PETER JOSEPH TAORMINA
Alkali-Stressed *Listeria monocytogenes*: Survival, Thermotolerance, and Behavior on Beef Frankfurters
(Under the Direction of LARRY R. BEUCHAT)

Listeria monocytogenes was more resistant to heat treatment following incubation for 45 min in tryptose phosphate broth (TPB) at pH 12.0 compared to pH 7.3. Cells survived at least 6 days in TPB at pH 9.0, 10.0, and 11.0 stored at 4 or 21°C, and cells stored in TPB at pH 10.0 for 48 h were more resistant to treatment at 56°C than were cells stored in TPB at pH 7.3. Cells were suspended in 1% solutions of eight food processing plant cleaners (pH 7.1 – 12.5) or in water (control) and incubated at 4ºC for 30 min or 48 h. In only three alkaline cleaning solutions (pH 11.6 – 12.4) were populations reduced significantly ($P \leq 0.05$) after 30 min compared to reductions in water. After 48 h, populations were significantly higher in one cleaning solution (pH 10.4) than in water, while populations in six other cleaning solutions were reduced by ≥ 4.72 $\log_{10}$ CFU/ml. Cells exposed to cleaning solutions for 30 min became sensitive to 4.0 or 6.0 ppm free chlorine, and to 50 or 100 ppm benzalkonium chloride and cetylpyridinium chloride, common components of quaternary ammonium sanitizers. Cells exposed at 4°C to 1% solutions of two alkaline cleaners or alkali-adapted in TPB (pH 10.0) at 37°C for 45 min, then 4°C for 48 h, were inoculated onto beef frankfurters containing high fat (16 g) and high sodium (550 mg) (HFHS) or low fat (8 g) and low sodium (250 mg) (LFLS) per 57-g serving. Frankfurters were surface-inoculated (2.0 $\log_{10}$ CFU/g), vacuum packaged, stored at –20, 4, or 12°C, and analyzed for populations over time. Populations remained stable at -20°C for up to 12 weeks. After storage at 4°C for 6 weeks, populations of control cells and cells exposed to alkaline cleaners were ca. 6.0 $\log_{10}$ CFU/g of LFLS frankfurters and ca. 3.5 $\log_{10}$ CFU/g of HFHS frankfurters, but growth of alkali-adapted cells on both types of frankfurters was retarded. Growth at 12°C was more rapid, but growth of alkali-adapted cells on HFHS and LFLS frankfurters after storage for 9 and 6 days, respectively, was also delayed. Alkaline stress of *L. monocytogenes* induces

tolerance to heat, sensitivity to sanitizer, and changes in growth characteristics on frankfurters.

INDEX WORDS: *Listeria monocytogenes,* Alkali, Thermotolerance, pH, Alkaline Cleaners, Frankfurters, Cross-Protection, Food Processing Environment

ALKALI-STRESSED *LISTERIA MONOCYTOGENES*: SURVIVAL, THERMOTOLERANCE, AND BEHAVIOR ON BEEF FRANKFURTERS

by

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DEDICATION

This dissertation and all the hard work that went into it is dedicated to my loving, trustworthy, faithful, and God-fearing wife:

Sharon L. T. Taormina

Proverbs 31:10 - A good wife who can find? She is far more precious than jewels.

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CHAPTER 1

LITERATURE REVIEW

Introduction

The modern approach to producing safe and wholesome foods emphasizes refrigeration as an essential hurdle against microbiological decomposition. Refrigeration also provides extension of quality and shelf life of foods and food ingredients by hindering the rate of deteriorative chemical reactions. Preservation by low temperature is used at various points throughout the chain of food production, from farm to table. Although, overall, refrigeration is beneficial to the microbiological quality and safety of temperature-sensitive foods, this technology has come with a cost. Certain microorganisms designated as "psychrotrophs" are provided a selective advantage in refrigerated food systems. A widely accepted definition of a psychrotroph (*psychros*, cold, and *trephein*, to nourish or develop) is an organism that can grow at temperatures between 0ºC and 7ºC and produce visible colonies (or turbidity) within 7-10 days (Jay, 2000). The bacterial pathogen, *Listeria monocytogenes*, exhibits such characteristics and can flourish in refrigerated foods and in environments where they are produced.

The implications of *L. monocytogenes* in refrigerated foods are serious, since populations can reach high levels while competing microflora are inhibited. The unhindered survival and growth of *L. monocytogenes* in ready-to-eat or ready-to-heat-and-eat foods are of particular concern since these products are typically not subjected to a listericidal step prior to consumption. Attempts of food processors to eliminate or limit the incidence of *L. monocytogenes* in these products are not always successful. Eradication of this pathogen from food processing environments is extremely difficult, and its presence in the environment makes contamination of at least some food products inevitable. The challenge is to understand every aspect of this pathogen that relates to

food production in an attempt to minimize its impact on humans. In achieving better protection of foods contaminated with *L. monocytogenes*, it is necessary to study not only survival and growth, but also environmental conditions that might render cells more capable of causing illness. Those conditions would include the various stresses that *L. monocytogenes* may encounter in food processing environments prior to contaminating food products. It is of interest to determine if stressful conditions encountered in food processing environments may render cells more tolerant to heating or increase their ability to survive and grow in foods. Of further interest is the ability of environmentally stressed cells to withstand exposure to sanitizers.

Understanding why and how *L. monocytogenes* can persist in various food production environments will facilitate devising better ways to control the occurrence of the pathogen in foods. As more is known regarding the behavior of *L. monocytogenes*, both in foods and in food production settings, appropriate changes can be made to make control measures more effective. This information, ultimately, would be applied to enhance the safety of food commodities and to do so with greater efficiency.

Listeria monocytogenes

General characteristics

Listeria monocytogenes is a short, motile, Gram-positive, nonsporeforming rod that is facultatively anaerobic, and nutritionally nonfastidious. Bergey's Manual of Determinative Bacteriology describes the organism as small coccoid rods, 0.4-0.5 by 0.5-2.0 μm, with rounded ends, slightly curved in some culture media, occurring singly, and in V-shaped or parallel pairs (Seeliger and Welshimer, 1954). The microorganism has flagella and is highly motile at low temperatures (Salyers and Whitt, 1994). Cell walls contain about 20% hexose (glucose and galactose), 5% hexosamine, and 5% protein with alanine, glutamic acid, diaminopimelic acid, aspartic acid, and leucine (Seeliger and Welshimer, 1954). The organism grows from 3°C to about 45°C, with

optimal growth between 30 and 37°C (Gray and Killinger, 1966), although it is considered by some to be a psychrotolerant foodborne pathogen (Wilkins *et al*., 1972). Growth of *L. monocytogenes* has been documented over a pH range of 5.0 to 9.6 (Gray and Killinger, 1966), but survival occurs within food products with wider pH ranges (Conner *et al*., 1986). Some strains are positive for acid production from D-galactose, melezitose, and rhamnose, while lactose and sucrose support slow acid production (3-7 days) in some strains. The species requires biotin, riboflavin, thiamine, and thioctic acid, and cysteine, glutamine, isoleucine, leucine, valine, and other amino acids for growth.

