. Bacteriophage typing of *L. monocytogenes* has revealed that many phage types can be found within an apparently homogeneous serovar (Seeliger and Langer, 1989). However, not all *L. monocytogenes* strains are typable. The low percentage of typable strains of the serogroup 1/2 and the lack of phages specific for some of the rarely isolated serovars (3, 4a, 4ab, 4c, 4d, 4e and 7) illustrate the main limitation to phage typing and the need for more bacteriophages (Rocourt *et al.*, 1985). Of 2470 *L. monocytogenes* strains examined in France from 1985-1987, 699/826 (84.6%) of serogroup 4 strains were typable, but only 809/1644 (50.8%) of serogroup 1 strains were typable using a set of 35 phage (Audurier and Martin, 1989). They state that the typability of strains varies according to sample origin, and that most of the isolates from food and dairy products were serovar 1/2. Bacteriophage typing has been used for characterization of strains of *L. monocytogenes* involved in outbreaks of disease (Audurier *et al.*, 1984; Fleming *et al.*, 1985; Linnan *et al.*, 1988).

2.3.5 Multilocus enzyme electrophoresis

Polymorphic variation of metabolic enzymes has been used to estimate the genetic diversity and structure in natural populations of bacteria (Sealander *et al.*, 1986). Bacterial isolates are characterized by the relative electrophoretic mobilities of a large number of enzymes, and mobility variants (electromorphs) of each enzyme are equated with differences in genotype (Selander *et al.*, 1986). Each unique combination of electromorphs defines an electrophoretic type (ET). Multilocus enzyme electrophoresis (MEE) has been used to characterize isolates of *L. monocytogenes*. Piffaretti *et al.* (1989) analyzed 175 isolates for allelic variarion at

16 loci and found 45 distinctive electrophoretic types which clustered into two primary divisions (I and II). All isolates of serotypes 4a, 4b and 1/2b were of ETs in division I, while the isolates of all ETs in division II were of serotypes 1/2a and 1/2c (Piffaretti *et al.*, 1989). Bibb *et al.* (1989) examined 310 strains of *L. monocytogenes* and found 56 ETs. Gellin *et al.* (1991) examined 136 isolates and found that they were distributed among 37 distinct ETs. MEE identified three space-time clusters of listeriosis which suggested possible common-source outbreaks (Gellin *et al.*, 1991).

In Canada, 26 electrophoretic types were identified among the 35 clinical isolates examined during 1989 indicating that cases of listeriosis were associated with a heterogeneous population of *L. monocytogenes* (Varughese *et al.*, 1991). MEE has been used to demonstrate relationships between clinical isolates of *L. monocytogenes* and isolates recovered from foods (Farber *et al.*, 1990; Varughese *et al.*, 1991).

2.3.6 Restriction fragment length polymorphism

Restriction endonuclease analysis of the chromosomal DNA of *L. monocytogenes* has been used to study the epidemiology of listeriosis outbreaks (Facinelli *et al.*, 1988; Kaczmarski and Jones, 1989; Nocera *et al.*, 1990; Wesley and Ashton, 1991). Facinelli *et al.* (1988) described a case of cross-infection involving two babies infected with *L. monocytogenes* serogroup 1. The isolates were non-typable using bacteriophages, however identical 'DNA fingerprints' were obtained for the two strains after agarose gel electrophoresis of BamHI, EcoRI and HindIII digests of chromosomal DNA (Facinelli *et al.*, 1988).

For each strain of *L. monocytogenes*, Nocera *et al.* (1990) extracted the total DNA, digested it with EcoRI, and separated the DNA fragments by agarose gel electrophoresis to get a restriction endonuclease analysis (REA) profile. *Listeria* strains from species other than *L. monocytogenes* gave REA profiles that were clearly distinct from those obtained with the *L. monocytogenes* strains, and the *L. monocytogenes* strains were classified into 10 groups according to their REA profiles between 23.1 and 13.6 kbp (Nocera *et al.*, 1990). Although the REA patterns did not completely discriminate between strains of serotype 4b and 1/2b, all 57 serotype 4b strains that were identified as Swiss epidemic strains by phage typing clustered into two closely related REA profiles (Nocera *et al.*, 1990).

Wesley and Ashton (1991) used REA with the endonuclease HhaI to differentiate strains of *L. monocytogenes* serotype 4b associated with three major food-borne epidemics of listeriosis. They found that isolates associated with each of the outbreaks exhibited a REA profile that was characteristic of the outbreak.

Carriere *et al.* (1991) used restriction endonucleases which cleave DNA at rare sequences (ApaI, NotI) to digest the genomic DNA of *Listeria monocytogenes* strains belonging to serovars 1/2a, 1/2b, 1/2c, and 4b. The resulting fragments were analyzed by pulsed-field gel electrophoresis. Restriction fragment length patterns varied among the different serovars with serovars 1/2a and 1/2b isolates showing very different electrophoretic patterns. Restriction polymorphism was less pronounced in serovar 4b isolates and all serovar 1/2c isolates exhibited identical electrophoretic patterns (Carriere *et al.*, 1991).

Polymorphism demonstrated by digestion of genomic DNA with restriction endonucleases appears to be one of the most useful methods for epidemiological studies of listeriosis.

2.4 Nature of the disease

2.4.1 Pathogenicity

L. monocytogenes is a facultative intracellular parasite that is able to survive and grow within macrophages and monocytes (Mackaness, 1962). Following injection of mice with sublethal numbers of *L. monocytogenes*, a well defined course of events occurs. The organism is rapidly taken up by resident macrophages in the liver and spleen, followed by rapid destruction of most bacteria. However, a limited number of bacteria regularly escape destruction and initiate intracellular growth (Mackaness, 1962). Growth in the liver and spleen is logarithmic for the next 2-3 d, followed by a rapid bacterial inactivation over the next 3-4 d, signalling recovery of the host (Mackaness, 1962; Chakraborty and Goebel, 1988). Convalescent mice are resistant to further challenge by *L. monocytogenes*, and this resistance can be adoptively transferred to naive recipients using T-lymphocytes from the resistant host (Chakraborty and Goebel, 1988). These findings triggered the concept of T cell-dependent macrophage activation and made *L. monocytogenes* a model for T cell mediated immunity against intracellular pathogens.

Hemolysin production by *L. monocytogenes* has been found to be associated with its virulence (Groves and Welshimer, 1977; Rocourt *et al.*, 1982; Seeliger, 1984). An extracellular hemolysin produced by *L. monocytogenes* was purified and characterized by Geoffroy *et al.* (1987). They demonstrated that *L. monocytogenes* hemolysin is a protein with a molecular weight of 60,000 daltons, the lytic activity of which is suppressed by oxidation and cholesterol. It is a sulfhydryl-activated toxin

that shows immunological cross-reactivity with streptolysin O and hence, has been called listeriolysin O (Geoffroy *et al.*, 1987). The importance of listeriolysin O in the virulence of *L. monocytogenes* has been shown by isolating non-hemolytic mutants of virulent strains of *L. monocytogenes*. Non-hemolytic mutants of *L. monocytogenes* generated by transposon mutagenesis were not capable of growth in host tissues and were rapidly eliminated from infected mice (Gaillard *et al.*, 1986, Kathariou *et al.*, 1987). Virulence was restored in hemolytic revertants demonstrating the importance of the hemolysin in pathogenicity of *L. monocytogenes* (Gaillard *et al.*, 1986, Kathariou *et al.*, 1987).

Two components of *L. monocytogenes* which affect the immune response of the host are the monocytosis-producing activity (MPA) and the immunosuppression activity (ISA) and have been suggested as virulence factors (Chakraborty and Goebel, 1988). The MPA appears to be a low molecular weight anionic component (1000 daltons) associated with the cytoplasmic membrane which exclusively stimulates cells of the mononuclear phagocytic lineage (monocytes) (Galsworthy, 1987). The ISA has a molecular weight of 150,000 daltons and contains, in addition to a protein moiety, carbohydrates, glycerol and phosphorus (Galsworthy, 1987).

Other virulence determinants of *L. monocytogenes* have been suggested and include a 60,000 dalton protein produced by invasive *Listeria* spp. (Chakraborty and Goebel, 1988)

The enzymes catalase and superoxide dismutase have also been suggested as virulence factors (Welch, 1987). During the respiratory burst in macrophages, toxic oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·) are produced. Catalase and superoxide dismutase detoxify these oxygen radicals improving the chances of intracellular survival of the pathogen (Chakraborty and Goebel, 1988).

2.4.2 Clinical manifestations of listeriosis

Listeriosis is a bacterial infection affecting man, domesticated and wild animals. *L. monocytogenes* is capable of causing a wide range of disease in man including: meningo-encephalitis, septicemia, pneumonia, endocarditis, localised abcesses, cutaneous lesions, conjunctivitis, urethritis, an infectious mononucleosis-like syndrome, hepatitis and arthritis (Gray and Killinger, 1966; Newman *et al.*, 1979; Hardie and Roberts, 1984). The clinical manifestations of human listeriosis differ among the high risk groups (pregnant women, neonates and persons with immunocompromising conditions) (Gellin and Broome, 1989).

2.4.2.1 Listeriosis during pregnancy

Pregnant women who develop listeriosis may have a low grade septicemia that appears clinically as an influenza-like illness, with fever, headache and myalgia (Gray and Killinger, 1966; Gellin and Broome, 1989). Gastrointestinal symptoms such as diarrhea and abdominal cramping are less common. Trans-placental infection of the fetus may result in the premature delivery of a stillborn or acutely ill infant (McLauchlin *et al.*, 1986). For the pregnant woman, the infection is usually self-limited because the bulk of the infection is eliminated with the birth of the infected fetus and delivery of the intrauterine contents (Boucher and Yonekura, 1986).

2.4.2.2 Neonatal listeriosis

Neonatal listeriosis accounts for the largest recognizable group of individuals infected by *L. monocytogenes* (Albritton *et al.*, 1984). Two distinct clinical syndromes are seen in neonatal listeriosis: early onset and late onset (Albritton *et al.*, 1984; Gellin and Broome, 1989). The early onset predominantly septicemic form of the disease occurs in infants which were infected *in utero*. The infants are commonly of low birth weight and the manifestations of the disease are apparent either at birth, within a few hours of birth or within a few days after birth, with the mean onset of symptoms apparent at 1.5 d of life (Gellin and Broome, 1989). This syndrome, granulomatosis infantisepticum, is characterized by widely disseminated lesions, which are most common in the liver and placenta; however the brain, spleen, adrenal glands, kidney, lungs and gastrointestinal tract may also be affected (Gellin and Broome, 1989). Late onset neonatal listeriosis usually affects full term, normal weight infants which appear healthy at birth, with the onset of symptoms occurring several days to several weeks after birth with a mean onset of symptoms apparent at 14.3 d of life (Gellin and Broome, 1989). The source of infection for this predominantly meningitic form is less clear; however post-partum acquisition of the pathogen is presumed (Gellin and Broome, 1989). The overall mortality rate for the early-onset form is about 30%, while the late-onset form is about 10% (Albritton *et al.*, 1984).

2.4.2.3 Nonperinatal listeriosis

In nonpregnant adults and children listeriosis is principally an infection of persons with predisposing factors such as malignancy, immunosuppressive therapy, transplant operations and old age (Nieman and Lorber, 1980). Other disorders associated with increased susceptability to *L. monocytogenes* include diabetes, alcoholic and nonalcoholic liver disease, chronic renal disease, collagen-vascular diseases and conditions associated with iron overload (Gellin and Broome, 1989). Ho

et al., (1986) suggested that patients with diminished gastric acidity may also be at increased risk. However, up to 30% of adults and 54% of children and young adults with listeriosis have no apparent immunocompromising condition (Gellin and Broome, 1989).

Nonpregnant adults with listerosis usually exhibit meningitis or septicemia (Nieman and Lorber, 1980). When *L. monocytogenes* attacks the central nervous system, meningitis is the usual result (Gray and Killinger, 1966). In persons with listerial meningitis, the cerebrospinal fluid is usually purulent, with elevated leukocyte levels (Gellin and Broome, 1989). The clinical features of such an infection range from subtle personality changes, to ataxia, tremors, seizures and coma (Gellin and Broome, 1989). Septicemia is diagnosed when *L. monocytogenes* is isolated from the patient's blood without documented infection elsewhere. Most patients experience fever, but may also experience other nonspecific symptoms including fatigue, malaise and abdominal complaints such as nausea, cramps, vomiting and diarrhea (Nieman and Lorber, 1980).

Focal infections by *L. monocytogenes* are rare, occur mainly in immunocompromised persons and are generally thought to be the result of seeding during an initial bacteremic phase (Gellin and Broome, 1989). However, cutaneous infections without systemic involvement have been reported for abattoir workers, veterinarians and accidentally exposed laboratory workers (Clegg, 1975; Gellin and Broome, 1989).

In farm animals, there are five separate diseases or syndromes (which seldom overlap): (1) infection of the pregnant uterus with subsequent abortion, stillbirth or neonatal death, (2) septicemia with miliary lesions, mainly in unweaned animals (3) encephalitis, (4) purulent conjuctivitis and keratitis, and (5) mastitis (Gitter, 1985).

The only reliable procedures for diagnosing listeriosis are bacteriological isolation methods (Hudak *et al.*, 1984). Treatment of listeriosis involves drug therapy. Administration of ampicillin and gentamicin in combination is generally recommended as the treatment of choice (Gellin and Broome, 1989).

2.4.3 Incidence in humans

Between 1951 and January 1972, 101 cases of human listeriosis were confirmed bacteriologically in Canada (Bowmer *et al.*, 1973). Listeriosis was found to occur during all seasons, afflict persons of all ages and both sexes, but particularly the very young and the elderly. Bowmer *et al.* (1973) found that the mortality rate was 32% in patients with meningitis and 36% in those with septicemia.

In 1988, a total of 60 cases of human listeriosis were identified across Canada, giving a rate of 2.3 cases per million population, with 6 occurring in Alberta (2.5 cases per million population) (Varughese and Carter, 1989). A similar situation was observed in 1989 when 63 cases of human listeriosis were identified, with 5 cases occurring in Alberta (Varughese *et al.*, 1991).

In the United States, *L. monocytogenes* has been estimated to cause 1700 serious infections and contribute to approximately 450 deaths and 100 stillbirths annually (Gellin *et al.*, 1991).

2.4.4 Reservoirs and transmission

L. monocytogenes is widespread in nature; humans, animals and the environment can be reservoirs of the organism. In Alberta, during the years 1951 to 1970, listeric infection was diagnosed in 106 chinchillas, 50 sheep, 42 chickens, 33 cattle, 18 swine and 2 rabbits (MacDonald *et al.*, 1972). During the survey period, isolation of *L. monocytogenes* was made from each of the following: dog, cat, mink, goat, turkey, goose, duck, canary and hungarian partridge (MacDonald *et al.*, 1972). *L. monocytogenes* has been isolated from dust, soil, water, sewage and decaying vegetation, and animal feed and silage (Skovgaard and Morgen, 1988; Weis and Seeliger, 1975; Fenlon, 1985). Fecal carriage of *L. monocytogenes* has been reported in clinically healthy humans and animals: egg products factory workers 29.1%, office personel 11.9%, slaughterhouse personel 13.3%; 15.3% of cattle feces in the province of Friesland and 6% of cattle feces in the province of Overijssel (Kampelmacher and van Noorle Jansen, 1969).

L. monocytogenes can invade humans and other animals by oral, ocular, cutaneous, respiratory or urogenital routes (Ralovich, 1984). Among the animals, the most important vehicles of infection are silage, grass, surface waters and dust (Ralovich, 1984).

The direct transmission of listeriosis from animal to man has been documented in farm workers, veterinarians and in abattoir workers (Schlech, 1984; Hird, 1987). However, most cases of human listeriosis do not appear to result from contact with infected animals (Gray and Killinger, 1966). Healthy carriers exist among human and animal populations, and they may play the predominant role in perpetuation and transmission of the disease (Gray and Killinger, 1966). However, it has been proposed that the indirect transmission from animal to man from contaminated food may be the most important link between animal and human listeriosis (Schlech, 1984).

2.4.5 Food-borne outbreaks of listeriosis

2.4.5.1 Epidemic listeriosis

Potel established an epidemiological link between the consumption of raw milk and human listeriosis in the 1950's when he isolated *L. monocytogenes* from a cow with listerial mastitis and from a pregnant woman who had ingested raw milk from the same cow during the pregnancy. The same serotype was recovered from the cow and the woman's stillborn twins (Seeliger, 1961). This incident was considered to be part of an epidemic of listeriosis which occurred between 1949 and 1957 in Halle, Germany where many human cases of listeriosis were thought to be due to the consumption of raw milk. Sour milk, creams and cottage cheese were also considered as possible sources of *Listeria.* (Seeliger, 1961).

During September and October 1979, an outbreak of *L. monocytogenes* serotype 4b infection involving patients from 8 Boston hospitals was reported (Ho *et al.*, 1986). The authors were able to make an association between the use of antacids or cimetidine and the development of listeriosis, suggesting that neutralized gastric acidity enabled *L. monocytogenes* to survive digestion and establish infection. A food preference survey implicated several foods (chicken salad, tuna fish and cheese) as possible sources of infection. Raw vegetables (tomatoes, celery and lettuce) were usually served with the implicated food items and may have been the vehicle of transmission of *L. monocytogenes* (Ho *et al.*, 1986). However, the authors were unable to exclude pasteurized milk or cheese made from pasteurized milk as a source of the outbreak.

Between March and September, 1981, an outbreak of adult (7 cases) and perinatal (34 cases) infection due to *L. monocytogenes* serotype 4b occurred in the Canadian Maritime provinces (Schlech *et al.*, 1983). A case-control study identified contaminated coleslaw as the probable vehicle of transmission. *L. monocytogenes* of the epidemic serotype and phage type was isolated from coleslaw, but none of the other food items, from the refrigerator of one of the patients (Schlech *et al.*, 1983). Restriction fragment length analysis of 31 clinical isolates revealed that 28 of the 31 clinical isolates had identical restriction patterns and that they were identical to the reference isolate (81-861) which was recovered from a patient's refrigerated coleslaw (Wesley and Ashton, 1991). In addition, *L. monocytogenes* serotype 4b was isolated from several unopened packages of the commercially produced coleslaw. A list of cases of veterinary disease in the Maritimes revealed the name of a farmer with two

documented cases of listeriosis in his flock of sheep, one in 1979, and the other in 1980. This farmer was also on the list of commercial cabbage growers and used raw sheep manure to fertilize his cabbage crop (Schlech, 1984). Cabbage from the last crop had been distributed throughout the maritimes and at least one 5000 lb lot had been supplied to a regional coleslaw processer who produced the product that the outbreak victims had consumed (Schlech, 1984).

Between June 30 and August 30, 1983, 49 cases of listeriosis were reported in Massachusetts. This episode was considered to be an epidemic and an epidemiological study was initiated. Pasteurized whole or 2% milk from a single dairy processing plant was implicated as the source of infection (Fleming *et al.*, 1985). Inspection of the dairy plant did not reveal any breaches in the pasteurization process suggesting that the pathogen may have survived the heat treatment (Fleming *et al.*, 1985). Post-pasteurization contamination of the product was considered unlikely as skim milk which was not linked with the disease outbreak was also processed on the same equipment (Fleming *et al.*, 1985; Donnelley and Briggs, 1986). Although *L. monocytogenes* was not cultured from the pasteurized product, veterinary records revealed that several cases of bovine listerial encephalitis had occurred in dairy cows that supplied milk to the dairy (Fleming *et al.*, 1985). *L. monocytogenes* was isolated from 15/124 of raw milk samples taken from sources supplying the pasteurizer and several different serotypes were identified, including 1a, 3b, 4ab and significantly, 4b, the epidemic serotype (Fleming *et al.*,1985). Although *L. monocytogenes* of the relevant serotype was recovered from the raw milk samples tested, the phage type associated with the epidemic was not found (Fleming *et al.*,1985). Wesley and Ashton (1991) found that nine human isolates associated with the epidemic exhibited an identical restriction enzyme pattern (HhaI digest of chromosomal DNA), which differed markedly from the restriction enzyme profiles of the serotype 4b raw milk isolates. The differences in bacteriophage type and restriction enzyme pattern of the raw milk and clinical isolates clearly showed that the epidemic strain was not among the raw milk isolates. However, the REA pattern observed with the patients was similar to that seen in other 4b isolates (including three isolates recovered from ice cream, a bovine milk strain, and two strains isolated from sheep brains) collected from diverse geographical locations suggesting widespread environmental contamination with the epidemic strain (Wesley and Ashton, 1991). Therefore, *Listeria* contamination of raw milk may not have been the cause of the outbreak.