Serotyping and phage typing have led to the classification of 13 serovars of *L. monocytogenes*, but 95 % of human isolates are typed either 1/2a, 1/2b, or 4b (Rocourt and Cossart, 1997). Molecular typing methods have been developed to increase the stringency of strain typing and have become important tools for monitoring of environmental niches in food processing facilities, as well as for epidemiological investigations. Serovar 4b strains are responsible for 33 to 50 % of sporadic human cases of listeriosis worldwide and for all major foodborne outbreaks since 1981 (Rocourt and Cossart, 1997). Ribotyping, a molecular typing technique based on comparison of rRNA sequences of isolates, has distinguished two groups, each containing two serovars. One group consists of most serovar 4b and 1/2b strains of human origin, while the other group consists of serovar 1/2a and 1/2c strains, mainly from food and the environment (Rocourt and Cossart, 1997). One possible, yet unproven, explanation to this observation could be that different selective pressures administered to these strains exposed to different environmental conditions resulted in alteration of RNA sequences causing previously similar strains to be typed differently.

Listeriosis

Listeria monocytogenes is pathogenic to both humans and animals, but tends to be opportunistic. Infections in healthy adults are usually asymptomatic or lead to mild influenza-like symptoms, although diarrhea and abdominal discomfort can occur (Salyers and Whitt, 1994). Asymptomatic intestinal carriage of the organism has been estimated to occur in 5 to 10 % of the population (Salyers and Whitt, 1994) or higher for populations with some association with reservoirs of *L. monocytogenes* (Slutsker and Schuchat, 1999). It has been suggested that the general public is frequently exposed to low populations of *L. monocytogenes* by consumption of various foods (WHO Working Group, 1988; Farber et al., 1989; Pinner *et al*., 1992; Beuchat, 1996b); however, individuals with compromised immune systems are more likely to develop infection after exposure (Seeliger and Cherry, 1957; Gray and Killinger, 1966). Mead *et al.* (1999) estimated that *L. monocytogenes* was responsible for 3.8% of foodborne illness cases requiring hospitalizations, and 27.6 % of deaths cause by foodborne illness.

Human listeriosis usually manifests itself as encephalitis, meningitis, or sepsis, often with fatal outcome (Twedt, 1986). Infected pregnant women may or may not exhibit flulike symptoms. Unborn children may succumb to neonatal septicemia or meningitis and become spontaneously aborted, typically during or after the third trimester (Rocourt and Cossart, 1997). Among neonates and immunocompromised adults, mortality rates often exceed 30% (Twedt, 1986), but the mortality rate among the general population is closer to 20% (Gellin and Broome, 1989). Among non-pregnant adults, most affected persons have some unrelated underlying condition that predisposes them to consequences of infection (Salyers and Whitt, 1994; Rocourt and Cossart, 1997; Slutsker and Schuchat, 1999). Onset of clinical symptoms of infection varies, with incubation periods of 1 to 20 days. Late onset of symptoms has limited the availability of suspected food samples to testing for the pathogen, and thus made conclusively implicating food vehicles difficult.

Salyers and Whitt (1994) thoroughly described the etiological mechanisms of *L. monocytogenes*. Their review is the basis for the brief summary of the progressive stages of human infection that follows. Production of flagella by *L. monocytogenes* is not an important virulence factor in the human body. However, the ability of the *L monocytogenes* to rearrange actin plays an important role in cell-to-cell spread. Attachment to host cells is not clearly understood, but once bound, *L. monocytogenes* induces phagocytosis. Invasion is mediated by a membrane protein called internalin. A major virulence factor is the surface protein listeriolysin O (LLO), which is a sulfhydryl-activated, pore-forming cytotoxin, or hemolysin. LLO facilitates escape of cells from phagocytes. Host cell membrane lipid hydrolysis occurs via production of two phospholipases [phosphatidylinositol-specific phospholipase C (PI-PLC, *plcA*) and phosphatidylcholine-specific phospholipase C (PC-PLC, *plcB*)]. Movement of *L. monocytogenes* through host cells occurs via polymerization of actin to form long actin tails. Actin tail formation is catalyzed by ActA, which is a protein localized on the bacterial surface. Virulence genes are clustered in the same region of the chromosome and some of them are arranged in an operon. PrfA is a positive regulator of virulence genes, perhaps temperature sensitive, which transcribes itself, *plcA*, *hly*, and others.

Virulence variables

Several factors may influence the pathogenicity of *L. monocytogenes*. Laymann (1959) observed that cultures grown at 4°C were more pathogenic than those grown at 37°C. Although growth of the pathogen is slow at 3 to 4°C, turbidity in broth or growth on solid media can occur within 5 to 8 days. At 6°C, the log phase is reached in 10 to 11 days (Welshimer, 1960). These cultures are highly motile, possess well-developed flagella, and are pathogenic for laboratory animals (Seeliger, 1958). However, hemolysin production was reported to be very poor at 4°C (Kleikamp, 1959), and may be completely absent in old, laboratory-maintained strains. This may be due to the

frequent transfers into nutrient rich growth substrates and overall lack of environmental pressure. *L. monocytogenes* was only one-fourth to one-ninth as virulent for mice by the respiratory route when grown in a medium containing 1% glucose as when grown in one containing 0.2 to 0.6% glucose (Friedman *et al*., 1962; Friedman and Kautter, 1962). These findings indicate that culture conditions can have a direct effect on the virulence of the organism. Since virulence genes in *L. monocytogenes* have been closely associated with its response to stress (Mekalonas, 1992; Datta, 1994; Ripio *et al*., 1998), the question arises whether culture conditions can also play a role in the resistance of the pathogen to stressful conditions such as those encountered during laboratory challenge studies or antimicrobial efficacy testing. Environmental stress could increase the virulence of *L. monocytogenes*. Others have recognized the need for research for determining the influence that growth of *L. monocytogenes* in foods may have on the pathogenicity of the organism (Conner *et al*., 1986).

Exposure of *L. monocytogenes* to environmental stresses may select for more resistant strains. Since some stress response genes in *L. monocytogenes* also control the organism's virulence (Datta, 1994; Ripio *et al*., 1998), it is hypothesized that exposure to chemical stress, such as high pH, may actually destroy less resistant and hence less virulent strains. Therefore, chemical stresses could actually select for more virulent strains of the organism. These injured cells may begin to produce listeriolysin upon exposure to chemical stresses. Listeriolysin O is a water-soluble hemolysin which damages membranes and is cytotoxic. It is considered one of the major virulence factors of *Listeria* spp. Heat and cold shock has been shown to have an effect on the production of listeriolysin O and pathogenicity of *L. monocytogenes* (Buncic *et al*., 1996; Sampathkumar *et al*., 1999).

Agricultural reservoirs

Isolation of *L. monocytogenes* from more than 37 species of mammals, 17 species of birds, and aquatic life had been reported (Gray and Killinger, 1966) and has occurred within a diverse geographical distribution. The first reported isolation from domesticated farm animals occurred in 1929 in New Zealand when Gill (1933) observed a disease in sheep, which was in fact listeric encephalitis. Other early indications of the ubiquity of *L. monocytogenes* on the farm is evidenced by isolations from cattle, swine, and fowl as well as sheep (Hird and Genigeorgis, 1990). The shedding of *L. monocytogenes* in milk from cattle was correlated with mastitis, but the organism was also later isolated from the milk of clinically healthy cows (Hird and Genigeorgis, 1990; Ryser, 1999). Frequent isolations of *L. monocytogenes* from raw goat and cow milk have been subsequently reported (Hird and Genigeorgis, 1990). Spread of *L. monocytogenes* in agricultural environments is believed to be mostly through feces. The organism has often been recovered from feces, intestinal contents, and carcass swabs of chickens (Hird and Genigeorgis, 1990). Recovery of *L. monocytogenes* from the feces of 0.6% of healthy humans examined and from the carcasses of 53% of fresh and frozen commercial chickens has been reported (Welshimer, 1981).

Welshimer and Donker-Voet (1971) isolated *L. monocytogenes* from vegetation, soil, or both in 11 of 12 farm sites tested in April of consecutive years and from six of seven non-agricultural sites. They concluded that decaying, moist vegetation sustains the pathogen. A prior survey of decaying stalks, leaves, and tassels of corn taken from various farms supports that conclusion (Welshimer, 1968). In a survey conducted in various parts of southwest Germany, *L. monocytogenes* was detected in a high proportion of plants, soil samples, and feces of deer and stags as well as birds (Weis and Seeliger, 1975). In this study, faded and decayed grass was a direct indicator of the presence of *Listeria*. Unexpectedly, *L. monocytogenes* was isolated from the leaves of shrubs 50 cm above ground, as well as those on the ground. Welshimer (1960)

demonstrated that *L. monocytogenes* could survive in soil for up to 295 days. It is likely that the microflora of soil or fertilizer used to grow produce would become incorporated onto the surfaces and possibly the inner tissues of the roots, stems, leaves, and fruiting bodies of plants. In a study attempting to show this relationship, soil was inoculated with *L. monocytogenes* and used to grow vegetables (Van Renterghem *et al*., 1991). Three months after they were sown, 3 out of 6 radishes contained *L. monocytogenes*. Similarly, Al-Ghazali and Al-Azawi (1990) were able to detect *L. monocytogenes* on 10% of 50 alfalfa plants that had been grown on farmland soil treated with active sludge cake that contained the pathogen.

Incidence and Transmission of *L. monocytogenes* in Foods

Human disease caused by *L. monocytogenes* can be described as either epidemic or sporadic. Outbreak investigations have proven that both epidemic (Slutsker and Schuchat, 1999) and sporadic (WHO Working Group, 1988) listeriosis is foodborne. Several large outbreaks have occurred as a result of consumption of a wide variety of contaminated foods. However, the majority of cases of listeriosis is believed to be sporadic. Since these disease events are difficult to track and usually escape surveillance efforts, the occurrence of listeriosis worldwide is probably greatly underestimated.

In an attempt to identify the role of certain foods in sporadic listeriosis, Pinner *et al*. (1992) surveyed foods collected from refrigerators of patients with listeriosis identified through active laboratory-based surveillance. *L. monocytogenes* was isolated from 50 of 140 (36%) beef samples, 33 of 108 (31%) poultry samples, 26 of 95 (27%) pork samples, 18 of 98 (18%) lunch meats, 7 of 57 (12%) seafoods, 72 of 683 (11%) vegetables, 5 of 155 (3%) fruits, and 9 of 533 (2%) of dairy products taken from the refrigerators of infected patients. It is likely that sporadic cases of listeriosis continually occur, but the few infected persons that seek care are rarely asked to provide fecal specimens in order to confirm the cause of illness. Beuchat (1996b) proposed that as

long as consumers enjoy raw and minimally processed vegetables in their diet, they would also continue to consume vegetables containing low numbers of *L. monocytogenes*. Between the fiscal years 1994 and 1998, 813 food products were recalled because of discovery of contamination with *L. monocytogenes*, representing 61% of all microbial-related recalls during that time (Wong *et al*., 2000). With the occurrence of *L. monocytogenes* in many varieties of foods, it is likely that humans are exposed to low populations regardless of diet choices.

Milk and dairy products

The first recorded outbreaks of human listeriosis were attributed to ingestion of *L. monocytogenes* cells present in raw milk, sour milk, cream, and cottage cheese (Ryser, 1999). These outbreaks occurred in Germany between 1949 and 1957, while simultaneous outbreaks occurred throughout other parts of Eastern Europe. Between the late 1950's and 1980's several outbreaks of listeriosis of unknown origin occurred throughout the world. Surveys of raw milks in several countries, including selected states within the United States, reveal that between 0 and 7.0% of samples tested contained *L. monocytogenes* (Marth and Ryser, 1990). Other reviews have stated that closer to 3 to 4% of the milk supply worldwide probably contains *L. monocytogenes,* and that populations are likely to be less than 10 CFU/ml (Kozak *et al*., 1996).

In 1981, a small outbreak of listeriosis occurred in East Cambria, England as a result of consumption of contaminated cream (Gilbert *et al*., 1989). This outbreak, involving 11 persons, was followed by several other dairy associated outbreaks of larger magnitude. In the United States, pasteurized milk was suspected of being a source of a 1983 outbreak in Massachusetts affecting 49 persons (Flemming *et al*., 1985); 142 cases of listeriosis reported in Los Angeles, CA in 1985 were traced to Mexican-style cheese (Linnan *et al*., 1988). The nature of these outbreak scenarios suggested that post pasteurization contamination of milk with *L. monocytogenes* was occurring.

Consequently, there became increased awareness and interest by all parties involved, including governmental public health agencies.

The United States Food and Drug Administration during a period between 1986-1987 conducted an extensive survey of dairy products for the presence of *Listeria*. Samples of whole milk, chocolate milk, and various frozen dairy desserts tested positive for *L. monocytogenes* (Marth and Ryser, 1990; Kozak *et al*., 1996). The survey also revealed that *L. monocytogenes* was a contaminant of the food factory environments where positive product samples were produced (Kozak *et al*., 1996). During this survey, the FDA also examined 181 samples of more than 10 varieties of cheese made from raw milk and isolated *L. monocytogenes* from only one sample of Cheddar cheese (Marth and Ryser, 1990). However, the Mexican-style cheese outbreak in Los Angeles in 1985, and higher incidence rates of the pathogen in cheese produced in Europe, suggest this food is a likely vehicle for transmission.

Soft cheeses produced using unpasteurized milk, to retain flavor, continue to be produced in Europe despite presenting high-risk foods for exposure to *L. monocytogenes*. Relatively small outbreaks have occurred in the 1990's. French-produced cheese has been notorious; Brie de Meaux cheese was responsible for 33 cases of listeriosis in 1995, and 14 cases of illness were attributed to Pont l'Évêque cheese in 1997 (Ryser, 1999).

Meat, poultry, and meat products

Johnson *et al*. (1990) reviewed the presence and population of contamination of *L. monocytogenes* on meat and meat products. They concluded that the widespread distribution of this organism and its occasional association with domestic livestock makes contamination of raw meats virtually unavoidable. Since that time, surveys have confirmed the presence of *L. monocytogenes* on meat, poultry, fish, and related products to be occasional but also consistent.