From January 1st to August 15th, 1985 in Los Angeles County, California 142 cases of human listeriosis were reported; 93 of the cases occurred in pregnant women or their offspring and 49 in nonpregnant adults (Linnan *et al.*, 1988). In this outbreak there were 48 deaths: 20 fetuses, 10 neonates and 18 nonpregnant adults. Of the *L. monocytogenes* isolates available for study, 86 of 105 were serotype 4b and 63 of those 86 were of the same phage type. The outbreak was traced to the consumption of a particular brand of Mexican-style soft cheese. *L. monocytogenes* 4b of the epidemic phage type was isolated from 9 of 11 previously unopened samples of "Brand A" cheese that were purchased from markets in Los Angeles (Linnan *et al.*, 1988). The positive cheese samples were made by Jalisco and were of two varieties– queso fresco and cotija. The contaminated cheeses had different expiration dates ranging from June 28 to August 16, 1985 (James *et al.*, 1985). Examination of the cheese plant revealed that the pasteurizer was functional; however on several occasions, 10% more raw milk than could be pasteurized (given the capacity of the pasteurizer) was delivered. Excessive levels of phosphatase in 9/80 samples of cheese were detected, suggestive of insufficient pasteurization of the milk or the introduction of raw milk into the milk following pasteurization (Linnan *et al.*, 1988). Environmental samples at the cheese plant (vat condensates, insects and drain swabs) were found to be positive for the epidemic phage type and samples of products made at the creamery which supplied raw milk to the cheese plant were also contaminated (Linnan *et al.*, 1988). Despite several attempts, *L. monocytogenes* was not isolated from raw milk or milk filters from the 27 dairy farms that supplied milk to the creamery that supplied the cheese factory (Donnelly *et al.*, 1986; Linnan *et al.*,1988). Identical restriction enzyme profiles (HhaI digests of chromosomal DNA) were exhibited by 18 human isolates, 10 cheese isolates (from unopened packages of cheese confiscated from the factory), cheese isolates (from packages purchased in Los Angeles supermarkets), a cheese isolate recovered from a patient's refrigerator and cheese plant environmental isolates (recovered from cooler condensate, insects, cheese curd and the pasteurizer) (Wesley and Ashton, 1991).

In Switzerland, a locally made soft cheese was implicated in about 120 cases of human listeriosis between 1983 and 1987. Ninety-five percent of the human isolates were serotype 4b; however, two distinct phage types were involved in 80% of all cases (Nocera *et al.*, 1990). Restriction enzyme analysis (EcoRI digests of chromosomal DNA) of 57 serotype 4b strains that were identified as either the phage type I or phage type II Swiss epidemic strain clustered into two closely related REA profiles (Nocera *et al.*, 1990).

2.4.5.2 Sporadic listeriosis

The consumption of uncooked hot dogs and undercooked chicken were identified as risk factors for listeriosis in a study of 82 cases of listeriosis and 239 controls matched for age and underlying disease (Schwartz *et al.*, 1988).

In December, 1988, a cancer patient was hospitalized in Oklahoma with sepsis caused by *L. monocytogenes* serotype 1/2a. The vehicle of infection was found to be turkey frankfurters. The patient had eaten 1 frankfurter per day (heated in a microwave oven). *L. monocytogenes* of the same electrophoretic enzyme type as the patient isolate was isolated from turkey frankfurters in the patient's refrigerator and from two unopened packages of the same brand name from a local store. Cultures of other foods in the patient's refrigerator yielded *L. monocytogenes*, but unopened samples of those foods were found to be negative for *Listeria* (Barnes *et al.*, 1989).

In 1989, in Britain, ready-cooked chicken nuggets from a "take away" were implicated in septicaemia caused by *L. monocytogenes* type 1/2a in a 52 year old immunocompromised female patient in a hospital (on steroid treatment for systemic lupus) and in a mild, short-lived illness (characterized by malaise, myalgia, diarrhea, and vomiting) experienced by her 29 year old immunocompetent son (Kaczmarski and Jones, 1989). Culture of the son's stools yielded *L. monocytogenes* types 1/2a and 1/2c and *L. innocua*. Fragment length analysis of DNA from the *L. monocytogenes* isolates showed two patterns, one of which was identical to that of the blood culture isolate from the woman. Cooked chicken nuggets from the same source were *Listeria* negative, but *L. monocytogenes* and *L. innocua* were isolated from the uncooked product. The incubation period was between 3 and 5 d in both mother and son. Kaczmarski and Jones (1989) suggested that sporadic cases of listeriosis may be accompanied by mild, non-specific infections in immunocompetent individuals more often than is realised.

A fatal case of listeriosis was traced to the consumption of alfalfa tablets by a 55 year old man with multiple health problems (Farber *et al.*, 1990). The victim was seriously ill with multiple organ failure including chronic hepatitis. He was taking steroids as well as antacids and cimetidine, and because of his poor health was also on food supplements including alfalfa tablets. The patient's symptoms included fever, respiratory distress and meningoencephalitis. *L. monocytogenes* serotype 4b was isolated from the patient's blood and cerebrospinal fluid. *L. monocytogenes* 4b of the same electrophoretic type was isolated from an opened package of the alfalfa tablets. This incident demonstrates that *L. monocytogenes* can be found on a dry product

where it is unable to grow. It may be possible that growth on the alfalfa occurred prior to drying.

Farber *et al.* (1990) reported a case of listeriosis in a 66 year old man with a history of heart disease, diabetes, alcoholism, arthritis, and who had an artificial hip. The patient had eaten large portions of an imported soft cheese that had been recalled because of listerial contamination. *L. monocytogenes serotype* 1/2b was isolated from the blood and from synovial fluid from the artificial hip of the patient and from opened and unopened packages of the soft cheese. The isolates recovered from the patient and the food were of the same electrophoretic enzyme type.

In two unrelated incidents, immunocompetent individuals acquired listeriosis from the consumption of soft cheese (Bannister, 1987; Azadian *et al.*, 1989). Bannister (1987) reported that a 36 year old woman without any debilitating condition became ill with meningitis caused by *L. monocytogenes* serotype 4b; she had eaten soft cheese from which a similar organism was isolated. Azadian *et al.* (1989) reported that a 40 year old woman became ill with meningitis shown to be caused by *L. monocytogenes* serotype 4b. Isolates of the same serotype and phage type were isolated from the patient's cerebrospinal fluid (CSF) and feces and from four unopened packages of a medium fat whey cheese of the same type the patient had consumed 24 h before the onset of symptoms. The cheeses were found to contain *L. monocytogenes* at levels between 30 and 50 million cfu/g. The patient claimed to have eaten 85 g of the cheese before the onset of symptoms, and assuming that her package was contaminated to the same degree as the unopened packages, an oral dose of 2.5 to 4.3 x 10^9 cfu was estimated.

2.5 Isolation and identification

Most clinical isolations of *L. monocytogenes* from samples that are collected aseptically are accomplished by direct plating of the infected sample onto non-selective nutrient agars (Bortolussi *et al.*, 1985). However, early workers found that primary cultivation (direct plating) of suspect, infected material often failed to reveal the presence of *L. monocytogenes* (Murray *et al.*, 1926; Gill, 1937; Gray *et al.*, 1948; Seeliger, 1961; Gray and Killinger, 1966; Errebo Larsen, 1969).

Gray *et al.* (1948) found that clinical samples from suspected listeric infections that failed to yield *L. monocytogenes* after primary plating would do so after holding samples at 4°C. Gray *et al.* (1948) macerated brain samples (from cattle thought to be suffering from listeriosis) in tryptose broth and then stored them at 4°C for several weeks to several months prior to plating. They noted increases in the

numbers of *L. monocytogenes* as the samples were kept at 4°C for longer periods of time. Cold enrichment, as this technique has become known, has proven useful for the isolation of *L. monocytogenes* from a wide range of sources.

Gray *et al.* (1948) noted that it was possible to distinguish colonies of *L. monocytogenes* from other colonies by means of a binocular scanning microscope and the use of obliquely transmitted light. Oblique illumination, first described by Henry (1933), is conducted by reflecting a beamed white light source from a mirror so that the light strikes the bottom of a petri plate at a 45 degree angle. When illuminated in this manner, colonies of *L. monocytogenes* examined from above with a dissecting microscope appear as slightly raised, small, smooth, blue-gray colonies (Gray, 1957). Many workers have used this method to identify *Listeria* in heavily contaminated backgrounds that otherwise would not have been detected.

The combination of cold enrichment and the use of oblique illumination for the examination of colonies has been used by many workers for the isolation of *L. monocytogenes* from samples containing a mixed microflora and from samples in which the pathogen could not be isolated following primary culture. A major disadvantage of the cold enrichment technique is that the time involved is too long to make it practical for routine use. More recently the introduction of selective enrichment broths for *L. monocytogenes* has resulted in improved isolations of the organism from a variety of sources. Selective enrichment broths that have been used successfully for *Listeria* isolation include: thiocyanate nalidixic acid broth (Watkins and Sleath, 1981), UVM Listeria enrichment broth (Donnelley and Baigent, 1986), FDA Listeria enrichment broth (Lovett *et al.*, 1987), Fraser broth (Fraser and Sperber, 1988), L-PALCAMY broth (van Netten *et al.*, 1990). After incubation in selective enrichment broth, subcultures are made onto suitable selective agars. Many selective agars for *Listeria* have been developed, many of which require the use of oblique lighting for discrimination of the colonies.

Gray *et al.* (1950) added 0.05% potassium tellurite to tryptose agar as a differential ingredient. Even though colonies are black when grown on tryptose agar supplemented with tellurite, they retain some of their characteristic blue-green color at the margin of the colonies when examined by Henry illumination.

McBride and Girard (1960) developed a selective medium for *L. monocytogenes* based on the use of phenylethanol agar base, lithium chloride, glycine and blood. On this medium, McBride Listeria agar, *L. monocytogenes* appears as "dew drop" colonies surrounded by a faint zone of hemolysis. McBride Listeria agar has been modified by many workers. Lovett *et al.* (1987) omitted the blood and

added cycloheximide in their version of this agar, Modified McBride Listeria agar (MMA). MMA plates can be examined by Henry illumination, making it easier to differentiate *Listeria* from non-*Listeria* isolates. Lee and McClain developed lithium chloride-phenylethanol-moxalactam agar (LPM) by increasing the concentration of lithium chloride ten fold and adding moxalactam to a final concentration of 20 µg/ml (Lee and McClain, 1986). The presence of moxalactam, which has a broad spectrum of activity against many Gram-positive and Gram-negative bacteria including *Staphylococcus*, *Proteus*, and *Pseudomonas* spp. inhibited the growth of many bacteria which interfered with the recovery of *L. monocytogenes* from raw beef (Lee and McClain, 1986). Since there is no blood in this formulation, the plates can be examined by Henry illumination.

Beerens and Tahlon-Castle (1966) found the incorporation of 40 µg/mL of nalidixic acid into blood agar plates to be effective in suppressing Gram-negative organisms and recommended it for the isolation of streptococci, *L. monocytogenes*, and *Erysipelothrix*. Since that time, nalidixic acid, alone or in combination with other selective agents, has been widely used in selective media for *L. monocytogenes* (Ralovich *et al.*, 1971; Mavrothalassitis, 1977; Martin *et al.*, 1984; Dominguez Rodriguez, 1984; Donnelly and Baigent, 1986; Lovett *et al.*, 1987).

Ralovich *et al.* (1971) developed a selective medium for the isolation of *L. monocytogenes* which contained tryptaflavine and nalidixic acid as selective agents. This medium, tryptaflavine nalidixic acid serum agar (TNSA), was found to be useful for the isolation of *L. monocytogenes* from samples containing a mixed microflora.

Martin's medium (gum based nalidixic acid medium) is a solid medium of relatively low selectivity (Martin *et al.*, 1984). This medium was developed in order to improve the use of Henry illumination for the detection of *L. monocytogenes* on plates with mixed cultures. The replacement of agar with a hydrocolloid gum for thickening made the medium more transparent than agar thickened plating media.

Dominguez Rodriguez *et al.* (1984) used esculin in combination with ferric ammonium citrate to indicate colonies of *Listeria* spp. which turn the medium black due to the reaction of esculinetin, an esculin hydrolysis product and the ferric ions with the formation of a black precipitate. This isolation agar, isolation medium III, contains acriflavine and nalidixic acid as selective agents for *Listeria*. The authors state that an incubation temperature of 22°C is preferable to 37°C when examining growth from enrichment broths of fecal samples because of growth of non-*Listeria* contaminants.

ALPAMY agar (van Netten *et al.*, 1988) utilizes the esculin/ferric ammonium citrate indicator system of Dominguez Rodriguez *et al.* (1984) and improves upon it by the addition of mannitol and phenol red to the formula. As a result, *Listeria* spp. which are consistently esculinase +ve and mannitol -ve, give colonies which are differentiated from the background flora on the basis of two biochemical characters. *Enterococcus* spp. which are also esculin +ve can be differentiated from *Listeria* spp. because acid produced by the former from mannitol changes the color of the agar to yellow from red. ALPAMY agar which contains acriflavine, lithium chloride and phenylethanol as selective agents was considered to be too selective for recovery of injured cells, even when used on resuscitated populations of many *Listeria* strains and led to the development of PALCAM agar (van Netten *et al.*, 1989). PALCAM agar (van Netten *et al.*, 1989) is also both selective and differential for *L. monocytogenes*. PALCAM contains polymyxin B, acriflavine, lithium chloride and ceftazidime for selectivity and uses esculin/ferrous iron and mannitol/phenol red indicator systems for differentiation.

Oxford agar, a selective differential medium for the isolation of *L. monocytogenes* from clinical specimens with a mixed flora was developed by Curtis *et al.* (1989). The medium contains lithium chloride, cycloheximide, colistin sulfate, acriflavine, cefotetan, and fosfomycin as selective agents. The differential character of the agar is attained by incorporation of the esculin and ferrous iron indicator system into the medium. McClain and Lee (1989) modified Oxford agar by changing the combination of selective ingredients to lithium chloride, colistin sulfate and moxalactam.

Al-Zorkey and Sandine (1990) developed Al-Zorkey-Sandine listeria medium, a selective differential medium for *L. monocytogenes*. This medium contains acriflavin, ceftazidime and moxalactam as selective agents and esculin and ferric ammonium citrate for differentiation.

Both pathogenic and apathogenic species of listeria grow on the selective agars. The presence of the apathogenic listeriae reduces the probability of selecting pathogenic organisms from such mixed cultures. Cassiday *et al.* (1990) replica plated bacterial colonies from *Listeria*-selective agars to sheep blood agar to screen for beta-hemolysis. By using the replica plating method, the authors were able to recover *L. monocytogenes* from 59 of 142 *Listeria* -selective plates which contained colonies of hemolytic and nonhemolytic *Listeria* species that were considered negative when tested by conventional colony picks (3 to 10 per plate). Blanco *et al.* (1989) developed a technique in which sheep red blood cells agar was overlayed onto

selective plating medium after listeria growth in order to detect directly the hemolytic activity of the colonies. Using this technique on spiked raw milk containing 10^7 cfu/mL of natural microflora, Blanco *et al.* (1989) were able to differentiate three colonies of *L. monocytogenes* among 2520 colonies of *L. innocua.*

van Netten *et al.* (1991) devised an agar overlay method to improve the efficiency of recovering hemolytic listeriae from PALCAM agar isolation plates. Columbia blood agar (6% sheeps blood) containing polymyxin B, acriflavine, ceftazidime and 5% supernatant fluid from a culture of *Staphylococcus aureus* is poured onto inoculated PALCAM plates. Hemolytic listeriae (*L. monocytogenes*, *L. seeligeri* and *L. ivanovii*) were easily detected by clearly defined zones of hemolysis around esculin positive and mannitol negative colonies. In spiked food samples (400-800 cfu *L. monocytogenes* per gram of food with a 100-fold excess of non-hemolytic *L. welshimeri*) colonies of *L. monocytogenes* could be differentiated (van Netten *et al.*, 1991).

2.5.1 Enrichment and plating methods

The outbreaks of food-borne listeriosis in the 1980's stimulated development of a variety of isolation protocols and media, mainly based on cold enrichment, selective enrichment and selective differential plating. Ralovich (1984) described enrichment procedures to isolate *L. monocytogenes.* The method used depended upon the source of the sample. For clinical samples collected aseptically and most likely not contaminated with bacteria other than *Listeria* such as cerebrospinal fluid, blood, non-decomposing post-mortem samples (liver, spleen, brain, lymph node, placenta, meconium) neither a selective culture medium nor a selective culture is necessary. Samples can be cultivated in a liquid medium such as tryptose phosphate broth at 37°C and after the first and second day of incubation, streaked onto blo agar or serum agar plates incubated for 48 h at 37°C. However, for samples containing miscellaneous bacteria including feces, throat and vaginal secretion, food, fodder, sewage and soil samples, decomposing post mortem samples, as well as materials not collected under aseptic conditions, the author suggested selective enrichment in a liquid medium containing acriflavine and nalidixic acid (eg. Levinthal broth containing tryptaflavine and nalidixic acid). In this medium, the cultivation lasts 7 d, at 37°C for the first two days and at room temperature for the remaining 5 d. Subcultures are made onto selective plates at 2, 4 and 7 d of culture.

A combination of cold and selective enrichment was used in the investigation of the 1983 Massachusetts outbreak of listeriosis (Hayes *et al.*, 1986). Samples of

raw milk were added to nutrient broth and stored at 4°C. After one month the cold enriched culture was subcultured into nutrient broth supplemented with nalidixic acid and potassium thiocyanate, incubated at 35°C overnight, and plated onto selective media (Hayes *et al.*, 1986).

For the isolation of *Listeria* spp from raw milk, Slade and Collins-Thompson (1988) combined cold enrichment in a non-selective broth (tryptose broth) for 3, 7, or 14 d at 4°C, with secondary enrichment in a selective broth containing thiocyanate, nalidixic acid and acriflavine for 24 h at 37°C and plating on selective agar.

For the isolation of *L. monocytogenes* from heavily contaminated backgrounds, Doyle and Schoeni (1986) used a selective enrichment broth composed of 0.9% tryptose broth supplemented with sheep blood (with polymyxin B, acriflavine and nalidixic acid as selective agents) and incubation under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) with shaking at 100 rpm for 24 h at 37°C prior to plating on selective agar.

The U.S. Food and Drug Administration (FDA) method for the isolation of *L. monocytogenes* from dairy products involves a 1 and 2 d enrichment step followed by isolation on modified McBride Listeria agar (MMA) and Lithium chloride phenylethanol moxalactam agar (LPM) and confirmation through biochemical, serological and mouse pathogenecity testing (Lovett and Hitchins, 1988). In this procedure, 25 g of the sample is mixed with 225 mL of *Listeria* Enrichment Broth (LEB) which consists of trypticase soy broth supplemented with 0.6% yeast extract, to which 15 mg/L acriflavine-HCl, 40 mg/L nalidixic acid and 50 mg/L cycloheximide, are added to eliminate overgrowth of the culture by competing organisms. Samples are enriched at 30°C for 24 h and 48 h after which times the culture is streaked onto MMA and LPM plates and incubated at 30°C for 48 h. After incubation, the plates are examined with obliquely transmitted light for typical blue-grey colonies. Typical colonies are streaked onto TSA+0.6% yeast extract to ensure purity prior to biochemical testing. The method has been used successfully for isolating *Listeria* from milk, ice cream, cheese and environmental test samples (Lovett, 1988).

The U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) method to detect the presence of *Listeria* in processed meat and poultry products is a two step enrichment followed by isolation on modified Oxford agar (McClain, and Lee, 1989). The enrichment media are modifications of UVM broth (Donnelly and Baigent, 1986). The primary enrichment broth contains nalidixic acid (20mg/L) and acriflavin (12 mg/L) as selective agents, while the secondary

enrichment broth (Fraser Broth) contains nalidixic acid (20 mg/L), acriflavine (25 mg/L), lithium chloride (3 g/L), and ferric ammonium citrate (50 mg/mL). A 25 g sample is blended with 225 ml of the primary enrichment broth and incubated for 24 h at 30°C. A 0.1 mL aliquot of the primary enrichment broth culture is subcultured into 10 mL of Fraser broth and incubated for 26 h at 35°C. Esculin hydrolysis in Fraser broth results in the production of a black precipitate, the presence of which could be considered as a presumptive positive enrichment culture (Fraser and Sperber, 1988). Culture tubes that remain the original straw color are reported as negative for *L. monocytogenes* (McClain and Lee, 1989). Blackened or darkened tubes resulting from the hydrolysis of esculin are then streaked directly onto modified Oxford agar, incubated for 24-48 h at 30°C and examined for black colonies which are typical for *Listeria* spp. Colonies suspected of being *Listeria* are screened by first streaking for isolation on horse blood overlay agar plates. Hemolytic isolates are then inoculated into brain heart infusion broth and motility test medium. Gram-positive, short rods with tumbling motility are then identified by further biochemical testing.