In a Nationwide beef survey of steers and heifers conducted by the United States Department of Agriculture, rump, brisket, and flank surface tissue were excised from 2089 carcasses that were in the chiller for at least 12 h before they were sampled for *L monocytogenes* (FSIS, 1994; McNamara, 1995). Out of the pooled samples from three sites 4.1% were found to be positive for *L. monocytogenes*. These statistics indicate that contamination is not rampant, but, in fact, manageable. However, certain steps undertaken in meat production may amplify populations of *L. monocytogenes* present in meats. Additionally, contamination of some food processing environments with even small numbers of *L. monocytogenes* cells can lead to pervasive contamination, resulting in potential cross-contamination onto product. Jay (1996) summarized numerous international surveys for *L. monocytogenes* in meat and poultry products. Prevalence ranged from 0 of 62 positive samples of fresh beef France and 0 of 24 positive beef and lamb in Beijing, China, to as high as 17 of 22 positive samples of ground beef in Canada. The overall prevalence rate was about 16%, although that percentage represented various products of different microbiological susceptibilities subjected to different sampling routines. An Australian retail survey of vacuum-packed whole salami and sliced corned-beef, ham, and luncheon-style meats detected *Listeria* in 53% of 175 samples, with corned-beef having the highest incidence rate and *L. monocytogenes* being detected more frequently than *L. innocua* (Grau and Vanderlinde, 1992).

Poultry products are contaminated with *L. monocytogenes* relatively frequently (Bailey *et al*., 1989; Genigeorgis *et al*., 1989; Genigeorgis *et al*., 1990; Jay, 1996). Turkey wings, legs, and tails obtained in supermarkets in Davis, CA, USA, were analyzed for *L. monocytogenes* (Genigeorgis *et al*., 1990). The pathogen was detected on 20% of wings, 13.3% of legs, and 11.7% of tails. Further analyses resulted in isolation of *Listeria* spp. from several areas in a slaughterhouse from which these products were derived.

In a recent survey of retail foods in Japan, *L. monocytogenes* was isolated from 12.2, 20.6, 37.0, and 25% of 41 minced beef, 34 minced pork, 46 minced chicken, and 16 minced pork-beef mixture samples, respectively (Inoue *et al*., 2000). Highest numbers were recovered from minced chicken samples, which also contained the most 4b serovars. The pathogen was also present in 5.4% of 92 smoke salmon samples and in 3.3% of 213 ready-to-eat raw seafood samples. In Taiwan, examination of foods for *L. monocytogenes* turned up positives in 58.8% of pork samples, 50% of chicken carcass samples, 38% of turkey parts, 34% of frozen semiready foods, 24% of beefsteaks, and 10.5% of seafoods.

Reported cases of listeriosis in non-dairy foods of animal origin began in 1956 when 19 persons in the Soviet Union became ill with possible transmission from pork (Ryser, 1999). In Sweden in 1959, 4 cases of illness were documented and poultry was the suspected vehicle of transmission (Jay, 1996). After the outbreaks in late 1950's and up until the 1980's no more cases or outbreaks attributable to foods of animal origin were published, although numerous cases of unknown origin were documented (Ryser, 1999). In New Zealand in 1980, 22 cases were attributed to consumption of shellfish and raw fish. Later that decade, a large outbreak (366 cases) occurred in Northern Ireland and was traced to pâté (McLauchlin, *et al*., 1991). In subsequent years some isolated cases followed which were linked to cooked/chilled chicken (Kerr *et al*., 1988; Kaczmarski and Jones, 1989), turkey franks (MMWR, 1989), and pork sausage (Cantoni *et al*., 1989; Parodi *et al*., 1990). The 1990's began with another outbreak of listeriosis linked to pâté in Australia (Eyles, 1994). In France, jellied pork tongue was the vehicle for a 1992 outbreak involving 279 cases (Jacquet *et al*., 1995), followed by a 1993 outbreak involving 39 cases attributable to pork pâté. Cold-smoked rainbow trout was suspected as the causative agent of transmission of *L. monocytogenes* to nine persons in Sweden (Ericsson, 1997).

A severe outbreak of listeriosis occurred in the United States starting in August of 1998 (MMWR, 1998, 1999). At least 50 illnesses were reported and 15 adult deaths as well as 6 neonatal deaths occurred. The outbreak encompassed at least 11 states and was attributed to consumption of hot dogs and possibly deli meats. The company that produced the contaminated meat products instituted a massive recall. Investigations conducted by epidemiological investigators pointed to the role of a particular factory in producing contaminated product. A construction event took place in that particular factory shortly before illnesses started to occur. Investigators later concluded that the construction event probably created a disturbance of a community of *L. monocytogenes* cells somewhere within the plant, which caused dispersion of cells throughout the plant and ultimately contamination of cooked product.

Produce

A noticeable trend in recent years that is cause for concern has been an increase in the number of foodborne disease outbreaks associated with the consumption of fresh produce (Hedberg *et al*., 1994; Tauxe, 1997; Altekruse and Swerdlow, 1996; Bean and Griffin, 1990). Concurrently, the consumption of fresh produce contaminated with *L. monocytogenes* has resulted in three known outbreaks of listeriosis to date (Francis *et al*., 1999). The first outbreak, which occurred in 1976, affected 20 persons and was suspected to result from consumption of contaminated raw salad (Ho *et al*., 1986). In 1981, 41 people became ill and sought care after consuming coleslaw (Schlech et al., 1983). The coleslaw had been commercially prepared with cabbage and carrots obtained from wholesalers and local farmers. An investigation into the sources of the raw vegetables identified a farmer who had applied both composted and raw sheep manure to the fields in which cabbage was grown. An isolated case of *L. monocytogenes* septicemia occurred after consumption of salted mushrooms (Junttila and Brander, 1989). An 80-year old man had eaten homemade salted mushrooms that had been

washed, cooked, and salted before storage in a cold cellar for 5 months. Before consumption, the mushrooms were kept in a washing room of the barn for a couple of days. Carrots that were stored in the same room were also contaminated with *L. monocytogenes*, although fecal samples of the sheep and cattle on the premises did not reveal the pathogen. Another reported sporadic episode was associated with consumption of pickled olives (Casolari *et al*., 1994).

The ubiquity of *L. monocytogenes* and its demonstrated high prevalence in the agricultural environment highlight the potential for contamination of produce either while growing in the field or during harvesting, packing, and transportation. *L. monocytogenes* has been isolated from a variety of fresh intact vegetables and ready-to-eat minimally processed vegetables throughout the world (Beuchat, 1996a; Beuchat, 1996b; Francis *et al*., 1999). The frequency by which the organism has been isolated has varied according to the type of produce, the method of detection, and the developmental status of the country in which the produce surveys were conducted. For instance, a cabbage in Sri Lanka (Gunasena *et al*., 1995) was found to contain the pathogen in 6 of 18 (33%) samples, but a survey in the United States (Heisick *et al*., 1989b) detected fewer positive samples with only 1 out of 92 (1.1%) testing positive. In another survey conducted within the United States, a relatively high proportion of potatoes and radish samples were shown to harbor *L. monocytogenes* (Heisick *et al*., 1989a; Heisick *et al*., 1989b). Although it is logical that a microorganism prevalent in soil would often be found on tubers and roots, more surveys should be conducted before these particular vegetables are confirmed as high risk for presence of *L. monocytogenes*. Regardless of the variations in surveys, the presence of *L. monocytogenes* in a number of different types of vegetables is well established. Consequently, the potential for contamination of all types of produce by *L. monocytogenes* exists as does the risk of consuming the pathogen.

Discussion about *L. monocytogenes* on fresh produce would be remiss if the possibility of contamination during minimal processing were not mentioned. While many processing facilities take adequate steps to control microorganisms within the processing environment, others may be less thorough in cleaning and sanitation protocols. In a plant producing minimally processed fresh vegetables in England, 19% of the products examined contained *L. monocytogenes*, but only 1.8% of the individual ingredients entering the facility were contaminated (Velani and Roberts, 1991). The organism survives nicely in wet conditions, and has the ability to persist in plant environments and product contact surfaces, often by forming biofilms (Krysinski *et al*., 1992; Cox *et al*., 1989). Thus, *L. monocytogenes*, which is likely to be introduced into fresh produce-processing facilities by raw ingredients, can be transferred to the final product unless adequate control measures including effective sanitation are in place.

Behavior of *L. monocytogenes* in Foods

Milk and dairy products

Of major concern to dairy producers is the contamination of pasteurized milk with unpasteurized milk that may contain *L. monocytogenes*. Care should be taken to minimize the potential for mixing pasteurized and unpastuerized milk and milk products because *L. monocytogenes* is capable of growth in dairy foods held at refrigeration temperatures (Richter *et al*., 1992). Rosso *et al*. (1996) demonstrated the influence that storage temperature can have on the time required for *L. monocytogenes* to reach a certain population. Increasing storage temperature from 4 to 8°C decreased the time required for the inoculated population to reach a ten-fold increase by 60 to 65% for dairy products and 50% for soft cheeses (Camembert, Brick cheese). The consequence would be a reduction in time for *L. monocytogenes* to reach 100 cells per gram of dairy food after about 5 days at 8°C as opposed to 2 weeks at 4°C. Bactericidal factors in milk such as lactoperoxidase, lysozyme, lactoferrin, and agglutinins have been shown to inhibit

growth of *L. monocytogenes* (Champagne *et al*., 1994). However, low initial populations can grow to dangerous levels during refrigerated storage of milk. Reports on the behavior of *L. monocytogenes* in skim milks and yogurt both during refrigerated storage and during fermentation revealed that the pathogen finds conditions favorable for survival and growth in some cases (Schaack and Marth, 1988a; Schaack and Marth, 1988b). A great deal of research has been done showing the stability of *L. monocytogenes* in hard cheeses the pathogen's ability to grow in soft cheeses during manufacture and storage (Ryser and Marth, 1987a; Ryser and Marth, 1987b, Ryser et al., 1985).

Meat and meat products

Much of the research that addresses the survival and/or growth of *L. monocytogenes* on meat and meat products studied the effects of modified atmosphere or vacuum packaged meats. Inocula consisting of strains Scott A and V7 grown under microaerobic conditions were capable of surviving on ground beef in gas permeable and gas-impermeable packages at 4°C (Johnson *et al*., 1988). Sodium lactate, kappa-carrageenan, sodium erythorbate, and a combination of sodium alginate/lactic acid/calcium carbonate used as ground beef binding agents were evaluated for their effects on growth of *L. monocytogenes* (Harmayani *et al*., 1993). Vacuum packaged beef striplions supported the growth of *L. moncytogenes* at 0, 2, 5, and 10°C but not at -2°C, although growth rates were generally half that of spoilage microflora after relatively long lag periods (Gill and Reichel, 1989). Beef striploins packaged under CO_2, however, only supported growth of *L. monocytogenes* at 10°C. In a study on vacuum packed processed meats in Australia, *L. monocytogenes* grew fastest on corned beef and slowest on ham stored at a temperature range between 0 and 15°C (Grau and

Vanderlinde, 1992). The authors concluded that pH, salt, and residual nitrite may influence the rate of growth of the pathogen on chilled meats.

Glass and Doyle (1989) demonstrated product dependent variations in behaviors of *L. monocytogenes* on vacuum-packaged meats stored at 4.4°C. The organism survived but did not grow on summer sausage, grew only slightly on cooked roast beef, grew well on some wiener products but not on others, grew well on ham, bologna, and bratwurst, and grew the best on sliced chicken and turkey. The behavior of *L. moncytogenes* on vacuum packaged meat fillets stored at 5°C was affected by the permeability of the packaging film as well as the presence or absence of spoilage microflora (Tsigarida *et al*., 2000). Lower permeability film suppressed growth of the pathogen compared to higher permeability film and the presence of background flora limited its growth. After aerobic storage at 4°C, treatments with sodium lactate generally contained fewer *L. monocytogenes*, while treatments with sodium erythorbate generally had higher numbers.

The survival and growth of *L. monocytogenes* on frankfurters has been studied under a variety of modifications to experimental parameters. A relatively early study, which preceded frankfurter related transmission of *L. monocytogenes* to humans, investigated the antimicrobial activity of liquid smoke (Messina *et al*., 1988). Anti-listerial activity of phenols present in liquid smoke was demonstrated in saline buffer and one liquid smoke product dipped or sprayed onto frankfurters showed promise in controlling *L. monocytogenes* at 4 ± 1°C. In a related study, differences in survival and growth of *L. moncocytogenes* in exudate fluids from all beef frankfurters during storage at 4 or 25°C was attributed in part to concentration of phenols in exudates (Yousef *et al*., 1991). Marked differences were correlated between initial pH of exudates and behavior of the pathogen at 4°C, with exudates exhibiting higher initial pH able to support growth or increased survival at 25°C. Frankfurters made from beef and pork inoculated with

just *L. monocytogenes* or with *L. monocytogenes* and high or low populations of *Pediococcus acidilactici*, vacuum packaged, and stored at 4°C revealed interesting antagonistic relationships between the organisms on frankfurters (Berry *et al*., 1991). A high inoculum of the pediococcal strain inhibited growth of *L. monocytogenes* for up to 60 days, while a low population inoculum caused a lag in growth. Additionally, this study showed anaerobic storage conditions to be more inhibitory overall against *L. monocytogenes* on frankfurters.

The research dealing with *L. monocytogenes* on frankfurters points to several factors that commonly influence survival and or growth of the pathogen. The initial pH of the frankfurter is typically an important attribute, with more neutral pH frankfurters often supporting good growth with short or no initial lag. Other parameters affecting survival and growth include the salt content, population of lactic acid bacteria, storage temperature, and atmosphere. One other parameter that may help explain the ability of *L. monocytogenes* to grow on frankfurters is the dense layer of coagulated protein on surfaces of frankfurters (McKellar *et al*., 1994). Vacuum packaged frankfurters are packed tightly so that surfaces of frankfurters touch each other as well as the package. These interface areas probably contribute greatly to the behavior of *L. monocytogenes* in such products.