Even the shortened procedures (Lovett and Hitchins, 1988; McClain and Lee, 1989) for the isolation and identification of *L. monocytogenes* are tedious and time-consuming. For this reason, rapid methods including fluorescent antibody, enzyme-linked immunosorbent assays, and nucleic acid probes have been explored.

2.5.2 Fluorescent antibody techniques

Khan *et al.* (1977) described fluorescent antibody techniques for the rapid detection of *L. monocytogenes* in meat and milk. However, numerous difficulties were encountered including high background staining, cross-reactivity and false positive results. To improve upon this, Donnelley *et al.* (1986) examined the use of flow cytometry to automate the fluorescent antibody technique and to exclude false positive results based upon measurements of cellular morphology and DNA content. With this method, they were able to rapidly detect *L. monocytogenes* in raw milk, but continued to find a high degree of false positive samples (Donnelly *et al.*, 1988). McLauchlin and Pini (1989) were able to demonstrate the presence of listeria in samples of soft cheese by a direct immunofluorescence test using two monoclonal antibodies conjugated to fluorescein isothiocyanate.

2.5.3 ELISA

Monoclonal antibodies directed against the flagellar antigens of *L. innocua* which reacted with *Listeria* strains containing either the A, B or C flagellar antigen

have been developed (Farber and Spiers, 1987). Cheese and milk samples naturally contaminated with *L. monocytogenes* were found by this method to be positive for *Listeria* within 2 d (Farber and Spiers, 1987).

Butman *et al.* (1988) produced and characterized a series of monoclonal antibodies which identified a genus specific *Listeria* antigen which was characterized as a heat stable protein. Two of the monoclonal antibodies were used together to create an enzyme linked immunosorbent assay (ELISA) that could be used directly on enrichment broth samples (Mattingly *et al.*, 1988). ELISA techniques for the detection of *L. monocytogenes* without cross reaction from other *Listeria* spp. have as yet not been developed.

2.5.4 Nucleic acid probes

DNA probes have been developed for the detection of *L. monocytogenes* in foods. Klinger *et al.* (1988) developed a nucleic acid hybridization assay for *Listeria* spp. in foods which detects *Listeria* spp.–specific 16 S rRNA sequences by using a ^{32}P-labelled synthetic DNA probe. The commercially available test (Gene Trak®) uses a 'dipstick' and two probes, one of which is nonisotopically labelled. Both probes are directed to different regions of the target rRNA. The unlabelled 'capture' probe has a poly dA tail that will attach to the complementary poly dT segment that is attached to a solid matrix (dipstick) which is used to remove the hybrids from solution. If the *Listeria* specific sequences are present, then the detection probe (which also binds to the rRNA but at a different position) will also be removed from the reaction mixture. Thus, the fluorescein moieties which serve as reporter molecules will be removed from the reaction mixture only if the target rRNA is present. The system described above will not specifically detect *L. monocytogenes* and so better gene probes were sought.

More specific probes were developed from a 500 bp fragment of a presumptive β–hemolysin gene (Datta *et al.* 1987) and a 651 bp fragment containing a part of the hemolysin gene of *L. monocytogenes* (Chenevert *et al.* 1989). Datta *et al.* (1988) determined the DNA sequence of the 500 bp probe and from the sequencing information, several oligonucleotides were synthesized and used as synthetic probes. They found that synthetic oligodeoxyribonucleotides, 20 bp in length, were as effective as the 500 bp fragment for the detection of *L. monocytogenes* by colony hybridization, and successfully used the probes for the detection of *L. monocytogenes* from artificially contaminated samples of raw milk and soft cheese.

Peterkin *et al.* (1991) developed a DNA probe specific for *Listeria monocytogenes*. For detection of the organism, the probe was labelled with horseradish peroxidase and used in a colorimetric colony hybridization method on hydrophobic grid-membrane filters (Peterkin *et al.*, 1991). The method gave a positive reaction with 70 *L. monocytogenes* strains, while showing a negative reaction with 10 strains of other *Listeria* spp. and with 20 organisms belonging to other genera (Peterkin *et al.*, 1991).

A detection method for *L. monocytogenes* based upon use of the polymerase chain reaction (PCR) has been described (Bessesen et al., 1990). A pair of 24-mer oligonucleotide primers that define a 606-base-pair segment of the listeriolysin gene (Mengaud *et al.*, 1988) were used for PCR amplification. Thirty amplification cycles were performed, each consisting of denaturation at 94°C for 2 min, primer annealing at 51°C for 2 min and extension at 74°C for 2.5 min. The reaction products were separated by agarose gel electrophoresis which demonstrated that the 606-base-pair segment was successfully amplified and hybridized to a ^{32}P labelled oligonucleotide probe (that is internal to the primer pairs). The technique identified 95 of 95 *L. monocytogenes* strains, 0 of 12 other *Listeria* strains, and 0 of 12 non-*Listeria* strains. Commercial whole homogenized milk was inoculated with *L. monocytogenes* and the cells pelleted by centrifugation, washed in saline, then in water, then lysed by microwaving. Successful amplification of listerial DNA only occurred at cell concentrations in excess of 10^5 cfu/mL, therefore, the sensitivity of the assay would have to be improved before it could be used directly on clinical or food samples (Bessesen *et al.*, 1990).

2.6 Occurrence and behavior in foods

Many surveys on the occurrence of *L. monocytogenes* in foods have been conducted. Foods that have been found to be contaminated with *L. monocytogenes* in Canada include cheese, ice cream, raw milk, pâté, beef, ground beef, pork, ham, sausage, salami, smoked meat, lobster, shrimp, crab, smoked salmon, raw vegetables and alfalfa tablets (Varughese *et al.*, 1991).

2.6.1 Meat and Poultry

Johnson *et al.* (1990) reviewed the results of many surveys on the prevalence of *Listeria* spp. in meat and meat products. *L. monocytogenes* was isolated from 0 to 68% of samples of raw meat (beef, pork and lamb). The prevalence of *L. monocytogenes* in ground meats and sausage meats ranged fron 8-92%, and up to a

third of ready-to-eat meat products contained the pathogen (Johnson *et al.*, 1990). The presence of listeriae on carcasses has been attributed to contamination from feces, animal pelts and hides and from the slaughterhouse environment. It has also been suggested that meat handlers may contaminate the product by virtue of their being carriers. Post-processing contamination of ready-to-eat meat products poses a public health risk since *L. monocytogenes* has been shown to grow on many prepared meat products (Glass and Doyle, 1988).

Fresh poultry is often contaminated with *Listeria* spp. (Gitter, 1976; Pini and Gilbert, 1988; Skovgaard and Morgen, 1988; Bailey *et al.*, 1989). Pini and Gilbert (1988) found that 60 of 100 (60%) of raw chickens were contaminated with *L. monocytogenes*. Skovgaard and Morgen (1988) were able to isolate *Listeria* spp. from scald water and cooling water in a poultry plant. Cooked "ready-to-eat" chickens and cook-chill poultry dishes have been found to be contaminated with *L. monocytogenes* (Kerr, 1988a, 1988b). Siragusa and Johnson (1988) showed that cooked chicken slurries supported the growth of *L. monocytogenes* at 5°C with increases in population of 2 log cycles over 6d. Kaczmarski and Jones (1989) isolated *L. monocytogenes* from uncooked chicken "nuggets".

2.6.2 Vegetables

Food-borne listeriosis as a result of consuming contaminated vegetables has been linked to the consumption of cabbage (Schlech *et al.* 1983), and suspected for tomatoes, lettuce and celery (Ho *et al.*, 1986). Pelletized alfalfa was implicated in a case of listeriosis (Farber *et al.* 1990).

Sizmur and Walker (1988) isolated *L. monocytogenes* from 4 of 60 samples of prepackaged (ready-to-eat) salads purchased in British supermarkets. The salad varieties from which *L. monocytogenes* was isolated were of two types; one contained cabbage, celery, sultanas, onion, and raisins, while the other consisted of lettuce, cucumber, radish, fennel, watercress, and leeks (Sizmur and Walker, 1988). In a survey of fresh produce, *L. monocytogenes* in addition to other *Listeria* spp. were isolated from cabbage, cucumbers, potatoes and radishes (Heisick *et al.*, 1989). In addition, they found lettuce and mushrooms to be a source of *L. innocua*. Steinbruegge *et al.* (1988) isolated *L. monocytogenes* from lettuce purchased from retail outlets. Contamination of produce by soil (Heisick *et al.*, 1989) or by feces (Schlech *et al.*,1983) have been suggested as sources.

L. monocytogenes has been found to be able to survive and grow on refrigerated cabbage and cabbage juice, lettuce, broccoli, cauliflower and asparagus

(Conner *et al.*, 1986; Steinbruegge *et al.*, 1988; Berrang *et al.*, 1989). Berrang *et al.* (1989) found that although controlled atmosphere packaged vegetables had an increased shelf life, *L. monocytogenes* grew on the vegetables during the storage period.

2.6.3 Milk and dairy products

L. monocytogenes has been isolated from many dairy products. Because of their association with foodborne listeriosis, dairy products have been heavily scrutinized for the presence of *L. monocytogenes* and studies have also been conducted to see which dairy products support the growth of the pathogen. Liquid dairy products (whole milk, skimmed milk, chocolate milk, whipping cream and 11% nonfat milk solids) have all been shown to be capable of supporting the growth of *L. monocytogenes* at temperatures as low as 4°C (Donnelley and Briggs, 1986; Rosenow and Marth,1987). Rosenow and Marth (1987) inoculated liquid dairy products (skimmed milk, whole milk, chocolate milk and whipping cream) with *L. monocytogenes* and stored the products at temperatures ranging from 4°C to 35°C. They found that the organism was able to grow at all temperatures, with generation times of 35 h at 4°C, 4.5 - 6 h at 13°C, 2 h at 21°C and 41 min at 35°C. After three to four weeks, levels of the organism reached 10^7 cfu/ml in all of the products except chocolate milk where the levels approached 10^9 cfu/ml.

The presence of *L. monocytogenes* in soft cheeses has been reported by several authors. Pini and Gilbert (1988) found that 23 of 222 (10%) of soft cheeses were contaminated with *L. monocytogenes* and 19 of the cheeses were contaminated with *L. innocua*. Farber *et al.* (1987) found that 3 of 374 samples of soft and semi-soft cheeses contained the pathogen and although the cheeses were labelled as being produced from pasteurized milk, the cheeses were found to be phosphatase positive. The cheeses were found to contain 10^4 to 10^5 cfu/g of *Listeria*, and the contaminants shown to persist in the cheeses one year later. In two of the samples, there was a 1 to 2 log cycle decline in numbers but in the third, the levels of the listeria were unchanged. *L. monocytogenes* has been shown to survive the manufacture of cottage cheese (Ryser *et al.*, 1985), cheddar cheese (Ryser and Marth, 1987a) and colby cheese (Yousef and Marth, 1988) with the organism surviving ripening. In Camembert, *L. monocytogenes* showed no growth initially, but as the pH increased during storage, the levels of the organism increased (Ryser and Marth, 1987b). In feta cheese, *L. monocytogenes* grew during the first two days of ripening (i.e. before the pH had decreased to 4.6), and then growth of the organism ceased (Papageorgiou and

Marth, 1989). Terplan (1986) also found that the growth of *L. monocytogenes* in soft surface ripened cheeses paralleled increases in pH during ripening. *L. monocytogenes* has been isolated especially from soft and semi-soft cheeses of varying sources which were not involved in cases of listeriosis (Terplan, 1986).

2.6.4 Contamination of food

It is well established that *L. monocytogenes* can be found in raw milk (Lovett *et al.*, 1986; Farber *et al*,. 1987). Whether or not the organism makes its way into dairy products via raw milk or as a result of post-pasteurization contamination is less clear.

Cox *et al.* (1989) examined food processing environments, households and a sawmill for the presence of *Listeria* spp. The authors suggest that almost any moist environment will support the growth of listeriae provided that substrates for growth are present. They found that drains, floors, standing water residues and food contact surfaces were sources of *Listeria* spp. in food factories. In households, they found dishcloths to be a source of contamination. Everson (1988) reported that a 1986 Wisconson Dairies Cooperative program of surveillance did not find *Listeria* spp.in any environmental samples in any dairy plant except in raw milk and the floors and drains of the intake area. Charlton *et al.* (1990) surveyed 156 milk processing plants for *Listeria* and found that 46 plants (29.5%) yielded positive results for *Listeria* spp., of which 31 (19.9%) were positive for *L. monocytogenes*. The prevalence of *L. monocytogenes* was found to be 10.7% in packaging-filling areas, 9.8% in cold box cooler rooms, 3.2% in processing rooms and 1.9% in raw milk receiving rooms (Charlton *et al.*, 1990). Drains were found to be the most common source of *L. monocytogenes* in milk plants, with samples taken from conveyors, condensates, and utility tables also yielding the organism (Charlton *et al.* 1990). Walker *et al.* (1991) felt that raw milk was not a likely source of indirect contamination of frozen milk products since plants that only received pasteurized milk or pasteurized mixes were found to be contaminated with listeria as often as those receiving raw milk. These results would tend to support Terplan *et al.* (1986) who suggested that post-pasteurization contamination of cheese is responsible for the presence of *Listeria* .

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3. Incidence of *Listeria monocytogenes* in raw bulk milk in Alberta[1]

3.1 Introduction

Recent outbreaks of human listeriosis associated with the consumption of milk and dairy products (Fleming *et al.*, 1985; James *et al.*, 1985) have been and continue to be of concern to both dairy producers and dairy processers. The outbreaks have stimulated research on many aspects of the causative organism, *Listeria monocytogenes*, including the incidence of the organism in raw milk. Dominguez-Rodriguez *et al.* (1985) reported that 45% of 95 raw milk samples collected in Spain were positive for *L. monocytogenes*. Lovett *et al.* (1987) tested raw milk from three areas of the United States (East, Central, West) and found that the incidence of *L. monocytogenes* ranged from 0% in California to 7% in Massachusetts, with an overall incidence of 4.2%. Two studies conducted in Ontario reported incidences of 1.3% (Farber *et al.*, 1988) and 5.4% (Slade *et al.*, 1988). Farber *et al.* (1988) reported a lower incidence of *L. monocytogenes* in the winter than in the other seasons. Liewen and Plautz (1988) isolated the organism from 4% of raw milk samples collected in Nebraska. The same authors also reported a higher incidence in the winter months, in contrast to Farber *et al.* (1988). Davidson *et al.* (1989) found *L. monocytogenes* in 4% of 256 raw milk samples collected in Manitoba. Recently Fenlon and Wilson (1989) examined raw bulk tank milk in North-East Scotland and found the incidence of *L. monocytogenes* to range from 3.8% in the summer to 1% in the fall. They estimated the level of the organism in the milk to be less than 1 cell/mL prior to enrichment. This paper describes the results of a study on the incidence of *L. monocytogenes* in raw bulk milk in Alberta.

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3.2 Materials and Methods

3.2.1 Milk samples

Two types of milk samples were examined. The first set of samples was collected from milk transport tankers on arrival at a dairy processing facility. Thirty six of these samples, designated 'truck' samples, were collected in May, and the sampling was repeated in July-August. One of the original objectives of this sampling plan was to facilitate the identification of producer farms positive for *L. monocytogenes* . A second set of milk samples was collected over an 18 month period directly from the bulk tank milk of individual milk producers in Central and Southern Alberta. These samples designated 'bulk tank' samples were collected by licensed tanker truck drivers. A total of 426 'bulk tank' samples were collected in this way. All milk samples were transported to the laboratory in refrigerated containers.

3.2.2 Isolation and identification of *Listeria monocytogenes*

i) Tanker truck samples: twenty five millilitres of raw milk was added to 225 mL of Enrichment Broth (EB) (Lovett *et al.*, 1987), mixed by swirling and incubated at 30°C After incubation for both 24 h and 7 d, the EB cultures were streaked onto selective agars. In addition, replicate aliquots of the EB cultures were diluted 1:10 in 0.5% KOH prior to plating. The selective agars used were: a) lithium chloride-phenylethanol-moxalactam agar (LPM) (Lee and McClain, 1986); b) gum based nalidixic acid medium (GBNA) (BBL., Baltimore, U.S.A.) (Martin *et al.*, 1984). The plates were incubated at 30°C for 48 h and examined by Henry oblique transillumination. If present, a minimum of two typical colonies were picked from each type of selective agar, with and without KOH treatment for further identification. In most cases twelve isolates in all were selected.

ii) Bulk tank milk samples: 25 mL of raw milk was added to 225 mL of EB, mixed by swirling and incubated at 30°C. After incubation for both 24 and 48 h the EB cultures were plated directly onto three selective agars: a) lithium chloride-phenylethanol-moxalactam agar (LPM) (Lee and McClain, 1986); b) acriflavin-lithium chloride-phenylethanol-aesculin-mannitol-egg yolk emulsion-fosfomycin (ALPAMY) (van Netten *et al.*, 1988); and c) Oxford agar (OA) (Curtis *et al.*, 1989). The EB cultures were also subcultured into Fraser broth (Fraser and Sperber, 1988) after 24 h incubation and incubated for 26 h at 35°C, after which time they were examined for blackening - i.e. esculin hydrolysis. The selective plates were examined and typical colonies were streaked onto plates of Tryptic Soy Agar (Difco Ltd.,

Detroit, U.S.A.) plus 0.6% yeast extract. If present a minimum of two typical colonies were picked from each type of selective agar for further identification. In most cases a total of 12 isolates were selected. Identification of *L. monocytogenes* from both truck samples and bulk tank samples was determined from the results of the following tests: Gram stain (+rods), catalase (+), oxidase (-), hemolysis on sheep blood agar (+), tumbling motility, umbrella motility in motility test medium, methyl red/Voges Proskauer (+/+), triple sugar iron slant (acid/acid, no gas), xylose (-), rhamnose (+), mannitol (-), and serology, using Listeria O antisera Type 1, Type 4 and Poly (Difco Ltd., Detroit).

3.3 Results and Discussion

Of the first 36 truck samples collected in May 1988 only one (2.8%) was positive for *L. monocytogenes*. Four samples (11.1%) were positive when the testing was repeated in June of the same year. It is not possible to ascertain, with any degree of certainty, seasonal variation from these data. It should be noted that neither Oxford agar nor LPAMY agar were used for testing truck samples as these were not available at the start of the study. The use of a KOH treatment on the EB cultures improved isolation on both LPM and GBNA by reducing the number of non-*Listeria* contaminants. The effect was most noticeable with GBNA.

Eight (1.9%) of the 426 bulk tank samples were positive for *L. monocytogenes* and no seasonal pattern of incidence was evident. The figure of 1.9% is comparable to the findings of 1.6% in Manitoba (Davidson *et al.*, 1989), and 1.3% in Ontario (Farber *et al.*, 1988), but somewhat lower than the 5.4% found in Ontario by Slade *et al.* (1988). All of the isolates in the present study were identified as serotype 1. Slade *et al.* (1988, 1989) reported that the predominant serotype in their studies was also serotype 1, three isolates being identified as type 4. The results from the tanker truck samples stress the need for high standards of operation in dairy processing plants to prevent the spread of the organism from raw to processed products. The incidence of the organism in truck samples compared with bulk tank samples is no doubt due to the fact that truck samples are usually made up of the milk from several individual milk producers. Thus, even one positive bulk tank sample can result in a positive truck sample. Dairy processors should assume that the organism is in all raw milk supplies and act accordingly.

Fraser broth was included in the protocol for isolation of *L. monocytogenes* from bulk tank samples to assess its value as a potential indicator medium for the presence of this organism. In all cases when *L. monocytogenes* was isolated from a

milk sample, the Fraser broth was positive (black) after 26 h incubation. It should be noted however that not all Fraser broth positives were positive for *L. monocytogenes*. From this it is concluded that Fraser broth could possibly be used as a screening medium in the isolation of *L. monocytogenes*, particularily in screening out negative samples. The efficacy of the three selective agars, Oxford agar, LPM and ALPAMY, used to isolate *L. monocytogenes* from the bulk milk samples was comparable. However some differences were noted. ALPAMY agar gave rise to colonies of *L. monocytogenes* that were the most easily recognizable. LPM agar was the most selective of the three media, giving rise to the least total background colonies.