Produce

The behavior of *L. monocytogenes* on fresh produce has been well studied. Populations of *L. monocytogenes* increased on broad-leaved endives and butterhead lettuce by 1.5 log and by 0.5 log on curly-leaved endives, but decreased by l log on lamb's lettuce during storage at 10ºC for 7 days (Carlin and Nguyen-The, 1994). The pathogen was able to multiply by up to 7-fold in white cabbage, Chinese cabbage, and celery after inoculation and storage at 4°C (Breer and Baumgertner, 1992). However, a decrease in population of *L. monocytogenes* of approximately 14-fold was observed on

inoculated fennel, carrots, red cabbage, savoy cabbage, and beetroot. Berrang *et al*. (1989) reported that controlled atmosphere storage of fresh asparagus, broccoli, and cauliflower at 4 or 15°C lengthened the time that each vegetable was considered acceptable for consumption by subjective inspection. However, they observed that populations of *L. monocytogenes* increased during storage, implying the produce may not look spoiled but still contain large numbers of *L. monocytogenes*. Growth of the pathogen was prolific on chicory endive stored under vacuum of 400mB at 6.5°C for up to 7 days when compared to atmospheric pressure (Aytac and Gorris, 1994). However, the same conditions caused populations of *L. monocytogenes* to decrease on mung-bean sprouts. Conner *et al*. (1986) determined that cabbage juice supports good growth of *L. monocytogenes* and that the organism is more tolerant of environmental stresses such as moderate NaCl concentrations and low-pH conditions than previously reported. Several studies have indicated that various types of produce can support the growth of *L. monocytogenes* at abusive tempertatures (>12°C) and that the population of the pathogen can remain stable on certain produce items stored at 4 to 5°C (Steinbruegge *et al*., 1988; Kallander *et al*., 1991; Farber *et al*., 1998; Garcia-Gimeno *et al*., 1998). However, some types of vegetables such as carrots have been shown to inhibit growth and at times reduce numbers of *L. monocytogenes* (Beuchat and Brackett, 1990; Nguyen-the and Lund, 1992).

Thermotolerance of *L. monocytogenes*

The thermotolerance of *L. monocytogenes* has been well studied since the late 1980's. The International Commission on Microbiological Specifications for Foods has summarized a large portion of that information (ICMSF, 1996). A vast majority of thermotolerance data collected was analyzed and presented as D values. The D value is equal to the absolute value of the reciprocal of the slope of the survivor curve and is a measure of the rate of death of an organism (Jay, 2000). It is numerically equal to the

number of minutes required for the survivor curve to traverse 1 log cycle, or the time required to destroy 90% of the population. The validity of heat survival data is often challenged regarding the conditions of the cell population when it was obtained, the technique used to heat cells, the technique used to recover heat-injured cells, and the statistical assumptions required for analysis with D values. Nonetheless, D values continue to be a popular tool with which food microbiologists describe heat inactivation of cell populations.

Significant differences in heat resistance both between serovars of *L. monocytogenes* and between strains belonging to the same serovar have been reported (Sörqvist, 1994). However, much of the heat inactivation data has been generated using a human isolate with serotype 4b, designated Scott A. *Listeria monocytogenes* strain Scott A was shown to become more sensitive to heating if incubated at low temperatures at neutral pH, but cells grown at low temperatures became more heat resistant if the pH during growth is 5.4 (Juneja *et al*., 1998). The same strain increases in heat resistance following heat shocking (Fedio and Jackson, 1989; Linton *et al*., 1990; Linton *et al*., 1992). The heat-shock induced thermotolerance phenomenon was also demonstrated using *L. monocytogenes* 13-249 (serotype 1) isolated from pasteurized cured ham (Jørgensen *et al*., 1999). Other factors may increase heat resistance of *L. monocytogenes*. Lou and Yousef (1996) added H_2O_2, ethanol, or hydrochloric acid to *L. monocytogenes* Scott A culture and measured $D_{56°C}$ values of cells. Adaptation of the cells to these stresses was accompanied by increased heat resistance.

Subjecting *L. monocytogenes* to cold temperatures appears to reduce the organism's capacity to tolerate heat. A mild heat treatment of 52°C for 1 h was more lethal for cells grown at ≤ 28°C than for cells grown at 37 or 42°C (Smith *et al*., 1991). Other studies have gone further to demonstrate the effects of exposure to cold environments on subsequent thermotolerance. Miller *et al*. (2000) observed an increased thermal (60°C) sensitivity of several strains of *L. monocytogenes* following cold shock at

0 to 15°C for 1 to 3 h as measured by D values. Cold shock for 3 h caused more sensitivity to heating than cold shock for 1 h. Another study concluded that ribosomal changes resulting from cold shock may be responsible for decrease in D value (Bayles *et al*., 2000).

Non-linear modeling of heat survivor curves

Plotting the heat inactivation of log populations of cells versus heating time typically results in a survivor curve that is not log-linear. The D value assumes that survivor curves fit log-linear inactivation kinetics, although, that is not always the case. There are six different shapes of heat survival curves, only one of which is linear (Xiong *et al*., 1999). Various non-linear microbial inactivation or survival models have been devised and they are summarized elsewhere (Körmendy and Körmendy, 1998; Xiong *et al*., 1999; Geeraerd *et al*., 2000). Logistic models appear to show the most promise, although other models are continuously under development. One of the more popular models is the Whiting-Buchanan (Whiting and Buchanan, 1992) because it fits each type of survivor curve and particularly addresses those curves with an initial lag in killing (shoulder) and/or a lag in killing at longer heating times (tail). The Whiting-Buchanan model is rather versatile, but some suggest that D values that are estimated with the model are not real (Xiong *et al*., 1999). Kamau *et al*. (1990) also devised rather useful models to address shoulders and tails in heat survivor curves. Moreover, a method to estimate true D values from these models was devised (Pruitt and Kamau, 1993).

Prevalence of *Listeria* in Food Processing Environments

A significant challenge to food processors is the hygienic maintenance of food processing environments, despite continuous production schedules and repeated introduction of microorganisms into those environments. Raw food ingredients and materials enter the processing environment daily and readily transfer indigenous

microflora to the environment, while equipment and workers that enter the plant can bring in a completely different yet important microflora. Plant traffic, maintenance, and even sanitation routines all serve to disperse microorganisms throughout the environment and eventually, certain microorganisms can find an ecological niche suitable for growth. Low temperatures and nutrient and water availability are characteristics of food processing environments that enhance the ability of *Listeria* spp. to establish and proliferate. Studies have been undertaken to assess the degree of *Listeria* contamination in factories processing various types of foods. Some more recent surveys are summarized here. For a further and more extensive review of the issue, refer to chapter 17 of the book *Listeria*, Listeriosis, and Food Safety which is entitled "Incidence and control of *Listeria* in food-processing facilities" (Gravani, 1999).

Samelis and Metaxopoulos (1999) made interesting observations in a meat processing plant in Greece producing 4,000 tons of processed meats annually. They observed that raw materials such as turkey necks and breasts, and mechanically deboned pork, pork trimmings, loins, and hind leg sections were the initial sources of *Listeria* spp. and *L. monocytogenes* contamination in the plant. Environmental sampling on a normal production day revealed that one of the cutters, a cutting machine used to cut down frozen pork shoulders, and tumbling machines all carried *Listeria* spp. and *L. monocytogenes* despite the use of what was described as excellent cleaning and sanitation. The authors indicated that equipment parts not made of stainless steel or parts not easily disassembled for daily cleaning created protected areas from which *L. monocytogenes* cells were consistently isolated. Contamination of product from tumblers used to make cured products was identified as another problem, and it was recommended that foaming and cleaning tumbling machines more than once or twice weekly would be beneficial.