From this study we found the following protocol most satisfactory for isolating *L. monocytogenes* from raw milk: enrichment in EB for 24 and 50 h at 30°C plus subculture from EB 24 h to Fraser broth for 26 h at 35°C. Samples showing positive in Fraser broth should be plated onto two types of selective agar–either LPM and Oxford or LPM and ALPAMY agar. The use of LPM gives selectivity, whereas the Oxford and ALPAMY agars give ease of colony recognition.

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4. A case of bovine mastitis caused by *Listeria monocytogenes*[1]

4.1 Introduction

Although *Listeria monocytogenes* is common in the feces of cattle (1) and widespread in the environment (2), only a few cases of listerial mastitis have been reported in the literature (3-5). These observations indicate that either the organism is not particularily invasive to the bovine udder, or that cases of listerial mastitis are not being detected in routine laboratory examination of mastitic milk samples. The following is a report of a case of subclinical mastitis caused by *L. monocytogenes* that may shed some light on this matter.

4.2 Materials and Methods

During the course of a study to determine the incidence of *L. monocytogenes* in raw bulk tank milk in Alberta (6), farms yielding positive results were visited for detailed investigations in an effort to determine the origin of the organism. All milk samples, including bulk milk, individual cow samples, and quarter samples were examined for the presence of *L. monocytogenes* by enrichment culturing in trypticase soy broth supplemented with 0.6% yeast extract, containing the selective agents acriflavine, nalidixic acid and cycloheximide as described by Lovett *et al.* (6a). Environmental samples such as feed, water, and feces were examined by primary enrichment in UVM broth (Becton Dickinson Canada Inc., Mississagua, Ontario) and secondary enrichment in Fraser broth (7). The enrichment cultures were streaked onto ALPAMY agar (7a), Oxford agar (Oxoid Canada Inc., Nepean, Ontario) and lithium chloride-phenylethanol-moxalactam agar (8) and incubated at 30°C for 48 h. Typical colonies were confirmed as *L. monocytogenes* as described by Fedio and Jackson (6). During the course of this investigation, one herd showing positive bulk milk samples was found to contain one animal with subclinical mastitis caused by *L. monocytogenes* serotype 1 in one quarter. The infected quarter was detected independently in two laboratories, one using the selective enrichment methods described by Fedio and _____

[1]A version of this chapter was previously published. W.M. Fedio, M. Schoonderwoerd, R.H. Shute, and H. Jackson. 1990. A case of bovine mastitis caused by *Listeria monocytogenes*. Canadian Veterinary Journal 31:773-775.

Jackson (6), and the other using routine plating on blood agar (5% ovine, Columbia agar base). Somatic cell counts of the milk samples were made using the Fossomatic automatic cell counter (Fossfood Technology Canada Inc., Brampton, Ontario). The detailed findings of this investigation are presented in this paper.

4.3 Results and Discussion

The infected animal, identified as 56UA, freshened on June 18, 1989 and appeared to have mastitis at that time in the left hind quarter; this quarter subsequently dried up. Initial tests on the animal on December 18, 1989 indicated the presence of *L. monocytogenes* and a somatic cell count (SCC) of 3,500,000/mL. The total aerobic plate count was 3,600/mL. It was decided at this time to treat the animal with antibiotics. Antimicrobial susceptibility tests (Kirby-Bauer) showed that the organism was sensitive to cephalothin, erythromycin, penicillin, tetracycline, neomycin, and novobiocin; it was resistant to oxacillin. The penicillin-sensitive/oxacillin-resistant pattern was confirmed by minimum inhibitory concentration (MIC) determination. Immediately prior to starting the treatment on January 4, 1990 further tests of the infected quarter showed heavy growth of *L. monocytogenes* on SBA and a SCC of 4,500,000/mL. The treatment given was 300 mg erythromycin (Erythro-36, Abbott Laboratories, Montreal, Quebec) twice daily for three days, followed by 200 mg cephapirin (Cefa-lak, Ayerst Laboratories, Montreal, Quebec) twice daily for three days. Subsequent testing on January 12 indicated that the quarter was negative for *L. monocytogenes* and the SCC was 1,000,000/mL. Five days later, the SCC was 4,000,000/mL and *L. monocytogenes* was detected. Final tests were carried out on January 26, 1990 at which time the SCC was 2,500,000/mL and light growth of *L. monocytogenes* was apparent. The milk was never visibly abnormal throughout the course of the investigation. However milk from the infected quarter was shown to contain about 2,000 to 5,000 organisms per mL during the infection. The results are summarized in Table 4.1.

The animal was slaughtered on February 9, 1990 and the udder collected. On macroscopic examination, the right hind mammary gland was slightly firm and on cross section there was evidence of mild fibrosis in areas with absence of lactogenesis, mainly along the ventral region of the gland. On microscopic examination, both active and inactive lobules were present within the gland. Within the active lobules there was evidence of a multifocal inflamatory reaction characterized by alveoli mainly filled with neutrophils, macrophages, lymphocytes,

Table 4.1 Somatic cell count and *L. monocytogenes* in milk from the infected quarter before and after treatment[a]

Day	SCC x 10^6/mL	*L. monocytogenes*	
1	3.5	+[b]	ND[c]
11	5.3	+	1800[d]
18	4.5	+	4600
Daily intramammary treatment, day 18 -day 23			
26	1.0	-	<10
31	4.0	+	970
40	2.5	+	3300

[a] Daily intramammary treatment with 300 mg erythromycin twice daily for three days, followed by 200 mg cephapirin twice daily for three days, day 18-day 23.

[b] On sheep blood agar plates.

[c] ND = not determined.

[d] Numbers of organisms/mL based upon typical colonies from plate counts on Oxford agar.

sloughed epithelial cells and fat droplets. In some alveoli, epithelial lining cells were reactive, characterized by cytoplasmic and nuclear enlargement, and many contained fat droplets. Some alveoli were devoid of epithelial cells and, in some locations, mucosal hyperplasia and erosion co-existed. In addition, there was considerable proteinaceous debris located mainly within the alveolar lumen, but also some within intralobular connective tissue. The intralobular connective tissue contained a low-to-moderate number of lymphocytes. There were multifocal-to-diffuse areas of inactive lobules, characterized by shrunken parenchyma which consisted mainly of the duct system. The increased amount of connective tissue within and around these lobules contained a few neutrophils, eosinophils, and some leukocytes.

The finding of lymphocytes and neutrophils to be the predominant leukocytes in the infected quarter is in contrast to the findings of Gitter *et al.* (3) who reported eosinophils to be predominant.

The detailed studies on the animal infected with *L. monocytogenes* were supplemented with observations on milking practices used, an investigation of the overall incidence of udder infections, and a study of the incidence of *L. monocytogenes* in the environment. These findings can be summarized as follows: 1) The milking herd consisted of 72 animals housed in a free stall barn. When the whole herd was tested by the California Mastitis Test (CMT) 15 of the animals (21%) showed a reaction to the CMT in at least one quarter. Culture results of individual quarter samples showed that 14 of these animals were infected with *Staphylococcus aureus* and one with *Streptococcus uberis*. Records showed that the average bulk tank SCC for the month of December 1989 had increased to 350,000/mL. 2) Milking practices were inadequate. In particular the operators used excessive water for washing udders and failed to dry the udder before attaching the teat clusters. This practice likely contributed to the considerable slippage of teat cups that was observed, resulting in air being sucked into the units. There was also a significant time lapse between udder preparation and attatchment of the milking units; delays of over two minutes were frequent. It should also be noted that the animals were exposed to significant physical contamination with manure in the free stall housing. 3) *L. monocytogenes* was isolated from one of 9 samples of feces in the free stall barn, although a rectal fecal sample from cow 56UA was negative. *L. monocytogenes* was also isolated from a sample of pelletized sugar beet pulp, although five samples of silage, one of feed grain and two of drinking water were negative. It is of interest to note that *Listeria* spp. other than *L. monocytogenes* were present in eight of the 26 environmental samples, six of which were negative for *L. monocytogenes*.

These data indicate that *L. monocytogenes* is not a particularily invasive organism for the bovine udder. Even in this herd with a serious mastitis problem, poor milking practices conducive to udder infection, and the presence of *L. monocytogenes* in the environment (feces and feed), only one infected quarter was found. The fact that the listerial infection was detected by routine laboratory plating of a quarter milk sample on blood agar shows that listerial udder infections would be detected in most laboratories; this view is supported by Sharp (5). Together these findings support the view that mastitis due to *L. monocytogenes* is likely to be rare, and probably only occurs under conditions conducive to all forms of udder infection. The failure of antibiotic therapy in this case is consistent with the intracellular nature of *L. monocytogenes* and is in agreement with the findings of other workers (3-5). However, it must be noted that one of the antibiotics used, erythromycin, is actively concentrated intracellularly (9). Culling infected animals would appear to be the only solution to the problem.

The isolation of *L. monocytogenes* from sugar beet pulp would appear to merit further study. Sugar beet pulp was implicated in an outbreak of listeriosis in chinchillas in Nova Scotia in 1977, although the organism was not isolated from the feed (10). The possibility that the organism could grow and flourish in beet pulp at various stages of production would not be suprising, in view of its ability to grow in poor quality silage (11) and raw cabbage (12). Although the isolates of *L. monocytogenes* from the udder and from the sugar beet pulp in this study were both identified as serotype 1, more detailed serology or phage typing would be necessary to confirm the relationship between them. Studies on this point could be useful in elucidating the complex epidemiology of *L. monocytogenes*.

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5. On the origin of *Listeria monocytogenes* in raw bulk milk[1]

5.1 Introduction

Outbreaks of human listeriosis associated with the consumption of milk and dairy products have been of major concern to the dairy industry (Fleming *et al.*, 1985; Linnan *et al.*, 1988). These incidents have stimulated research on many facets of the causative organism, *Listeria monocytogenes*, including the prevalence of the organism in the raw milk supply. Data in the literature show the prevalence to range from 0 to 45%, with most findings in the 1 - 8% range (Dominguez Rodriguez *et al.*, 1985; Lovett *et al.*, 1987; Farber *et al.*, 1988; Fenlon and Wilson, 1989; Fedio and Jackson, 1990; Tiwari and Aldenrath, 1990). Although the prevalence of the organism in raw milk is now well documented across a broad geographic base, there is little direct evidence available on the sources of the organism.

The possible sources of the organism on dairy farms are many and varied. *L. monocytogenes* is commonly found in the environment and plant material appears to be an important ecological niche and is suggested as a common reservoir (Welshimer, 1968; Welshimer and Donker-Voet, 1971; Weis and Seeliger, 1975). In particular, silage has been identified as a common source (Gray, 1960; Fenlon, 1985; Perry and Donnelly, 1990) and feeding of contaminated silage is thought to be the main mode of infection for listeriosis in farm animals (Schlech, 1984). The feeding of silage contaminated with *L. monocytogenes* can lead to asymptomatic shedding of the organism in the milk of dairy cows (Gitter *et al.*, 1980; Donnelly, 1986). The organism has also been found in the farm environment as a causitive agent of bovine mastitis (Gitter *et al.*, 1980; Sharp, 1989; van Dalen, 1988; Fedio *et al.*, 1990) and can be shed in both the milk and feces of apparently healthy cows (Hyslop and Osborne, 1959; Hyslop, 1975; Kampelmacher and van Noorle Jansen, 1969, 1980; Hofer, 1983; Husu, 1990).

It is evident from the above discussion that *L. monocytogenes* in raw bulk tank milk could originate from many sources. Direct contamination of bulk milk would occur as a result of udder infections or shedding from the udder; indirect contamination could occur if the organism were present in feed, feces and other environmental sources such as bedding and udder surfaces, under conditions of unsanitary milking

[1] A version of this chapter has been submitted for publication in the International Dairy Journal by: W.M. Fedio and H. Jackson. 1991.

practices.

The primary objective of this study was to attempt to determine the relative importance of the various potential sources of contamination on dairy farms with a known history of shipping milk contaminated with *L. monocytogenes*. Four such farms were selected for this study based on previous work (Fedio and Jackson, 1990). A secondary objective was the characterization of the isolates of the organism to determine if there were any epidemiological relationships.

5.2 Materials and methods

5.2.1 Samples

Three types of milk samples were examined: bulk tank milk (bulk), individual cow milk (cow) and aseptic quarter milk (quarter). Bulk samples were collected aseptically from the bulk milk tank and placed into sterile plastic containers. Cow samples were obtained as follows: udders were washed according to procedures in use on the farm and the foremilk discarded; a milk sample was collected either by direct milking of each quarter (with a portion of the sample being derived from each teat) or taken from the milk line after milking was completed. It must be noted that these samples were not aseptic and that the samples could be contaminated from teat surfaces, or carry-over in the milk line from one animal to the next. However, this approach was taken as a screening method in order to minimize the amount of aseptic quarter sampling necessary to detect cows that were shedding *L. monocytogenes* in their milk. Thus in the main, only cow samples found to be positive for *L. monocytogenes* were subject to subsequent aseptic sampling. Quarter samples were collected aseptically after washing the udder with Hibitane (4% chlorhexidine gluconate w/v) (Ayerst Laboratories, Montreal, Quebec), drying, rinsing with 70% ethanol and drying with a paper towel.

Environmental samples were collected in several ways. Milking equipment, drain, and surface samples were collected as swab samples. Representative samples of feed, manure, soil and similar materials were collected into sterile plastic bottles or sterile plastic bags. Silage samples of approximately 500g were collected directly into stomacher bags. Fecal samples, in contrast to manure samples, were taken directly from the rectum of cows using a plastic glove sleeve.

All samples were stored on ice until they were cultured for the presence of *Listeria*, usually within 18h of sampling.

5.2.2 Isolation of *Listeria* spp.

5.2.2.1 Milk samples

For all types of milk samples, 25 mL of milk was added to 225 mL of Listeria Enrichment Broth (FDA-LEB) (Lovett *et al.*, 1987), mixed by swirling and incubated at 30°C. After incubation for both 24 and 48h the FDA-LEB cultures were plated directly onto three selective agars: lithium chloride-phenylethanol-moxalactam agar (LPM) (Lee and McClain, 1986), acriflavin-lithium chloride-phenylethanol-aesculin-mannitol-egg yolk emulsion-fosfomycin (ALPAMY) (van Netten *et al* ., 1988) and Oxford agar (OX) (Oxoid Canada Ltd., Nepean, Ontario) (Curtis *et al.*, 1989). The plates were incubated at 30°C for 48 h.

After 24h incubation at 30°C, the FDA-LEB cultures were also subcultured into Fraser Broth (FB) (Fraser and Sperber, 1988). The FB cultures were incubated for 26 h at 35°C and examined for blackening, indicating esculin hydrolysis.

5.2.2.2 Environmental samples

Environmental samples were first inoculated into UVM modified Listeria enrichment broth (UVM-LEB) (Becton Dickinson Canada Inc., Mississagua, Ontario). Swab samples were inoculated into 10 mL of the broth, whereas 25g of all other samples were inoculated into 225 mL of UVM-LEB. After incubation for 24h at 30°C, 0.1 mL aliquots were subcultured into Fraser broth. The FB cultures were incubated for 26h at 35°C and examined for blackening prior to plating on the three selective agars.

Two typical colonies from each selective agar were picked and purified on Tryptic soy agar (Difco Ltd., Detroit, USA) plus 0.6% yeast extract.

5.2.3 *Listeria* confirmation

Presumptive *Listeria* colonies were screened by first performing catalase and oxidase tests. Catalase +ve, oxidase -ve isolates were inoculated into TSB + 0.6% yeast extract, incubated for 24h at 35°C and further examined. Identification of *Listeria* spp. was determined from the results of the following additional tests: Gram stain, hemolysis on sheep blood agar, growth and blackening on bile esculin agar, tumbling motility, umbrella motility in motility test medium, Methyl Red/Voges-Proskauer, triple sugar iron agar slant, carbohydrate utilization: (xylose, rhamnose, mannitol) and serology: *Listeria* O antisera type 1, type 4 and poly (Difco Ltd., Detroit , USA).

5.2.4 Antimicrobial susceptibility

Antimicrobial susceptibilities were determined, according to the manufacturers' instructions, using the Sceptor System (Becton Dickinson, Towson, MD, USA). Results were recorded as the minimal inhibitory concentration (MIC) of antimicrobial agent to inhibit growth of the test organisms.

5.2.5 Biochemical characterization

Additional biochemical characterization of isolates was performed using API20S™ (API Laboratory Products Ltd., St. Laurent, Quebec), MicroID® (Organon Teknika Corporation, Morris Plains, NJ) and Biolog (Biolog, Inc., Hayward, CA) test systems. The manufacturers' instructions were followed when any microbiological test system was used.

5.2.6 Plasmid DNA

L. monocytogenes isolates were examined for the presence of plasmids based upon the method of Anderson and McKay (1983) with some modifications. An overnight culture of the *Listeria* isolate was grown in Brain Heart Infusion Broth (BHIB) (Difco Ltd., Detroit) at 37°C in a gyrotary shaking incubator at 125 rpm (New Brunswick Scientific Co., Inc., Edison, NJ). A 10% inoculum of the overnight culture was subcultured into fresh BHIB and incubated as before for 4 h. A 1.5 ml aliquot of the 4 h culture was transferred to a 1.5 mL Eppendorf centrifuge tube and centrifuged for 2 min at 7000 x g in a microcentrifuge (BIG Hermie National Labnet Co., Woodbridge, NJ). After discarding the supernatant, an additional 1.5 mL of the 4 h culture was centrifuged in the same tube. The resulting pellet was resuspended in 400 µL of 6.7% sucrose (w/v) in 50mM Tris-1mM EDTA, pH 8.0, and warmed to 37°C. A 100 µL quantity of freshly prepared lysozyme solution (Sigma, 10 mg/mL in 25 mM Tris, pH 8.0) was added and the mixture was incubated at 37°C for 5 min. After incubation, 50 µL of 0.25 M EDTA in 50 mM Tris, pH 8.0, was added, followed by the addition of 30 µL of 20% SDS (w/v) in 50 mM Tris-20 mM EDTA, pH 8.0. The sample was mixed by inversion and incubated at 37°C for 10 min to complete lysis. Thirty µL of a freshly prepared 3 N NaOH solution was then added and the tube gently mixed by intermittent inversion for 10 min. Next, 50 µL of 2 M Tris was added and the tube gently mixed for 3 min, followed by the addition of 75 µL of 5.0 M NaCl and 700 µL of 3% NaCl-saturated phenol. The sample was mixed by inversion and centrifuged for 5 min at room temperature. The upper aqueous phase was transferred to a new Eppendorf tube and extracted with 700 µL of

chloroform:isoamyl alcohol (24:1 v/v). The tube was centrifuged for 1 min at room temperature and the upper layer was transferred to a new Eppendorf tube and 1 mL of cold (-70°C) ethanol was added. After an overnight incubation at -20°C, the DNA pellet was obtained by centrifugation of the sample for 30 min. The ethanol was poured off and the sides of the tube dried with cotton tipped applicators and the tube allowed to dry for 1 h in a biosafety hood. The pellet was resuspended in 15 µL of TE buffer containing 20 mg/mL RNAase. This was prepared by adding 2 µL of RNAase stock (10 mg/mL) to 998 µl of TE buffer (10 mMTris, 1 mM EDTA, pH 8.0). The mixture was allowed to stand at room temperature for 30 min before the addition of tracking dye (0.05% bromophenol blue). The sample was examined by agarose gel electrophoresis on 0.7% agarose in TAE buffer to which ethidium bromide had been added after boiling to a final concentration of 0.5 µg/mL. Fifteen µL samples were loaded into the wells and subjected to a constant voltage of 60V from an Electrophoresis Power Supply EPS (Pharmacia (Canada) Inc., Baie d'Urfé, Quebec). After 3 h the gel was removed and viewed under UV light at 300 nm from a Foto UV 300 DNA Transilluminator (Fotodyne Inc., New Berlin, WI). The DNA from *Escherichia coli* V517 was isolated in the same manner and used as a standard for estimating the size of the plasmids (Macrina *et al.*, 1978).

5.3 Results

The incidence of *L. monocytogenes* in raw milk samples from farms testing positive in bulk milk is shown in Table 5.1. In order to correctly interpret the data it should be noted that the different samples were collected sequentially over a period of time; 'bulk tank' samples preceeding 'individual cow' samples which in turn preceeded individual 'quarter samples'. This procedure was followed in order to minimize the amount of aseptic quarter sampling required to detect animals shedding *L. monocytogenes* from the udder. Although 21 of the 401 individual milk samples were found to contain *L. monocytogenes*, only one quarter of one animal was positive following aseptic sampling. This animal was subsequently shown to have subclinical listerial mastitis (Fedio *et al.*, 1990).

Table 5.2 shows the incidence of *Listeria* spp. in non-milk samples taken from farms which shipped milk contaminated with *L. monocytogenes*. Overall, 73 of the 186 samples examined (39.2%) were found to contain *Listeria* spp. and, of those, 17 (9.1%) were found to be *L. monocytogenes*.

Table 5.1 Incidence of *Listeria monocytogenes* and *Listeria innocua* in milk samples from '*Listeria* positive' farms.

	Bulk Tank Samples			Individual Cow Samples			Aseptic Quarter Samples		
Farm	L. spp.	L. i.	L.m.	L. spp.	L. i.	L.m.	L. spp.	L.i.	L.m.
1	9/10	3/10	6/10	9/181	3/181	6/181	0/160	0/160	0/160
2	2/4	0/4	2/4	1/97	0/97	1/97	ND	ND	ND
3	1/3	0/3	1/3	10/51	9/51	1/51	0/55	0/55	0/55
4	3/3	0/3	3/3	13/72	0/72	13/72	1/47	0/47	1/47*
Total	15/20	3/20	12/20	33/401	12/401	21/401	1/262	0/262	1/262

L.spp. = *Listeria* species; L.i. = *Listeria innocua*; L.m. = *Listeria monocytogenes*

*confirmed case of listerial mastitis (Fedio *et al.*, 1990. Can. Vet. J. 31:773-775)

TABLE 5.2 Incidence of *Listeria monocytogenes* and *Listeria innocua* in non-milk samples from '*Listeria* positive' farms.

	RECTAL FECAL SAMPLES			SILAGE/ FEED *			ENVIRONMENTAL **		
Farm	L. spp.	L. i.	L. m.	L. spp.	L. i.	L. m.	L. spp.	L. i.	L. m.
1	2/24	2/24	1/24	3/19	3/19	0/19	7/28	7/28	0/28
2	8/19	8/19	0/19	8/12	8/12	1/12	15/16	15/16	1/16
3	10/16	3/16	8/16	4/10	3/10	2/10	8/16	6/16	2/16
4	3/10	3/10	1/10	3/10	3/10	1/10	2/6	2/6	0/6
Total	23/69	16/69	10/69	18/51	17/51	4/51	32/66	30/66	3/66

L.spp. = *Listeria* species; L.i. = *Listeria innocua*; L.m. = *Listeria monocytogenes*

* includes silage, feed supplements, silage effluent

** environmental samples include manure, soil, straw, swabs

Silage/feed samples (Table 5.2) included silage, hay, grain and other feed supplements. On farm 1, *L. monocytogenes* was not isolated from any of the feed samples. However, *Listeria* spp. were isolated from one silage sample (pH 5.5). Fourteen other silage samples with pH ranging from 4.4 to 7.7 were found to be negative for *Listeria* spp. *Listeria* spp. were also isolated from alfalfa hay. All of the feed related isolates recovered from Farm 1 were found to be *L. innocua*. On Farm 2, *Listeria* spp. were recovered from silage samples (pH 5.2 and pH 5.3). A drainage problem at the site where the silage was made resulted in accumulations of silage effluent which tested positive for both *L. monocytogenes* and *L. innocua*. Only *L. innocua* was recovered from samples of grain taken from the grainery, chopped grain in the barn, the inside of fresh bales of hay and other samples of silage effluent. On Farm 3, fresh silage samples, with pH values ranging from 3.6 to 4.1 were found to be free of *Listeria* spp. However, air dried silage samples that had accumulated near the outlets of the automatic feeders were found to be contaminated with *L. monocytogenes* and/or *L. innocua*. *L. innocua* was also isolated from samples of hay taken from the feed bunk. On Farm 4, *Listeria* spp. were not detected in fresh silage samples (pH 5.1 to 5.7), but were detected in silage which had been supplemented with grain and beet pellets. Upon further sampling, the grain supplement was found to contain *L. innocua* and the beet pellet suplement was contaminated with both *L. monocytogenes* and *L. innocua*.

Environmental sources (Table 5.2) included soil, manure, calf pen samples (soil, manure, straw, milk residues on feeders), water samples, and equipment swabs. On Farm 1, listeriae were isolated from straw, soil, manure and water samples taken from the calf pen. Two samples of fresh herbage were also found to be contaminated, but since the animals were kept indoors all year and could not possibly eat the fresh plant material they were considered as environmental samples rather than feed. This designation was used for samples of fresh herbage taken from other farms as well. On Farm 2, 15 of 16 environmental samples were found to contain listeriae. Rotting straw, soil, dried manure and soil from the calf pen, manure and the drain in the milking parlour yielded *L. innocua*. *L. monocytogenes* and *L. innocua* were isolated from one sample of herbage, while *L. innocua* only was isolated from a further two samples of fresh plant material. Environmental samples from Farm 3 that yielded *L. mononcytogenes* included swabs of water bowls, while *L. innocua* was isolated from the manure scraper, straw bedding, flies and cobwebs. On Farm 4, *L. monocytogenes* was not isolated from environmental samples, but *L. innocua* was isolated from trough water and manure collected from the manure scraper. On the four *Listeria*

positive farms, 33.3% of the rectal fecal samples were found to contain *Listeria* spp. as shown in Table 5.3. Of those animals, 10 (14.5%) were found to carry *Listeria monocytogenes* in their feces. The incidence of *Listeria* spp. varied from 4.2% to 62.5% and the prevalence of *L. monocytogenes* from 0% on Farm 2 to 50% on Farm 3. On farms that had consistently tested negative for *Listeria* spp. in bulk milk, the overall incidence of fecal carriage of *Listeria* spp. was found to be 24.6% with 13.2% of the samples containing *L. monocytogenes*. The incidence of *Listeria* spp. varied between 15.8% to 32.7 % and *L. monocytogenes* from 5.3% to 16.4%.

The persistence of *L. monocytogenes* in rectal fecal samples of cow feces was followed on *Listeria*-negative Farm 1. On original sampling, rectal fecal samples from 9 cows yielded *L. monocytogenes* (Table 5.4). Repeat samplings of the same 9 cows demonstrated that fecal shedding of the organism was sporadic. Fecal samples from two of the cows did not yield listeriae on any of the four repeat samplings. Four of the cows yielded listeriae on one of the four repeat samplings; and three of the cow fecal samples yielded listeriae on two of the repeat samplings. Of the two cows yielding *L. monocytogenes* on resampling, one did so on the first retest with two negative samples followed by the isolation of *L. innocua* on the fourth retest, while the other yielded *L. monocytogenes* only on the fourth retest with the three intervening samplings being negative for listeriae.

L. monocytogenes farm isolates were found to have the same antibiotic resistance/sensitivity pattern as *Listeria monocytogenes* Scott A and *Listeria innocua* ATCC 33090. That is, they were resistant to oxacillin and sensitive to cephalothin, penicillin, ampicillin, erythromycin, tetracycline, chloramphenicol, gentamicin, ciprofloxacin, amoxacillin, clindamycin, nitrofurantoin, vancomycin, norfloxacin and trimethoprim/sulfamethoxazole. Although the results agree with previously published antibiotic resistance patterns (Wiggins *et al.*, 1978; Rocourt and Catimel, 1985), no unique resistance marker could be found on any of the raw milk, fecal, or environmental isolates examined.

Plasmid DNA was not detected in *L. monocytogenes* milk isolates. Thus, plasmid profiles were not effective in establishing any relationships between milk and other isolates.

During this investigation, Fraser broth was used as an indicator broth when milk samples were examined for *Listeria* spp. and as both an indicator broth and a secondary enrichment broth in the isolation of *Listeria* spp. from environmental samples. When used for milk samples in conjunction with the FDA *Listeria* enrichment broth, 130/683 (19.0%) of the tubes turned black, with 49 of those later

Table 5.3 Comparison of incidence of *Listeria monocytogenes* and *Listeria innocua* in rectal fecal samples between '*Listeria* positive' and '*Listeria* negative' farms.

	BULK TANK MILK			RECTAL FECAL SAMPLES		
L.m. + Farm	L. spp.	L. i.	L.m.	L. spp. (%)	L. i. (%)	L.m. (%)
1	9/10	4/10	6/10	2/24 (8.3)	2/24 (8.3)	1/24 (4.2)
2	2/4	0/4	2/4	8/19 (42.1)	8/19 (42.1)	0/19 (0.0)
3	1/3	0/3	1/3	10/16 (62.5)	3/16 (18.8)	8/16 (50.0)
4	3/3	0/3	3/3	3/10 (30.0)	3/10 (30.0)	1/10 (10.0)
Total	15/20	4/20	12/20	23/69 (33.3)	16/69 (23.2)	10/69 (14.5)
L.m.- Farm						
-1	0/3	0/3	0/3	18/55 (32.7)	11/55 (20.0)	9/55 (16.4)
-2	0/4	0/4	0/4	3/19 (15.8)	2/19 (10.5)	1/19 (5.3)
-3	0/4	0/4	0/4	7/40 (17.5)	3/40 (7.5)	5/40 (12.5)
Total	0/11	0/11	0/11	28/114 (24.6)	16/114 (14.0)	15/114 (13.2)

L.spp. = *Listeria* species; L.i. = *Listeria innocua*; L.m. = *Listeria monocytogenes*

Table 5.4 Persistence of fecal excretion of *Listeria monocytogenes*

cow	initial isolate(s)	First retest (0.5 months)	Second retest (1 month)	Third retest (2 months)	Fourth retest (3 months)
702	Lm[a],Li[b]	-[c]	-	Li	Li
932	Lm	Lm	-	-	Li
665	Lm	-	-	Li	LiRh-[d]
615	Lm	-	-	-	Li
580	Lm	-	-	-	-
704	Lm	-	-	-	-
613	Lm	Li	-	-	-
647	Lm	-	-	-	Lm
315	Lm,Li	-	-	Li	-

[a] *Listeria monocytogenes*

[b] *Listeria innocua* (Rhamnose +ve)

[c] not detected in 25g sample

[d] *Listeria innocua* (Rhamnose -ve)

shown to contain *Listeria* spp. (Table 5.5). The 553 samples that did not turn black were all found to be negative for *Listeria* spp. No Fraser broth cultures originating from milk examining fecal samples, 129/252 Fraser broth cultures blackened (Table 5.6), 81 of which were shown to contain *Listeria* spp. Of those cultures that did not change color (123/252), 4 were found to contain *Listeria* spp. A similar result was seen when silage/feed-related and other environmental samples were tested (Table 5.7). Blackening in the Fraser broth with *Listeria* spp. present was seen in 24/67 (35.8%), blackening without *Listeria* spp. accounted for 17/67 (25.4%). No color change with no *Listeria* was observed in 24/67 (35.8%) of the samples, but 2/67 (3.0%) of the samples did not change color yet *Listeria* spp. were isolated from them. The color of Fraser broth cultures was also examined after 48h and every tube originally considered as false negative cultures had turned black.

5.4 Discussion

Data on *Listeria* spp. other than *L. monocytogenes* were collected and are included in the results as the incidence of both types is favored by similar environmental conditions. The isolation procedure involved enrichment culture followed by the picking of 'Listeria-like' colonies from selective agar plates. *L. monocytogenes* cannot be distinguished from other *Listeria* species on the selective agars used in this study. In some instances both *L. monocytogenes* and non-pathogenic *Listeria* spp. were isolated from the same sample. Thus the probability of selecting *L. monocytogenes* or other *Listeria* spp. from the selective agars is going to be dependent on their relative proportions in the population. By picking a limited number of isolates for confirmation, *L. monocytogenes* might be missed if the sample contained non-pathogenic listeriae in addition to *L. monocytogenes* (Cassiday *et al.*, 1990). Therefore, those sources which were found to be contaminated by non-pathogenic *Listeria* species should be considered as potentially positive for *L. monocytogenes*.

The animals (milk or feces), milking equipment, feeds and barn environment were all found to be sources of listeriae on dairy farms.

The high incidence of *L. monocytogenes* in the bulk milk samples (33-100%) is not unexpected, as the farms were selected on the basis of their previous history in this regard. However, the difference in the incidence of the organism in cow and quarter samples is evident. Listerial mastitis is generally considered to be rare and not the main mode of listerial contamination of raw milk (Gitter *et al.*, 1980; Fernandez Garayzabal *et al.*, 1987; van Dalen, 1988; Sharp, 1989). The results of this study

Table 5.5 Comparison of Fraser Broth and the FDA method for the detection of *Listeria* spp. in raw milk samples.

Fraser Broth Blackening	Detection of *Listeria* spp. by modified FDA procedure	Raw Milk Samples	
		Number	Percent
+	+	49	7.2
-	-	553	81.0
+	-	81	11.9
-	+	0	0
	Total:	683	100

Table 5.6 Utility of Fraser Broth in a modified USDA procedure for the detection of *Listeria* spp. in fecal samples.

Fraser Broth Blackening	Detection of *Listeria* spp. by modified USDA procedure	Fecal Samples	
		Number	Percent
+	+	81	32.1
-	-	119	47.2
+	-	48	19.1
-	+	4	1.6
	Total:	252	100

Table 5.7 Utility of Fraser Broth in a modified USDA procedure for the detection of *Listeria* spp. in silage/feed related and environmental samples.

Fraser broth Blackening	Detection of *Listeria* spp. by modified USDA procedure	Environmental	
		Number	Percent
+	+	24	35.8
-	-	24	35.8
+	-	17	25.4
-	+	2	3.0
	Total:	67	100

show that although the organism was virtually absent from the interior of the udder, it could still be found in approximately 5% of the non-aseptic cow samples. This would indicate that the organism entered the cow samples from the outer surfaces of the teats or the milking equipment. Further support for this is evident from the data for Farm 4 which had the highest incidence of *L. monocytogenes* in the cow samples. The presence of the animal with listerial mastitis would certainly lead to dissemination of the organism to other animals during milking. Contamination of milking equipment by the shedder likely caused some of the positive individual cow milk samples since six of the 13 positive samples were from cows that were milked with the same milking cluster as the cow with listerial mastitis. The other seven positive samples were from cows milked earlier and on different units than the cow with mastitis. In addition, the other three farms where cow samples were positive, yet no quarter sample was positive, would indicate the importance of sources other than the interior of the udder although intermittent shedding of *Listeria* in the milk can not be ruled out. Thus, the organism is getting into the individual cow samples as a result of shedding in the milk, from contaminated milking equipmen or from the c .ter surfaces of the teats.

Although both *L. monocytogenes* and *L. innocua* were widespread in the farm environment, major differences in incidence are evident between farms. The highest overall incidence of *L. monocytogenes* was found in the rectal fecal samples, 10/69 (15%). This finding is similar to that of Hofer (1983) who reported 18% for stool samples of healthy beef cattle. The finding of the organism in feces makes it appear likely that fecal contamination may contribute to *Listeria* getting into the milk. Persistence of the organisms in feces does occur, but it is somewhat sporadic. Further studies on this aspect, using more animals and a longer period of testing, would appear to be of interest.

The soil, a natural reservoir for animal excreta, has been suggested as being the propagation means for *Listeria*, thus contaminating grass, plants and water supplies (Hofer, 1983). Fecal contamination of grass used for silage by wild birds has been suggested as one way of introducing the pathogen into silage (Fenlon, 1985). Dijkstra (1971) found that listeria in naturally infected suspensions of brain tissue, silage, feces and milk in tryptose phosphate broth stored at 5°C maintained their viability for years.

A saprophytic life cycle has been suggested for *Listeria* (Welshimer and Donker-Voet, 1971; Weis and Seeliger, 1975) with man and other animals being continuously exposed. Given the ubiquitous nature of *L. monocytogenes* it is not

surprising to find the organism in feed. Alfalfa hay, grain, feed supplements (beet pellets) and silage were found to contain listeriae.

On Farm 3, fresh samples of silage were not found to contain *L. monocytogenes*, but dried up accumulations of silage yielded the organism. The organisms either survived the silage making process or were reintroduced onto the silage. The latter possibility seems more likely considering the numerous isolations of *L. monocytogenes* from fecal samples in the barn.

On Farm 4, silage appears to have been contaminated when feed supplements were added to it. Inoculation of the silage with the pathogen (via feed supplements) prior to feed-out could account for the sample of supplemented silage which was found to contain listeria. Feed grain was found to contain *L. innocua* and beet pellets *L. monocytogenes* and *L. innocua*.

On Farm 2, *Listeria* spp. were isolated from silage and in effluent from the silage. Both the method used in silage preparation and the location of the silage pit likely contributed to the presence of listeriae in the silage. The silage was composed of a mixture of barley and oats which was prepared in a loose pile on the ground, surrounded by bales of straw. The pile was covered with plastic held down by tires. The silage was prepared in an area of the farm where drainage was poor and as a result, accumulations of water which included effluent from the silage were present. *Listeria* spp. were recovered in silage that was sampled from the bottom or the side of the pile however, when the interior of the pile was examined, *Listeria* spp. were not recovered. Considerable contamination of the silage from dirt, mud and water during transport before feed-out was common.

Deacidification of silage during aerobic deterioration has been suggested as one way to make silage more likely to support the growth of *Listeria*. (Woolford, 1990). Lehnert (1964) found that *L. monocytogenes* died during the first 5 days when inoculated into silage effluent. However, inoculation of the effluent with *L. monocytogenes*, a pseudomonad and a yeast (*Torulopsis* spp.) resulted in an increase in pH to 6 to 7.2 and growth of the *Listeria*.

The high incidence of *L. monocytogenes* in the environmental samples, as compared with the incidence in aseptic milk samples, would indicate that the farm environment is the most likely source of the organism in raw bulk tank milk. However, this finding in itself does not answer the question as to why some farms regularly have *L. monocytogenes* in the bulk milk, as evidenced by the 'Listeria positive' farms and others do not ('Listeria negative' farms) (Fedio and Jackson 1990). Is the environment of 'Listeria negative' farms free from this organism? The

ubiquitous nature of the organism would indicate that this is unlikely. In order to answer this question it was decided to examine rectal fecal samples of cows on three farms with no history of *L. monocytogenes* in the bulk milk –'Listeria negative' farms. Rectal fecal samples were chosen because these had shown the highest incidence of the organism on the 'Listeria positive' farms. It is apparent that the incidence of both *L. monocytogenes* and *L. innocua* is essentially the same on the two types of farm. The importance of this finding is clear. It must be concluded from these data that although *L. monocytogenes* is widespread in the dairy farm environment, it is not inevitable that the organism will find its way into the bulk milk. It follows that good sanitation and milking technique can and do play a vital role in preventing or minimizing the presence of the organism in bulk milk. Further support for this assertion comes from observations of milking practices on the 'Listeria positive' farms. All of the farms could be characterized as following poor milking practices. On Farm 1, as an example, a common sponge was used for udder washing, udders were wet when the milking units were attached, leading to slippage, and the units were not removed promptly at the end of milking. The milking procedures were conducive to mastitis and excessive milk contamination from the environment. The former was confirmed by the detection of high levels of both pseudomonal and coliform mastitis on this farm during this study. In contrast, the milking procedures on the 'Listeria negative' farms were rated as good.

The use of Fraser Broth in this study proved to be a valuable screening test as an indicator for the presence of *Listeria* spp., especially in milk samples. For raw milk samples, FB gave 0% false negative results and only 12% false positives when compared with the cultural method. The results with fecal and environmental samples were less reliable, with about 2-3% false negatives and 19-25% false positives, presumably due to the presence of greater numbers and types of competing micro-organisms, as compared with raw milk.

Only two species of *Listeria* were isolated in the course of this study, namely *L. monocytogenes* and *L. innocua* and all of the isolates of *L. monocytogenes* proved to be members of serogroup 1. This would appear to be a somewhat limited representation of the genus, considering the number and the diverse nature of the samples. Further attempts to differentiate the isolates of *L. monocytogenes* using more extensive biochemical tests (API20S, MicroID and Biolog), antimicrobic susceptibility tests (MIC's) and plasmid profiling were unsuccessful, although some very minor differences were noted in MIC's. Accordingly, no conclusions can be reached regarding the epidemiological significance of the findings. Further analysis

of the isolates using restriction enzyme analysis (Wesley and Ashton, 1991), or multilocus enzyme electrophoresis (Bibb *et al.*, 1989) would appear to be necessary to achieve definitive characterization. The former method is being investigated at this time.