A survey of a poultry-processing plant in Galicia, Spain revealed substantial environmental contamination (Franco *et al.*, 1995). All conveyor belt and table surfaces

sampled in the quartering room contained *Listeria* spp. while circular saws were slightly less contaminated. Tables and belts in the quartering room were sampled immediately after they had been cleaned and disinfected, and enriched samples were positive for *Listeria* spp. The processing plant had cleaned with several types of detergents and chlorine disinfectants using a delivery system that can apply water, detergent, and disinfectant solutions at up to 70 kg/cm^2 of pressure. These researchers concluded that disinfection procedures used in this plant did not effectively control *Listeria* spp. A separate investigation involving seven Danish abattoirs demonstrated the prevalence of *L. monocytogenes* throughout processing lines and that certain production steps may increase cross-contamination and spreading of the pathogen to other poultry products (Ojeniyi *et al*., 1996)

Pritchard *et al.* (1995) tested 21 different processing plants all producing varieties of dairy products. Equipment in 6 of those plants tested positive for *Listeria* spp. while there were positive environmental samples in 19 of the plants. Thirty-five percent of the positive tests were identified as *L. monocytogenes*. While *L. innocua* was the predominant organism found overall and on environmental sites (such as coolers, receiving and storage areas, areas below machinery, and foot baths), *L. monocytogenes* was most often isolated from the equipment sites (including tops of table tops, conveyor/chain systems, and filler machines). The authors indicated a very large overall difference in amount of environmental versus equipment contamination, and that the mere presence of *Listeria* spp. in the environment of dairy processing plants does not necessarily translate into prolonged contamination of the resident machinery. However, concern was expressed regarding the proximity of *Listeria* to finished product. Five of the holding tank and table-top samples were positive for *L. monocytogenes*, thus making post-processing contamination a very real threat. Of further significance was the number of sites underneath equipment that were positive for *Listeria* spp. The authors proposed

the danger of using pressurized wash hoses under equipment that could aerosolize *Listeria* and subsequently contaminate equipment.

Cox *et al*. (1989) surveyed for *Listeria* spp. in a variety of food-processing environments as well in non-food and domestic environments. Total samples positive for *Listeria* spp. from different areas in food factories were 23% in a frozen food factory, 15% in a liquid dairy factory, 25% in a factory producing Italian-style cheese specialties, 50% in an ice-cream production environment, 50% in a potato processing factory, and 0% in 2 dry culinary food factories. *L. monocytogenes* was detected with lower frequencies in these areas, although species sampling procedures may have limited its detection. In all types of food processing factories combined, the frequency of isolation in ranked order was drains, stagnant water and floors > residues > equipment surfaces. *Listeria* spp. and *L. monocytogenes* were also isolated from dishcloths, dustbins, and refrigerators of domestic environments.

The primary locations in food factories found to harbor *Listeria* are, in order: floors, drains, cleaning aids (i.e., brushes and sponges), product and/or equipment wash areas, food contact surfaces, condensate, walls and ceilings, and compressed air (Bernard and Sveum, 1994; Gravani, 1999). Ready-to-eat foods that do not receive an in package cook sufficient to eliminate pathogens are susceptible to contamination from exposure to the environment (Bernard and Sveum, 1994). The success of measures to control post-processing contamination of ready-to-eat foods are contingent upon a complete assessment of the processing and packaging environment with respect to presence of *Listeria*. Certain areas should receive more attention than others based upon vulnerability they impose upon products at various stages of production. Areas considered as primary points of contamination of ready-to-eat foods include direct product contact surfaces, personnel who handle products between the listericidal step and final packaging, and items such as clothing or gloves, which may come into direct contact with product (Bernard and Sveum, 1994).

Environmental harborages of *Listeria* are areas that repeatedly test positive for contamination despite efforts to reduce or eliminate the pathogen. Items consistently found to harbor *Listeria* include rollers for conveyors (especially hollow types), on/off control switches, rubber seals around doors, fibrous or porous type conveyor belts, open bearings within equipment (such as slicers and strippers), hollow implements (including box cutters), certain pieces of ancillary equipment (such as trash cans), and standing water in production areas (Bernard and Sveum, 1994). These harborages typically contain nooks where nutrient and water can become trapped and escape effective cleaning and sanitizing. They are frequently not discovered until equipment parts, which usually remain intact, are dismantled, inspected and sampled (Kornacki, 2000). Consequently, biofilm formation is highly associated with such areas (Mattila-Sandholm and Wirtanen, 1992). A biofilm consists of a community of microorganisms bound together by an extracellular matrix that is attached to a solid surface. Attachment is facilitated by the microbial excretion of exopolysaccharide, which is also termed glycocalyx. Once biofilms form, they are difficult to eradicate from food processing environments. This is due to the adhesiveness of the complex matrix and the protection it provides microorganisms against cleaning and sanitizing. *Listeria monocytogenes* is often associated with biofilms in the food industry. A possible reason that *Listeria* spp. are consistently isolated from food processing facilities and from food products could be related to this association. Periodic sloughing off of portions of *Listeria*-containing biofilm present in, on, or near processing equipment may occur as a result of running food through processing lines or as a result of cleaning and sanitation.

Controlling *Listeria* in Food Processing Enviroments

Effective control measures designed to ultimately reduce incidence of *L. monocytogenes* in foods must involve extensive review of all factors involved in the production of food. Various published sources have suggested and addressed steps that should be taken by

food processors implementing programs to control microorganisms such as *Listeria* (FDA-Milk Ind. Found. Intl. Ice Cream Assoc., 1988; Gabis and Faust, 1988; Bernard and Sveum, 1994). The use of Hazard Analysis Critical Control Point (HACCP) programs is essential to safe food production (NACMCF, 1998). However, in order for HACCP programs to be effective, prerequisite programs must be in place. Prerequisites programs may include facility design and maintenance, supplier control, specifications, production equipment, cleaning and sanitation, personal hygiene, training, chemical control, raw material transport, product traceability, and pest control. Some prerequisites to HACCP are also recognized as crucial to control of *Listeria* in the production environment. While all are important, cleaning and sanitation will be the focus of the following discussion.

Effectiveness of sanitation against L. monocytogenes

Although much scientific data exist verifying the efficacy of various sanitizers and disinfectants against *L. monocytogenes*, application of these findings to the food industry remain limited. Numerous factors influence sanitizer efficacy when applied in actual food production environments, which are difficult to sufficiently and consistently simulate in laboratory experiments. Despite abundant laboratory data showing efficacy of sanitizers against *L. monocytogenes* in factory settings, the pathogen is often isolated following cleaning and sanitizing of food processing equipment sufaces (Ojeniyi *et al*., 1996; Samelis and Metaxopoulos, 1999; Franco *et al*., 1995). Additionally, the frequency of food product recalls due to contamination with *L. monocytogenes* (Wong et al., 2000) attests to the ability of the organism to persist in food processing environments despite sanitation measures.

Typical cleaning processes applied to food production environments utilize a series of operations, each with a specific purpose (Tamplin, 1980). First, a pre-rinse is performed with water to remove gross, loose soil. Then, a cleaning step utilizes suitable

detergents (often alkaline) for removal of residual soils. Next, an inter-rinse removes all detergent and soil. Sanitizing is then performed by application of antimicrobial chemicals either with or without heat. Finally, a post-rinse using potable water washes away the antimicrobial chemicals. Although the cleaning step is necessary to remove fat and protein residues, cells of *L. monocytogenes* could perhaps become dislodged from surfaces during this step and remain in nooks within the processing environment that are protected from subsequent treatment with sanitizer. These cells may eventually contaminate foods and be more resistant to subsequent stress.