The results of this study support the following conclusions: a) *L. monocytogenes* and *L. innocua* are common in the dairy farm environment; b) the most likely sources of the organism in raw bulk tank milk are environmental in nature, with feces/manure playing the major role; c) contamination by *L. monocytogenes* from within the udder is likely to be rare; d) in most cases, control of the contamination of raw bulk tank milk should be readily achieved by good sanitation and milking practices; e) restriction enzyme analysis or multilocus enzyme electrophoresis of *L. monocytogenes* would appear to be necessary in order to reach meaningful conclusions on the epidemiology of this organism.

The findings also raise two other points for consideration. The first relates to the presence of pathogenic bacteria in raw bulk tank milk. In recent years, in addition to *L. monocytogenes,* organisms of the genera *Salmonella, Campylobacter*, and *Yersinia* have become increasingly important as causes of food borne illness related to dairy products. The environmental origins of *L. monocytogenes,* as detailed in this report, would almost certainly apply to these other genera. In essence, these organisms represent 'environmental' pathogens of raw milk, rather than traditional 'udder' pathogens of raw milk such as streptococci and staphylococci. These 'environmental' pathogens must be entering the milk supply, not from the udder, but from the environment, primarily due to unsanitary milking practices. The coliform testing of raw bulk milk was used for many years as a measure of farm hygiene and sanitation but has generally fallen by the wayside in recent times . In view of the factors stated above it is possible that the test should be re-examined to see if it has a place in controlling the incidence of pathogens of environmental origin. Having said this, it is still obvious that the best approach to control pathogens in raw milk will continue to be via education and enforcement of good milking practices.

A second and probably more important point for consideration is related to investigations of outbreaks of human listeriosis associated with the consumption of dairy products. A normal part of such investigations is the identification of the source of the contaminated raw milk. This latter process usually involves testing raw bulk tank milk samples, followed by testing of individual cow samples or pooled cow samples. The results presented in this paper would indicate that the absence of the organism from milk samples alone cannot in itself be considered proof that the

particular raw milk supply was not the original source. In order to prove this conclusively it would be necessary to demonstrate that the causative organism was not present anywhere in the farm environment, including not only milk but also environmental samples.

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6. The effect of tempering on the heat resistance of *Listeria monocytogenes* [1]

6.1 Introduction

Recent outbreaks of human listeriosis associated with the consumption of 'pasteurized' milk and milk products made from 'pasteurized' milk have been a cause for alarm in the dairy industry (Fleming *et al.*, 1985; James *et al.*, 1985). This stimulated a re-examination of the heat resistance of the causitive organism, *Listeria monocytogenes* (Bradshaw *et al*, 1985; Bunning *et al.*, 1988; Donnelly *et al.*, 1987; Doyle *et al.*, 1987; Fernandez Garayzabal *et al.*, 1987). In summary, the results of these studies confirmed that *L. monocytogenes* would be destroyed in milk by normal pasteurization procedures. Only the presence of very large and extraordinary numbers of the organism in the raw milk could result in survivors in the pasteurized product. It may be concluded, .herefore, that the presence of *L. monocytogenes* in pasteurized dairy products is most probably due to underpasteurization or post-pasteurization contamination. One other reason that would explain the organism surviving pasteurization is suggested by the results of Tsuchido *et al.* (1974, 1982) and Mackey & Derrick (1986, 1987a,b). These workers were able to increase the heat resistance of bacteria by means of mild heat treatments. Tsuchido *et al.* (1974) were able to increase the heat resistance of *Escherichia coli* K12 by raising the temperature of the cell suspension from 0-50°C at various rates prior to isothermal heating at 50°C. More recently, Mackey & Derrick (1986) increased the heat resistance of *Salmonella typhimurium* by exposing cultures to sublethal heat shock at 42, 45, or 48°C before exposing the organism to higher temperatures. They also demonstrated the same effect with *Salmonella thompson* when the organism was preheated at 48°C and then heated in food (Mackey & Derrick, 1987b).

The purpose of the work presented here was to determine whether it would be possible to increase the heat resistance of *L. monocytogenes* by mild preheating.

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6.2 Materials and Methods

6.2.1 Cultures and preparation of inoculum

One strain of *L. monocytogenes* was used throughout this study–Scott A, serotype 4b. Stock cultures were maintained on slants of Tryptic Soy Agar (Difco Ltd., Detroit) plus 0.6% Yeast Extract (Difco Ltd., Detriot) (TSA-YE) at 4°C. Subcultures were prepared by inoculating 250 ml of Tryptic Soy Broth (Difco Ltd., Detroit) plus 0.6% YE (Difco Ltd., Detroit) (TSB-YE) in 500 ml Erlenmeyer flasks. The subcultures were incubated for 24 h at 37°C in a gyrotary shaking incubator at 125 rpm (New Brunswick Scientific Co., Inc., Edison, NJ). One ml of the subculture was again inoculated into TSB-YE and incubated for a further 24 h. The second subculture was used as the test culture in the heating experiments.

6.2.2 Preheating and heat treatment

The inoculum, prepared as above, was centrifuged at 10000 x g for 20 min. The pellet was resuspended in 225 ml of TSB-YE. The resulting cell concentration was approximately 3 x 10^9 cfu/ml. A 9% (30 ml) inoculum was added to a 2 L Erlenmeyer side arm flask containing 300 ml of TSB-YE preheated to 60°C. Thermal death at 60°C was monitored by surface plating the heated suspension at intervals onto TSA-YE and LPM agar (lithium chloride–phenylethanol–moxalactam medium) (Lee & McClain, 1986). Plates were incubated at 37°C for 48 h before counting. This experiment was repeated with a test suspension that had been held at 48°C for 1 h before heating at 60°C.

The whole experiment was repeated with Ultra High Temperature (UHT) milk as the suspending medium in place of TSB-YE.

6.3 Results and Discussion

The survival curves of *L. monocytogenes* heated at 60°C in TSB-YE, with and without preheating, are shown in Fig. 6.1. It can be seen that preheating the suspension at 48°C results in a marked increase in heat resistance, as evidenced by differences in survival of 3 or more log cycles after 10–20 min. The lower survival on the more inhibitory medium, LPM, demonstrates that *L. monocytogenes* is subject to thermal injury as a result of the heating process (Clark & Ordal 1969). When the organism was preheated at 48°C in UHT milk the results were quite similar to those obtained in TSB-YE, although the effect of the preheating appears to be less marked

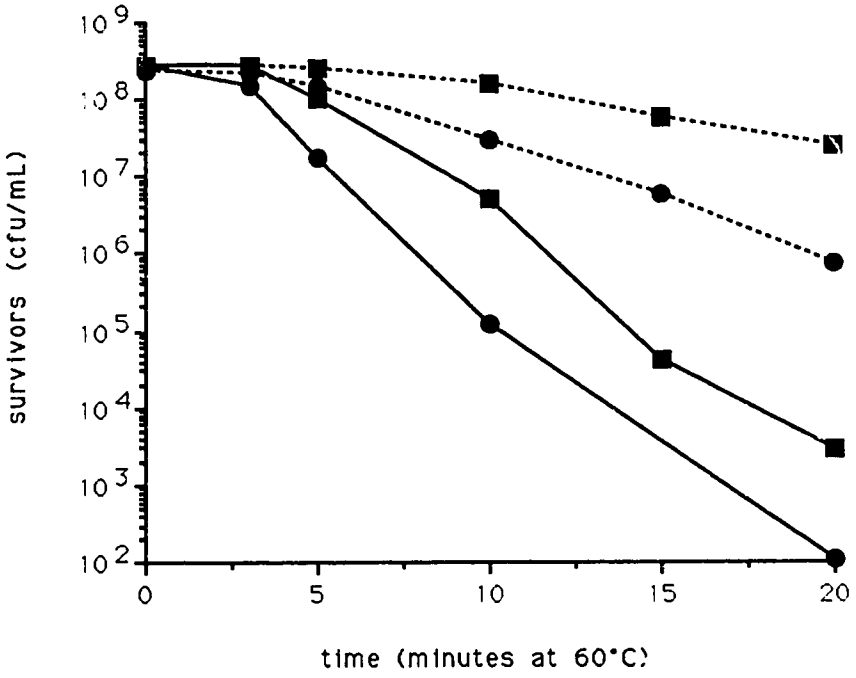

Figure 6.1 Effect of preheating in TSB-YE at 48°C/1 h on the survival of *L. monocytogenes* heated at 60°C in TSB-YE. Each point represents the geometric mean of three replicates. Media: ■, TSA-YE; ●, LPM. —, without preheating; ---,with preheating.

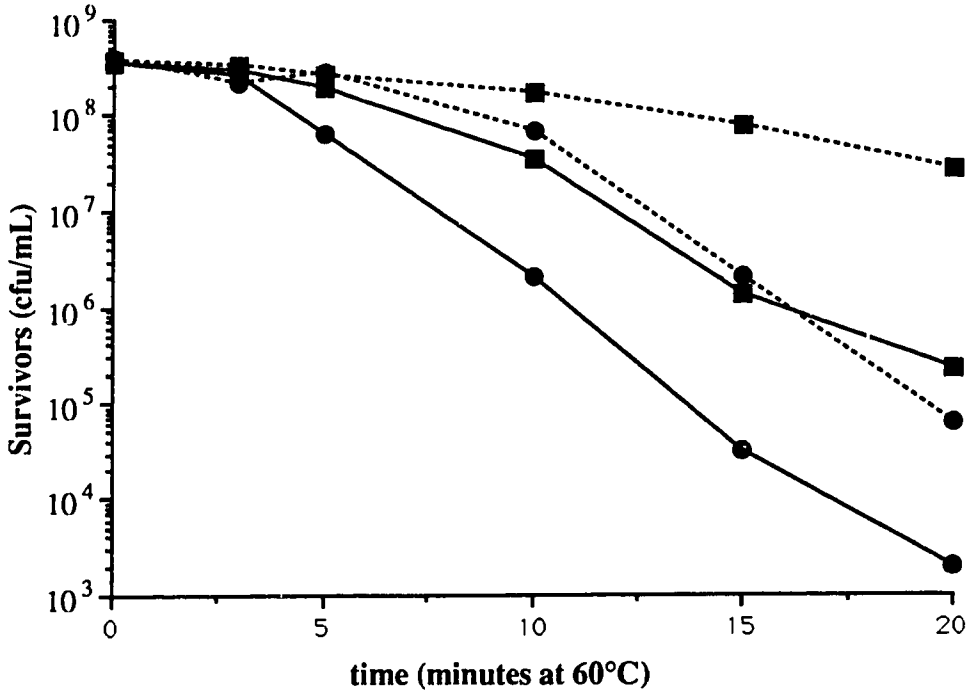

Figure 6.2 Effect of preheating in UHT milk at 48°C/1 h on the survival of *L. monocytogenes* heated at 60°C in UHT milk. Each point represents the geometric mean of three replicates. Media: ■, TSA-YE; ●,LPM. —, without preheating; ---, with preheating.

(Fig. 6.2). These results demonstrate that preheating *L. monocytogenes* in both broth and milk at 48°C for 1 h increases the heat-resistance of the organism when subsequently exposed to 60°C. This finding is supported by the data of Crawford *et al.* (1989). It is clearly not possible to say whether preheating has been a significant factor leading to the survival of this organism during commercial pasteurization. Obviously deliberate or inadvertant preheating can occur in processing milk and other foods. It would appear prudent to recognize the phenomenon of increased heat-resistance due to preheating as a contributing factor in the overall heat resistance of microorganisms. Further work is in progress to elucidate the cellular changes that result in increased heat resistance. Preliminary results indicate that protein synthesis occurs during preheating, suggesting that 'heat shock proteins' may be involved. We propose the term 'temper' (temper vb, tempered; tempered; tempering) be used to denote the phenomenon of heat-induced elevation of heat resistance. This is to avoid confusion with the terms 'sublethal heating' and 'heat shock', both of which have other microbiological connotations, and preheating which is commonly applied to food processing treatments that have no microbiological significance. *Temper* is proposed in the sense of 'to make stronger and more resilient through hardship'.

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7. Effects of tempering menstrua and the ribosomal response to tempering

7.1 Introduction

Fedio and Jackson (1989) proposed that the term 'temper' (tempering) be used to denote the phenomenon of heat-induced elevation of heat resistance. Use of the term tempering would avoid confusion with the terms 'sublethal heating' and 'heat shock', both of which have other microbiological connotations, and preheating which is commonly applied to food processing treatments that have no microbiological significance. *Temper* was proposed in the sense of 'to make stronger and more resilient through hardship'. Thus, tempering will be used in this context in this section.

Increased thermotolerance of bacteria due to tempering was first reported by Tsuchido *et al.* (1974) who found that cells of *Escherichia coli* pre-incubated at higher non-lethal temperatures (37°C to 45°C) for 10 min prior to heat challenge at 50°C showed more survivors than cells pre-incubated at lower temperatures (10°C to 20°C).

An increase in the thermal resistance of *Salmonella thompson* was reported for cells that were incubated at 48°C for 30 min prior to heating at 54°C and 60°C (Mackey and Derrick, 1987). Similarly, increases in the heat resistance of *Listeria monocytogenes* have been reported following a mild heat treatment of the organism (Fedio and Jackson, 1989; Quintavalla and Barbuti, 1989; Farber and Brown, 1990; Knabel *et al.*, 1990; Linton *et al.*, 1990; Bunning *et al.*, 1990).

Although most studies have examined increased thermotolerance following tempering in bacteriological media, the phenomenon has also been observed following tempering in foods. Mackey and Derrick (1987) observed increases in the heat resistance of *Salmonella thompson* following tempering in liquid whole egg, 10 and 40% milk solids, and minced beef. Increases in the heat resistance of *L. monocytogenes* have been shown following tempering in 2% UHT milk (Fedio and Jackson, 1989), cured meat (Farber, 1989), and raw sausage mix (Farber and Brown, 1990).

Previous work on thermal injury in vegetative bacteria has shown that heating may affect a number of sites in the cell including the cytoplasmic membrane, the cell wall, the outer membrane in Gram-negative organisms, teichoic acid in Gram-positive organisms, DNA, and ribosomes and ribosomal RNA (Tomlins and Ordal, 1976). A commonly observed heat-induced lesion in bacteria is the degradation of

ribosomes and ribosomal RNA (Sogin and Ordal, 1967; Tomlins and Ordal, 1971; Pierson *et al.*, 1978). In addition, as a response to mild thermal stress, a specific set of proteins known as heat-shock proteins are produced in a wide variety of organisms and have been suggested as being responsible for increased thermal resistance (Yamamori and Yura, 1982; Lindquist, 1986)

The purpose of the work described here was to look in detail at some of these factors to determine if they are related to the phenomenon of tempering.

7.2 Materials and Methods

7.2.1 Bacterial strains

Listeria monocytogenes Scott A was the test organism used for tempering experiments. *Staphylococcus aureus* ATCC 35152 and *Escherichia coli* D10 (a gift of Dr. P.C. Tai, Boston Biomedical Research Institute, Boston, Mass.) were used for comparison of ribosome patterns obtained following sucrose gradient centrifugation of cell extracts.

Stock cultures were maintained on Tryptic soy agar (Difco) plus 0.6% yeast extract (TSA-YE) at 4°C.

7.2.2 Prepartion of cells for heating

A loopful of *L. monocytogenes* Scott A was inoculated into 3 mL of Trypticase Soy Broth with 0.6% yeast extract (TSB-YE) and incubated at 37°C with shaking (125 rpm) for 24h. One mL of this culture was inoculated into 225 mL of fresh TSB-YE and incubated in the same manner. The culture was then harvested by centrifugation at 4°C and resuspended in 225 mL of tempering menstruum. The resuspended cells were centrifuged again and the pellet resuspended in 225 mL of tempering menstruum; this suspension was the control or untempered cells used for heating experiments. Tempered cells were prepared by distributing 50 mL aliquots of the untempered cell suspension into 250 mL Erlenmeyer flasks and placing the flask into a 48°C waterbath for 1 h prior to heat inactivation studies.

7.2.3 Inhibitors

Using TSB-YE as the menstruum, resuspended, untempered cells were prepared. Untempered cells were subjected to heat inactivation directly and tempered cells were incubated at 48°C for 1 h prior to heat challenge. When inhibitors were used, they were added to a portion of the untempered cell suspension and incubated at

37°C with shaking for 15 min prior to heating. Chloramphenicol (100 µg/mL), rifamycin (10 µg/mL) and 2,4-dinitrophenol (80 µg/mL) were the inhibitors used.

7.2.4 Tempering menstrua

The centrifuged cells were resuspended in various tempering menstrua: a) 0.1M potassium phosphate buffer (pH 7.0), b) 0.1M potassium phosphate buffer +1% glucose, c) 0.1M potassium phosphate buffer +1% casamino acids, d) 0.1M potassium phosphate buffer +1% glucose +1% casamino acids, e) 0.1M potassium phosphate buffer +1% yeast extract, f) 0.1M potassium phosphate buffer + 1% NaCl, g) 0.1M potassium phosphate buffer +1% NaCl +1% yeast extract, h) 0.1M potassium phosphate buffer +1% NaCl +1% yeast extract + 1% glucose+ 1% casamino acids. Part of the suspension was tempered (heated at 48°C for 1 h) prior to heating experiments.

7.2.5 Heating

For thermal inactivation studies, a 10% inoculum (25 mL) of either untempered or tempered cells was added to 225 mL of TSB-YE that had been preheated to 60°C and heated in a shaking water bath. At timed intervals aliquots of the heated suspension were removed, diluted in 0.1% peptone and plated onto TSA-YE, and incubated at 30°C for 48h before counting.

7.2.6 Preparation of cell lysates for sucrose gradient centrifugation

The cells used in the preparation of cell lysates for sucrose gradient centrifugation were of four types: 1) untempered cells, 2) untempered cells that had been heated at 60°C for 15 min, 3) tempered cells and 4) tempered cells that had been heated at 60°C for 15 min.

The cell lysates were prepared by alumina grinding according to the method of Davis *et al.* (1986). The cells were harvested by centrifugation and washed once with ice cold TKM buffer. TKM buffer contained 10mM Tris hydrochloride (pH 7.6), 50mM KCl, and 10mM magnesium acetate. The pellet was frozen with solid CO_2-ethanol and stored at -70°C. The cells were lysed by grinding with 1.5 times their weight of alumina, and DNase I was then added (2 µg/g of cells). The lysate was diluted with an equal weight of cold TKM buffer and centrifuged at 12,000 x g at 4°C for 10 min to remove cell debris and alumina. The supernatant was centrifuged at 30,000 x g at 4°C for 30 min and the resulting preparation was frozen in small

portions in solid CO_2-ethanol and stored at -70°C until used for sucrose gradient centrifugation.

7.2.7 Sucrose gradient centrifugation

Sucrose gradients (10-30%) were prepared in TKM buffer. From a stock solution of 60% (w/w) sucrose in TKM buffer (pH 7.6), five sucrose solutions in TKM buffer were prepared: 10%, 15%, 20%, 25% and 30%. Starting with the most concentrated, 6.8 mL of each was added to 34mL ultracentrifuge tubes (Ultraclear, Beckman Instruments Inc., Irvine, CA). The gradients were allowed to diffuse overnight at 1°C. Centrifugation to display the ribosome profiles was performed by applying the sample of the cell lysate to the top of the gradient. The gradients were centrifuged for 6.5 h at 4°C at 25,000 rpm in an SW27 rotor (Beckman Instruments Inc., Irvine, CA). The tubes were unloaded from the bottom of the tube with a peristaltic pump attached to a fraction collector and UV monitor (Pharmacia (Canada) Inc., Baie d'Urfé, Quebec). Absorbance at 254 nm was monitored.

7.2.8 Radiolabelling of bacteria for the examination of ribosomes

Labelled cultures of *L. monocytogenes* were prepared by the addition of 2 μCi/mL of [6-^{3}H]-uracil (NEN® Research Products, Du Pont Canada Inc., Markham, Ontario) to a mid-log phase culture, while labelled *E. coli* were prepared by growing cells with 0.2 μCi/mL of [2-^{14}C]-uracil (NEN® Research Products, Du Pont Canada Inc., Markham, Ontario). The cells were grown for an additional 2 h with shaking at 37°C at which time the cells were harvested by centrifugation (6000 rpm for 15 min in the GSA rotor of the Sorvall centrifuge) at 4°C and washed once in ice cold TKM buffer. The resulting pellet was frozen in solid CO_2-ethanol and ground with alumina as described above. Following ultracentrifugation at 25000 rpm in the SW27 rotor for 6.5 h at 4°C, 0.45 mL fractions were collected from the bottom of the tube and a 100 μL portion of each fraction was added to 10 mL of scintillation cocktail (Ecolite™, ICN Biomedicals, Inc., Irvine, CA) and counted in a Beckman LS 1801 liquid scintillation counter (Beckman Instruments Inc., Irvine, CA) using a ^{3}H/^{14}C dual label DPM program.

7.3 Results and Discussion

The data presented in Fig. 7.1 show that tempering *L. monocytogenes* for 1 h at 48°C yields cells with increased thermotolerance. There was an approximate 4 log decrease in the extent of heat induced death (after 20 min at 60°C) for those cells

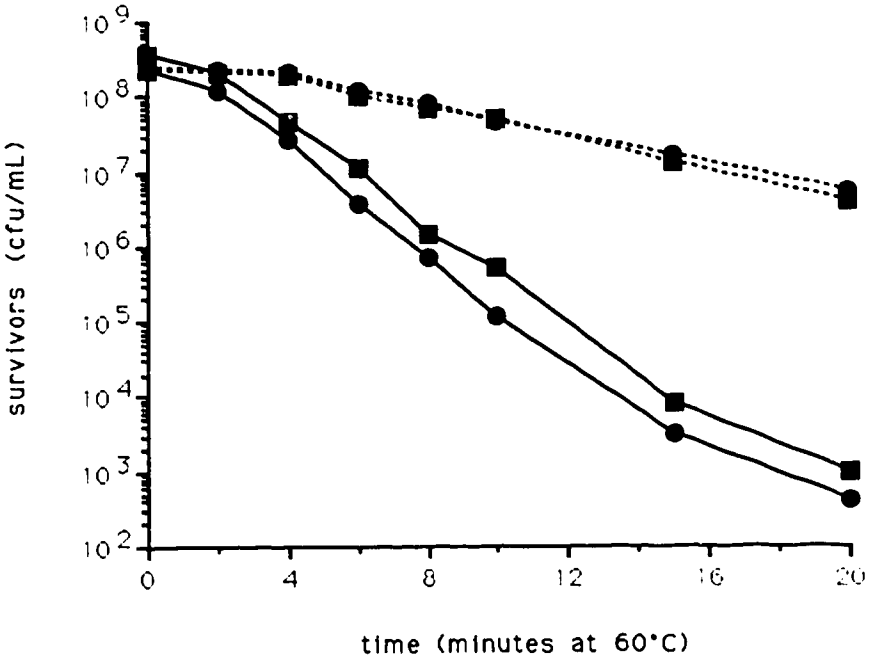

Figure 7.1 **Maintenance of increased thermotolerance during cold storage.**

(------) tempered cells

(——) untempered cells

■ heated immediately

● heated after 24 h at 1°C

given the tempering treatment. A similar response was seen for tempered cells that were stored at 1°C for 24 h prior to being challenged at 60°C.

The duration of acquired thermotolerance appears to be temperature dependent. Bunning *et al.* (1990) found that when cells tempered at 42°C were shifted to 35°C, the $D_{57.8°C}$ values returned to those of untempered cells within 1 h, but if kept at 42°C, the increased thermotolerance persisted for at least 4 h. Mackey and Derrick (1986) found that heat-shocked cells of *Salmonella typhimurium* maintained their increased thermotolerance for 10h when the cells were stored at 42, 45 or 48°C. Yamomouri and Yura (1982) found that increased thermotolerance in *E. coli* was lost within 1 h of shift back to a permissive growing temperature. In the present study, tempered cells held at 1°C retained their increased thermotolerance for 24 h. These results are supported by the work of Farber and Brown (1990) who found that cells of *L. monocytogenes* shocked at 48°C for 1 h in a fermented sausage mix maintained their increased thermotolerance after storage for 24 h at 4°C. Maintenence of the thermotolerant state for prolonged periods of time was shown in the work of Black and Jackson (1990), who found that tempered cells of *L. monocytogenes* held at 4°C in TSB-YE retained their thermotolerant state for at least 7 d, after which time the cells started to show loss of viability as did non-tempered control cells. These results indicate that the stability of the tempering phenomenon could be an important determinant for the survival of *L. monocytogenes* in foods even after storage at low temperature.

The composition of the tempering menstruum was an important determinant in the development of increased thermotolerance following heat-shock (Fig. 7.2a, 7.2b and 7.2c). *L. monocytogenes*, tempered in 0.1 M phosphate buffer (pH 7.0) (PB) showed a marked increase in killing at 60°C when compared with cells that were resuspended but not tempered in 0.1 M phosphate buffer (Fig 7.2a). In contrast, tempering in TSB-YE resulted in increased thermotolerance (Fig. 7.1, 7.2a). When *L. monocytogenes* was tempered in 0.1 M phosphate buffer supplemented with 1% casamino acids or 1% glucose the cells were less thermotolerant than untempered cells (Fig. 7.2b). However, tempering in 0.1 M phosphate buffer supplemented with 1% yeast extract resulted in cells that were more thermotolerant than the untempered cells (Fig. 7.2b). When *L. monocytogenes* was tempered in 0.1 M phosphate buffer + 1% NaCl (PBS), the cells became more sensitive to heating than untempered cells (Fig. 7.2c). Increases in thermotolerance were observed for bacteria tempered in PBS supplemented with 1% yeast extract and in PBS supplemented with 1% yeast extract, 1% casamino acids and 1% glucose (Fig. 7.2c).

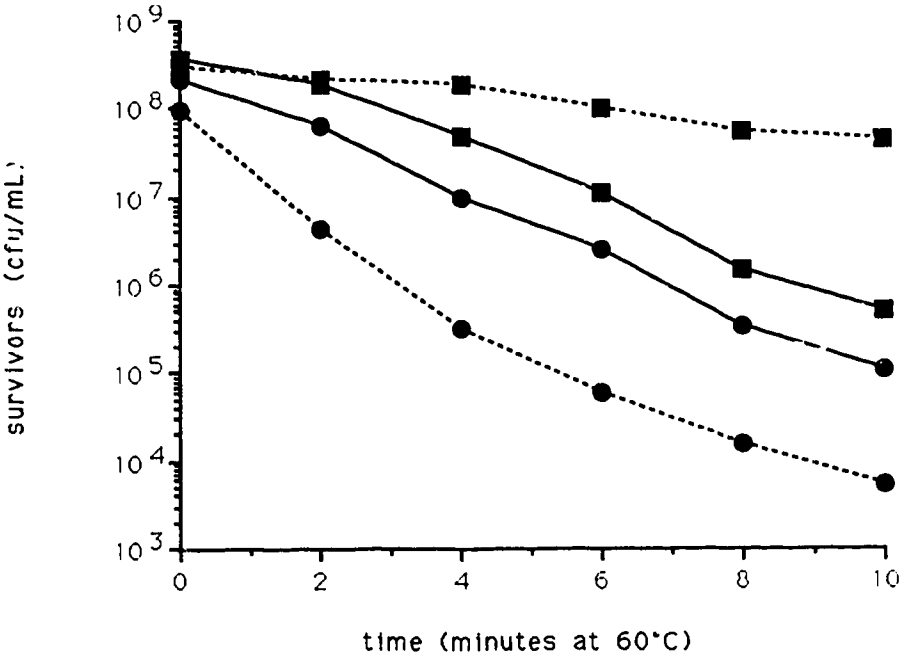

Figure 7.2a **Effect of tempering menstruum on the heat resistance of *L. monocytogenes* Scott A.**

(------) tempered cells

(——) untempered cells

● 0.1 M phosphate buffer (pH 7.0)

■ Trypticase Soy Broth + 0.6% yeast extract

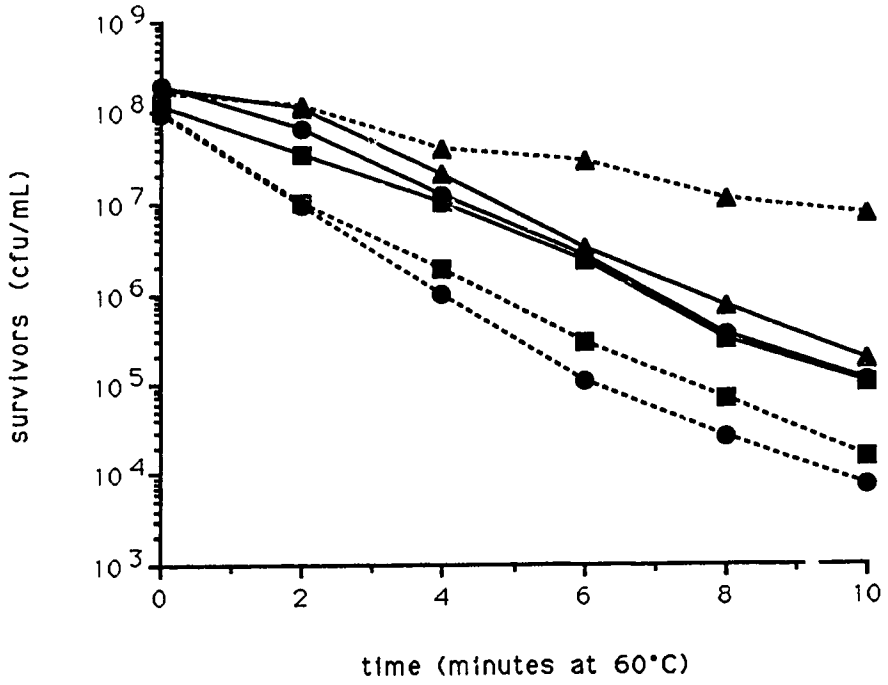

Figure 7.2b **Effect of tempering menstruum on the heat resistance of *L. monocytogenes* Scott A.**

(------) tempered cells

(——) untempered cells

■ 0.1 M phosphate buffer + 1% glucose

● 0.1M phosphate buffer + 1% casamino acids

▲ 0.1M phosphate buffer + 1% yeast extract

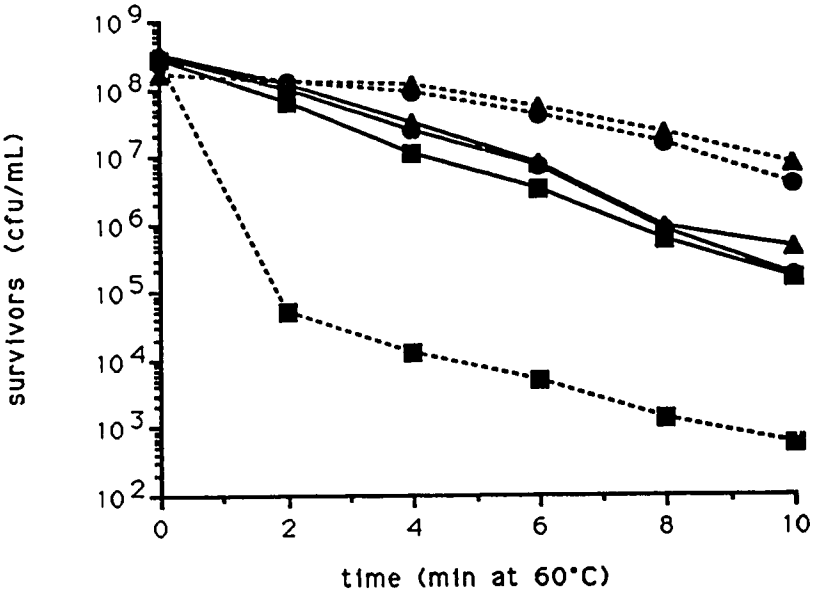

Figure 7.2c **Effect of tempering menstruum on the heat resistance of *L. monocytogenes* Scott A.**

(------) tempered cells

(——) untempered cells

■ 0.1M phosphate buffer + 1% NaCl

● 0.1M phosphate buffer + 1% NaCl + 1% yeast extract

▲ 0.1M phosphate buffer + 1% NaCl + 1% yeast extract +1% glucose + 1% casamino acids

The extent of killing in each of the tempering menstrua was compared by considering the $\log_{10}$kill after 10 min at 60°C. A significant tempering effect was observed for all tempering menstrua except 0.1 M phosphate buffer when *L. monocytogenes* was challenged at 60°C for 10 min (Table 7.1).

It is shown in Table 7.1 that when untempered cells were resuspended in PB before heat challenge in TSB-YE, the $\log_{10}$kill was significantly greater than when any other tempering menstruum was used. TSB-YE as a resuspension medium resulted in the lowest $\log_{10}$kill.

Among tempering menstrua, PB, PB+ 1% glucose, PB+1% casamino acids and PB + 1% glucose +1% casamino acids showed equal or slightly reduced thermotolerance, with no significant differences between them. However, tempering in PB +1% yeast extract, PBS +1% yeast extract, PBS +1% yeast extract +1% glucose +1% casamino acids and TSB-YE all increased the thermotolerance of *L. monocytogenes*, with TSB-YE producing the most pronounced effect.

Thus, the menstruum is important in determining whether or not tempering will result in increased thermotolerance. These results are in agreement with those of Mackey and Derrick (1990) who found that increased thermotolerance in *Salmonella* was most apparent in rich media. However, much more work is required to establish the principal molecular determinants in media that support tempering.

To obtain some indication of the mechanisms involved in acquired thermotolerance, selective metabolic inhibitors were added to the tempering menstruum. The effect of the addition of inhibitors to the tempering menstruum on the development of acquired thermotolerance is shown in Fig. 7.3. It can be seen that chloramphenicol, rifamycin and 2,4-dinitrophenol all caused a decrease in the heat resistance of organisms tempered in their presence compared with the tempered control. However, in all cases the heat resistance was still greater than the untempered control. The involvement of protein synthesis in the acquired thermotolerance can be postulated, since chloramphenicol treatment of the cells during tempering lowered the heat resistance of the organisms. This result is in agreement with the work of others (Mackey and Derrick, 1990). Partial inhibition of acquired thermotolerance was also observed when rifamycin was added to the tempering menstruum. Inhibition by rifamycin suggests that *de novo* RNA synthesis is necessary for the full development of the response. When 2,4-dinitrophenol, an uncoupling agent, which releases electron transport from respiratory control, was added to the tempering menstruum, partial inhibition of acquired thermotolerance was also seen suggesting that the response is an

Table 7.1 Effect of tempering menstruum on the survival of *Listeria monocytogenes* Scott A following heating for 10 min at 60°C.

	Log_{10} kill at 60°C[1]		
tempering menstruum[2]	untempered[3]	tempered[3]	LSD[4]
PBS	3.270[b]	5.193[a]	+
PB	3.657[a]	3.867[b]	ns
PB+glu+caa	3.213[b]	4.167[b]	+
PB+glu	3.010[bc]	3.867[b]	+
PB+caa	3.360[ab]	4.260[b]	+
PBS+ye	3.113[b]	1.870[c]	+
PBS+ye+glu+caa	3.203[b]	1.920[c]	+
PB+ye	3.257[b]	1.420[d]	+
TSB+ 0.6%ye	2.733[c]	0.713[e]	+

[1]Log_{10} kill = log_{10} cfu/mL (0 min) – log_{10} cfu/mL (10 min), results are the mean of three replicates

[2]tempering menstrua : PB=0.1M phosphate buffer (pH 7.0), PBS=0.1M phosphate buffer + 1% NaCl, glu=1% glucose, caa=1% casamino acids, ye=1% yeast extract

[3]means within columns followed by the same superscript letter are not statistically different ($p<0.05$, Duncan's Multiple Range Test)

[4] + = Log_{10} kill means for the untempered and tempered treatments are significantly different ($p<0.05$, Least Significant Difference)
ns = Log_{10} kill means for the untempered and tempered treatments are not significantly different ($p<0.05$, Least Significant Difference)

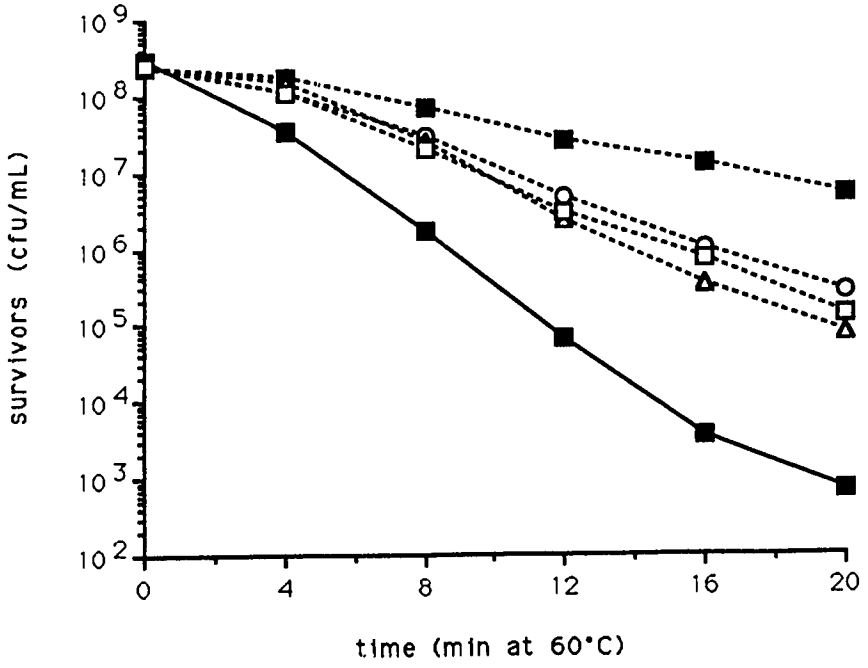

Figure 7.3 **Effect of the addition of metabolic inhibitors on the development of acquired thermotolerance.**

(------) tempered cells

(——) untempered cells

○ TSB-YE + chloramphenicol (100 μg/mL)

△ TSB-YE + rifamycin (10 μg/mL)

□ TSB-YE + 2,4-dinitrophenol (80 μg/mL)

■ TSB-YE with no inhibitors

active, energy requiring process. In summary, it can be concluded that protein synthesis, RNA synthesis and energy yielding mechanisms are all involved in the tempering phenomenon.

To determine the effect of heat on the ribosomes of *L. monocytogenes*, cell extracts were subjected to analysis on sucrose gradients. The results of the effect of tempering on ribosomal profiles of *L. monocytogenes* are shown in Fig. 7.4. Four peaks are seen in the absorbance trace of the extract from unheated and untempered cells of *L. monocytogenes* Scott A (Fig 7.4a). In Fig 7.4b, the profile of an extract from untempered cells that had been heated at 60°C for 15 minutes is shown. It can be seen that only one peak remains following heating of the cells. In the case of tempered cells (Fig. 7.4c) the ribosome profile is like that of the untempered and unheated cells (Fig. 7.4a). When the tempered cells are subjected to the 60°C for 15 minutes, all four of the ribosome peaks were retained (Fig. 7.4d). Thus, tempering of the cells resulted in increased thermal stability of the ribosomes.

Similarly, tempering resulted in increased thermal stability of the ribosomes of *S. aureus* (Fig. 7.5a-d). However, the ribosomes of untempered cells that had been heated at 60°C for 15 minutes were completely degraded (Fig 7.5b). Tempered cells that had been heated at 60°C for 15 minutes still maintained ribosome peaks (Fig. 7.5d).

E. coli D10, with a known ribosome profile, was provided by Dr. P.C. Tai (Boston Biomedical Research Institute, Boston Mass.) for confirmation of the sedimentation value of the ribosomal peaks for *L. monocytogenes*. In Fig 7.6a, the peaks marked 2, 3, and 4 represent 70S, 50S and 30S ribosomal subunits, respectively. When compared with *L. monocytogenes* (Fig 7.6b), it can be seen that three of the peaks have similar sedimentation properties as *E. coli* D10; however an additional peak at the bottom end of the tube could represent polymeric ribosomes. The ribosome profiles of ^{3}H labelled *L. monocytogenes* and ^{14}C labelled *E. coli* D10, were carried out to confirm that the peaks seen in the absorbance traces contained RNA, and are shown in Fig. 7.7.

Sublethal heating of bacterial suspensions has been found to cause the degradation of ribosomes, with the preferential destruction of the 30S ribosomal subunit and the destruction of its constituent 16S rRNA (Rosenthal *et al.*, 1972; Rosenthal and Iandolo, 1970). However, it would appear that ribosome damage during the sublethal heating of *S. aureus* is a consequence of the loss of Mg^{2+} from the cell and that ribosome destruction is a secondary effect (Hurst and Hughes, 1978). Thus, heat induced degradation of ribosomes has been attributed to destabilization of

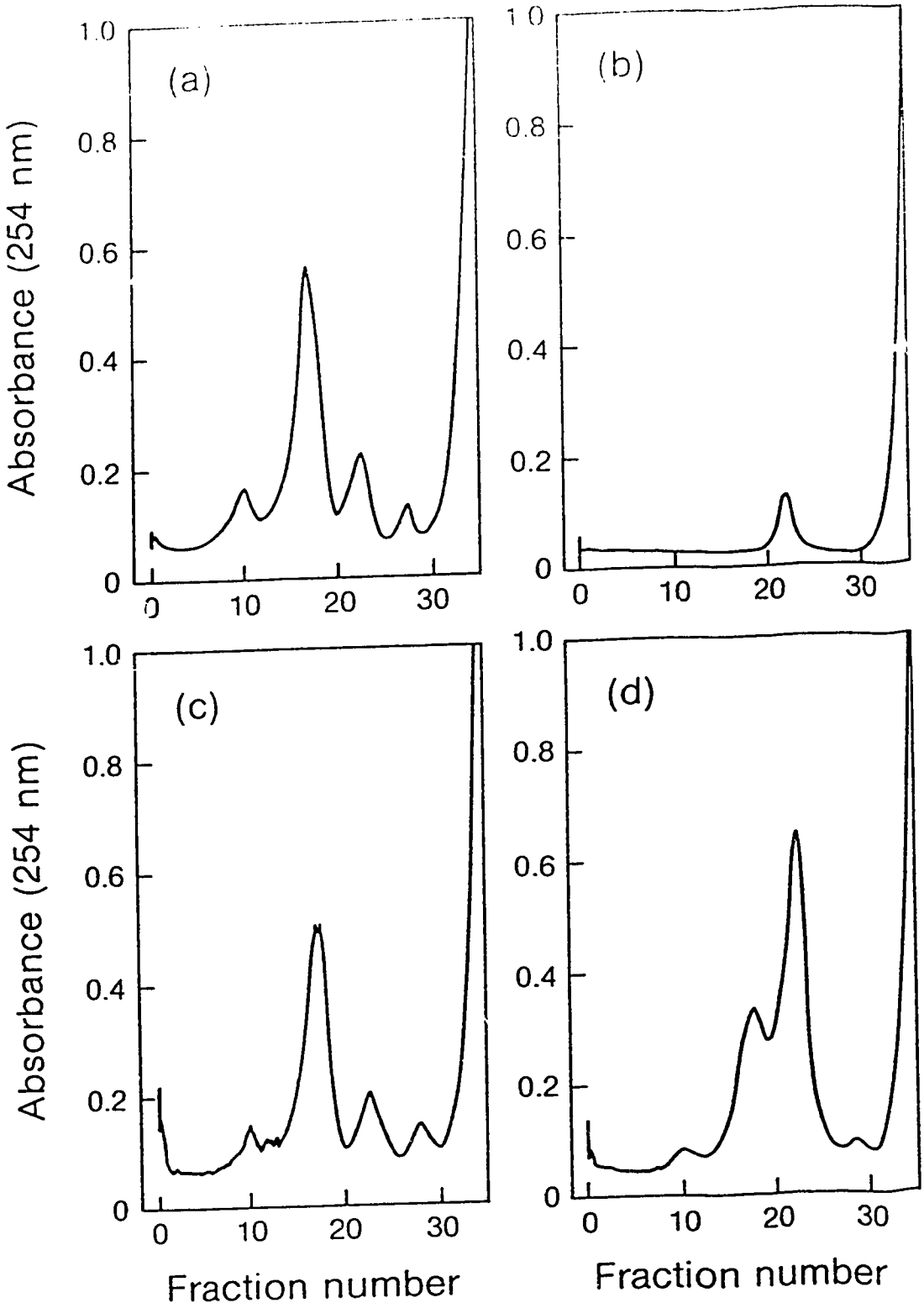

Figure 7.4 **Ribosome profiles of *Listeria monocytogenes*.**
a) untempered, unheated (b) untempered, heated (c) tempered, unheated (d) tempered, heated

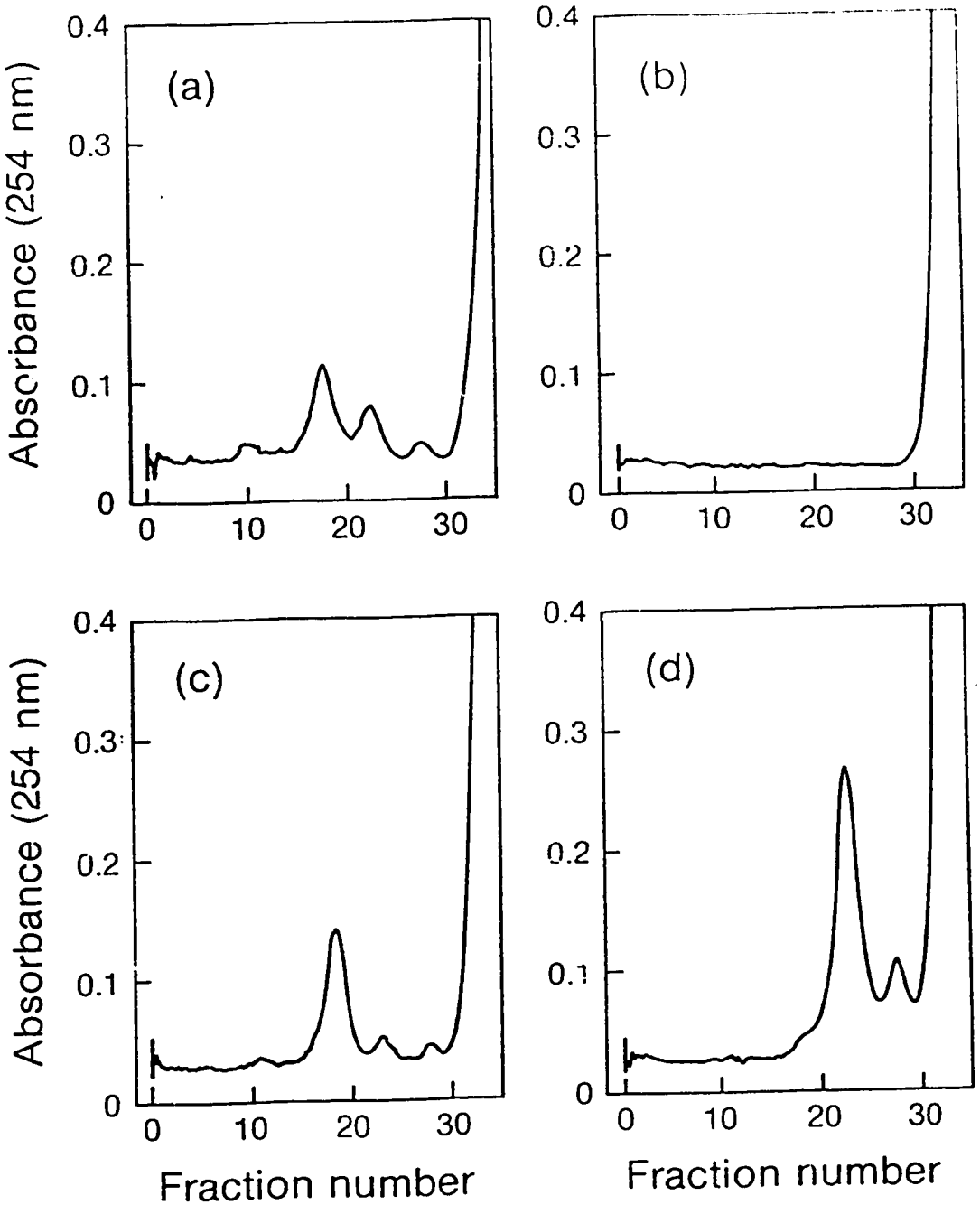

Figure 7.5 Ribosome profiles of *Staphylococcus aureus.*
(a) untempered, unheated (b) untempered, heated (c) tempered, unheated (d) tempered, heated

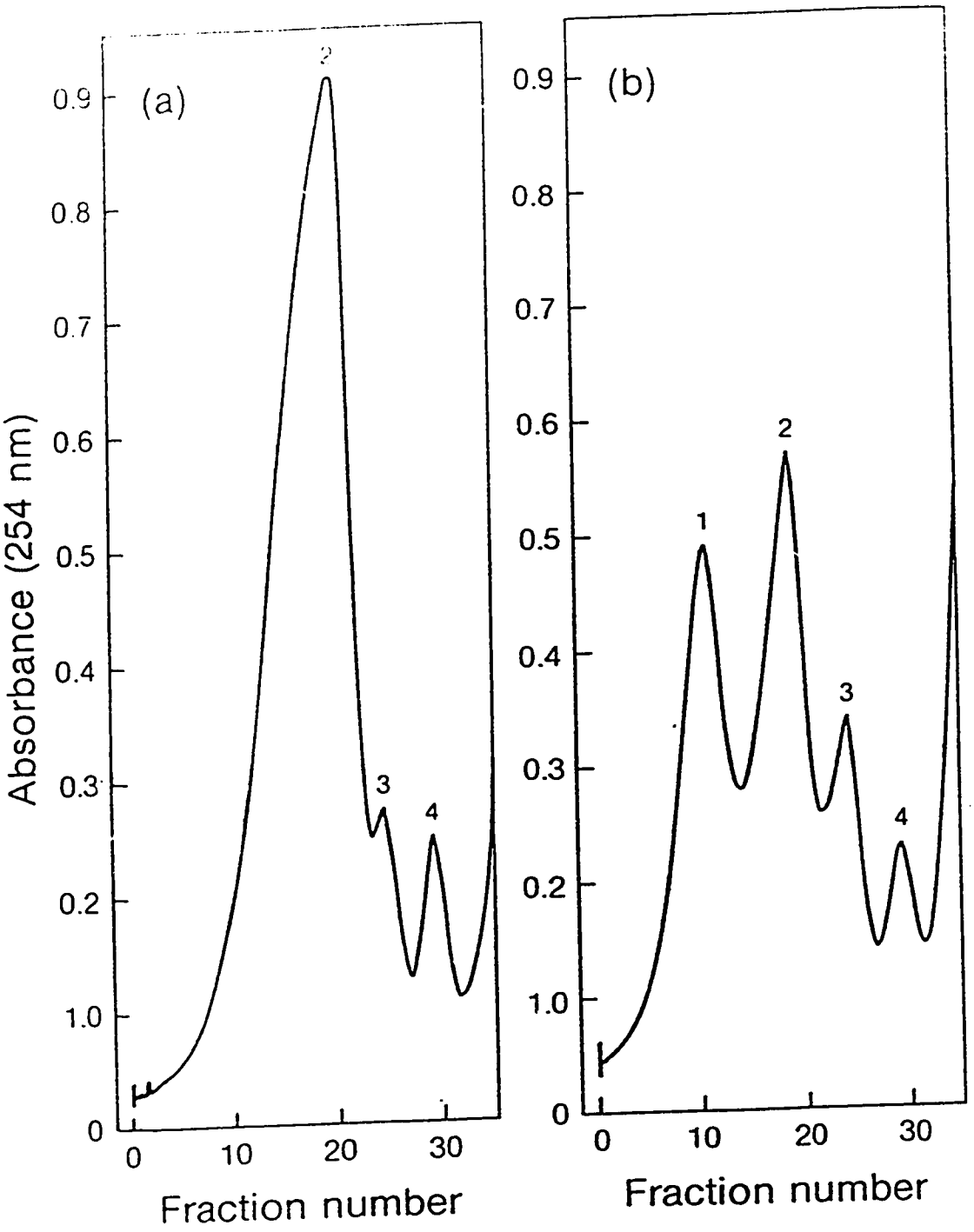

Figure 7.6 Comparison of the ribosome profiles of untempered, unheated cells of *Escherichia coli* (a) and *Listeria monocytogenes* (b).

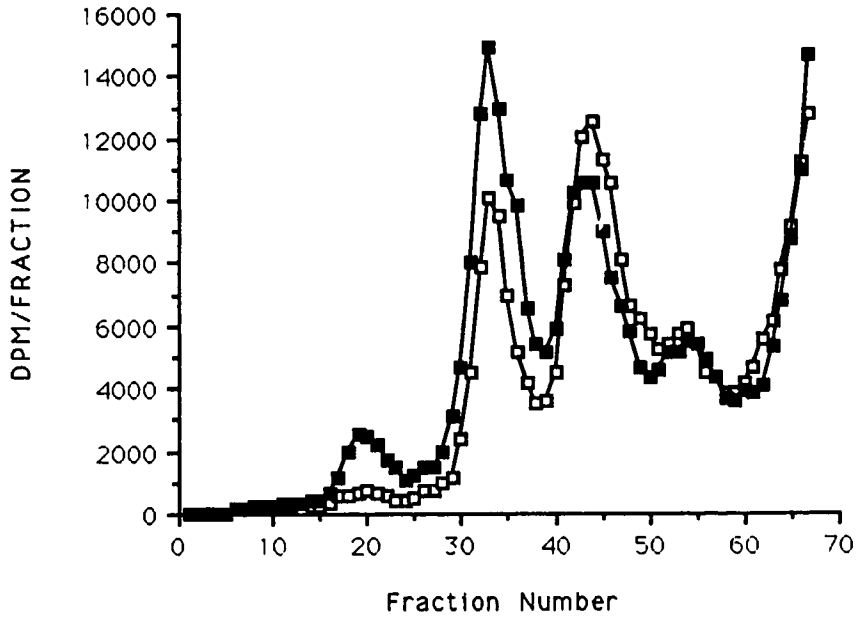

Figure 7.7 Comparison of the ribosome profiles of untempered, unheated labelled cells of *Escherichia coli* and *Listeria monocytogenes*.

□ ^{14}C labelled *Escherichia coli*. D10

■ ^{3}H labelled *L. monocytogenes* Scott A

Mg^{2+}:proteins:rRNA interactions (Hurst, 1977; Hurst and Hughes, 1978; Ray, 1986). Increased ribosome stability might then be achieved by preventing the loss of Mg^{2+} from the cell. It could be postulated that tempering acts to stabilize the ribosomes and minimizes subsequent injury during heating. This is supported by the data on the ribosome profiles of tempered and heated cells. It is possible that tempering stabilizes ribosomes by allowing some protein synthesis or reorganization that stabilizes the macromolecules.

The protein synthesis could in fact be associated with the induction of heat-shock proteins. Heat-shock proteins are generally defined as those whose synthesis is sharply and dramatically induced at high temperatures (Lindquist, 1986). *L. monocytogenes* has been shown to synthesize 12 to 14 heat-shock proteins ranging in size from 20 to 120 kilodaltons following incubation of the organism at 48°C (Sokolovic and Goebel, 1989). One of the proteins produced under heat-shock conditions was found to be listeriolysin, an essential virulence factor in *L. monocytogenes* (Sokolovic and Goebel, 1989). Listeriolysin was found in the supernatant of the heat-shocked cells, whereas the other heat-shock proteins remained associated with the cell (Sokolovic and Goebel, 1989). Functions of the other heat-shock proteins in *Listeria* have not been determined.

Increases in thermotolerance as a result of heat-shock proteins have been suggested (Yamamori and Yura, 1982; Lindquist, 1986). Factors other than heat, such as ethanol, hydrogen peroxide, glucose starvation and other stresses also induce the synthesis of heat-shock proteins (Morgan *et al.*, 1986; van Bogelen *et al.*, 1987; Jenkins *et al.*, 1988). Such treatments do not induce heat-shock proteins in all organisms, but in those in which they do, they also induce thermotolerance (Lindquist, 1986; Jenkins *et al.*, 1988). However, the specific protective mechanisms of heat-shock proteins have not been determined (Lindquist, 1986;).

As suggested by Lindquist (1986), other protective accommodations in cell physiology and structure might be made during heating that may be only coincidentally related to the induction of heat-shock proteins. Changes in membrane fluidity, membrane permeability, alterations in the cell wall, dehydration of the cells, and complexing of heat sensitive proteins in the cell during tempering may all affect the subsequent heat resistance of the organism, whether or not there is a contribution to the heat resistance provided heat-shock proteins.

In conclusion, although the results show that tempering has obvious effects on the ribosomal profile and ribosomal integrity during heating, it is not possible to state that this is a key factor in acquired thermotolerance.

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8. Conclusions

8.1 Incidence and origins of *L. monocytogenes* in raw milk

L. monocytogenes was found to be present in 1.9% of raw bulk tank milk samples taken in Alberta. The incidence in tanker truck samples was found to be 2.8% and 11.1 %. The higher incidence being due to pooling of positive and negative samples.

Sources of contamination of raw milk by *L. monocytogenes* were examined on four dairy farms with a history of shipping contaminated milk ('Listeria positive'). Feed materials, including silage and silage effluent, hay, grain and beet pellets were positive for listeriae. Silage, silage effluent and beet pellets yielded *L. monocytogenes*. Listeriae were also isolated from a wide range of environmental samples, such as straw bedding, soil, manure, flies, cobwebs, water, fresh herbage, water and feed bowls and drains. Fresh herbage and waterbowls yielded *L. monocytogenes*. Fecal shedding of listeriae, detected in rectal fecal specimens, was found to be 33% from 'Listeria positive' farms, with 14.5% of the samples containing *L. monocytogenes*. Similar samples from three farms with no history of shipping contaminated milk ('Listeria negative' farms) showed 24.6% positive for listeriae, and 13.2% positive for *L. monocytogenes*. Of non-aseptic individual cow milk samples, 8% contained listeriae, and 5% *L. monocytogenes*. In contrast, only one aseptic quarter sample out of 262 was positive for the organism. It can be concluded from these data that the farm environment and not the udder is the most likely source of the organism in raw milk. As a consequence of this, it can further be concluded that the incidence of *L. monocytogenes* in raw bulk tank milk could be minimized by implementation of accepted good milking practices.

L. monocytogenes as a causative agent of bovine mastitis was proven conclusively in this study. Although the milk was never visibly abnormal throughout the course of the investigation, *L. monocytogenes* was shed at levels of 2000 to 5000 cfu/mL. However, the data support the view that mastitis due to *L. monocytogenes* is likely to be rare, and probably only occurs under conditions conducive to all forms of udder infection. The infection was detected through routine microbiological testing procedures indicating that if listerial mastitis were common it would have been reported more frequently in the literature.

To better understand the origin of *L. monocytogenes* in milk, more refined methods of characterization of the organism must be employed than those used in this study. The use of bacteriophage typing, restriction endonuclease analysis of

chromosomal DNA, or multilocus enzyme electrophoresis would appear most likely to demonstrate as yet unclear relationships in the epidemiology of the disease.

8.2 Heat resistance of *L. monocytogenes*

Cultures of *L. monocytogenes* Scott A that were tempered at 48°C for 1 h in broth before heating at 60°C demonstrated a marked increase in heat resistance compared with untreated controls. Cells of *L. monocytogenes* were able to retain the increased thermotolerance even after storage at 1°C for 24 h. Acquired thermotolerance was also observed when the preheating and heating menstruum was 2% UHT milk. These results have implications for the microbial safety of foods and suggest that the tempering phenomenon could be important for the survival of *L. monocytogenes* during the thermal processing of foods.

The composition of the preheating menstruum was an important determinant in the development of acquired thermotolerance since not all tempering menstrua provided suitable conditions. Whereas 0.1M potassium phosphate buffer supplemented with 1% yeast extract supported development of acquired thermotolerance, 0.1M potassium phosphate buffer did not. However, the precise molecular determinants within the various menstrua were not determined and further work would be necessary for this purpose.

Cells treated with chloramphenicol, rifamycin and 2,4-dinitrophenol during tempering showed reduced enhancement of heat resistance following tempering, compared with untreated controls. However, the heat resistance was still greater than that of untempered controls. This shows that protein synthesis, RNA synthesis and energy yielding mechanisms are at least partially responsible for the tempering effect. These results are compatable with the notion that increased thermotolerance is due to the production of heat-shock proteins. Further work to characterize the tempering effect in *L. monocytogenes* with respect to heat-shock protein synthesis would be of interest.

Tempering at 48°C for 1 h stabilized the ribosomes of *L. monocytogenes* to subsequent heating, compared with untempered controls. The correlation between increased ribosome stability and increased thermotolerance might represent a causal relationship; however, other protective accommodations in the cell may be occurring.