Inactivation of L. monocytogenes *by chemicals.*

Inactivation of *L. monocytogenes* by various chemicals has been well studied. Brackett (1987) exposed cells of *L. monocytogenes* to various concentrations of reagent-grade NaOCl and household bleach for various times. Exposure to chlorine concentrations ≥ 50 ppm was lethal to the pathogen. Dipping Brussels sprouts containing ca. 6 $\log_{10}$ CFU of *L. monocytogenes*/g into ca. 200 ppm chlorine solution for 10 s reduced the number of viable cells by about 2 $\log_{10,}$ while dipping in water resulted in about a 1 $\log_{10}$ reduction. In a study conducted by Mustapha and Liewen (1989), NaOCl at 100 ppm was the observed minimum inhibitory concentration necessary to achieve a 4-log reduction *L. monocytogenes* in vitro. Several factors may contribute to the effectiveness of chemicals against *L. monocytogenes*. The lethality of NaOCl against *L. monocytogenes* was greater when used at 5ºC than at 25 or 35ºC, and was also greater when the chemical was used at pH 5 than at pH 7 or 9 (El Kest and Marth, 1988). This study also concluded that strain differences may affect inactivation of the pathogen by chlorine. Bunduki *et al*. (1995) proposed that in order for *L. monocytogenes* to repair from heat- or sanitizer-induced injury, cells require synthesis of mRNA and protein synthesis, as well as oxidative phosphorylation. They further concluded that the cell wall was not a site of damage due to injury by heat (56ºC for 20 min) or by a chlorine-based sanitizer (100 ppm for 2 min).

Although the efficacy of quaternary ammonium compounds (QAC) and chlorine against planktonic and attached *L. monocytogenes* cells has been demonstrated, several *Listeria* isolates, including 12 *L. monocytogenes* strains, have been shown to be resistant to antimicrobials such as benzalkonium chloride (Lemaitre *et al*., 1998).

Death of *L. monocytogenes* resulting from chemical treatment is influenced by other environmental factors. All indications are that cells of *L. monocytogenes* survive chemical stresses better when they are given time to colonize a suitable surface. Lee and Frank (1991) attached *L. monocytogenes* cells to surfaces of stainless steel by immersing stainless steel slides into a low nutrient medium inoculated with the pathogen. Incubation at 21°C for 4 days resulted in attached cells, while surface-adherent microcolonies of the pathogen were formed after 8 days of incubation. Surface-adherent microcolonies of *L. monocytogenes* were more resistant than attached cells to treatment with 200 ppm hypochlorite by immersing for 30 s. In a similar study, NaOCl at 100 to 800 ppm, overall, was less effective in reducing the number of *L. monocytogenes* on smooth stainless steel compared to treatment in vitro (Mustapha and Liewen, 1989). When the pathogen was allowed to attach to pitted stainless steel, treatment with NaOCl was even less effective.

Several studies have been devoted to observing the effects of chemical disinfectants, particularly chlorine, on inactivation of *L. monocytogenes* on produce. Beuchat and Brackett (1991) investigated the behavior of *L. monocytogenes* on whole and chopped raw tomatoes stored at 5, 10, and 21°C. They observed rates of death and growth of *L. monocytogenes* on tomatoes stored at 10 and 21°C were not influenced by prior treatment of tomatoes with 210 to 280 ppm free chlorine. Interestingly, the population grew on whole tomatoes held at 21 but not 10°C, while populations decreased on diced tomatoes stored at these temperatures. In a similar study on whole and shredded lettuce (Beuchat and Brackett, 1990), chlorine treatment (200 to 250 ppm dip for 1 min) or modified atmosphere (3% O_2, 97% N_2) did not influence the growth of

L. monocytogenes. These studies indicate that removal of some of the competitive microflora on these produce items by treatment with 200 to 280 ppm chlorine does not influence the rate of growth of *L. monocytogenes*.

Zhang and Farber (1996) tested the effectiveness of chlorine, chlorine dioxide, and trisodium phosphate in removing populations of *L. monocytogenes* (5.4 to 5.7 $\log_{10}$ CFU/g) on lettuce and cabbage. Exposure to 200 ppm chlorine for 10 min at 4 and 22°C, respectively, resulted in maximum reduction of 1.3 and 1.7 $\log_{10}$ CFU/g of lettuce and 0.9 and 1.2 $\log_{10}$ CFU/g of cabbage. Application of 5 ppm chlorine dioxide for 10 min at 4 and 22°C, respectively, resulted in 1.1 and 0.8 $\log_{10}$ reduction on lettuce and 0.4 and 0.8 $\log_{10}$ reduction on cabbage. The larger reductions on lettuce suggest that inactivation of *L. monocytogenes* using chlorine is influenced by the nature of the vegetable being treated. Trisodium phosphate was deemed ineffective at concentrations that did not affect the organoleptic quality of lettuce. Several studies have shown that exposure time in excess of 10 min provides only marginal improvement in reduction of populations of *L. monocytogenes* on vegetables (Brackett, 1987; El-Kest and Marth, 1988; Zhang and Farber, 1996).

Chlorine is the most widely used disinfectant for fresh produce due to its cost and its effectiveness (Elphick, 1998). The use of 60 to 80 ppm hypochlorite in produce wash water has been recommended for inactivation of enteric pathogens which might be present on fruits and vegetables (Hobbs, and Gilbert, 1978). The suggested exposure time was 30 s. In a survey of food processors handling raw produce that included questions about microbial wash and decontamination processes, 76% of the participants used chlorine to disinfect their products (Seymour, 1999). However, the survey also revealed that 65% of the processors do not pre-wash the produce in potable water prior to disinfection procedures. The lack of a pre-wash in potable water may hinder efficacy of chlorine because the more organic matter in the wash water, the more active chlorine is lost (Kotula *et al.,* 1997). Microbicidal activity depends on the amount of free

available chlorine (as hypochlorous acid, HOCl) in the water that comes into contact with microbial cells (Beuchat, 1992). The loss of effectiveness of chlorine results when the active component, HOCl, is consumed as it contacts other organic matter such as fruit, leaves, and dirt in the wash water. Failure to pre-wash produce to remove loose organic matter may contribute to the exposure of pathogenic bacteria on produce to sublethal doses of chlorine. Other chemicals may be affected as well, especially when dirt, leaves, or other plant tissues physically block access to the site of microbial colonization.

The use of chemical sanitizers in the production of minimally processed fruits and vegetables is not limited to chlorine. Several chemicals are currently being evaluated as disinfectants for produce. There is a need to determine the effects these chemicals have on *L. monocytogenes* in order to use them safely and efficiently. There is also a need to understand how injury of *L. monocytogenes* affects the pathogen's resistance to additional stresses.

Stress Responses in *L. monocytogenes*

Cross protection has been induced in *L. monocytogenes* as a result of shocking with heat and acid, starvation, and other environmental factors (Farber and Pagotto, 1992; Lou and Yousef, 1996; Lou and Yousef, 1997; van Schaik *et al*., 1999). If exposure of *L. moncytogenes* to sub-lethal concentrations of sanitizers causes the induction of a stress response that would enable cross protection against heat, the pathogen may survive a mild cooking-step and viable cells could reach the gastrointestinal tract. Due to prior exposure to harsh conditions, these cells may exhibit characteristics that enable them to better survive within the gastrointestinal tract. It has been suggested that stresses encountered by bacterial cells present in foods prior to reaching the host may increase in virulence (Archer, 1996).

Cross protection

Lou and Yousef (1996) observed an increase in heat resistance of *L. monocytogenes* as the duration of starvation in 0.1M phosphate buffer at 30°C increased. Recovery of previously starved cells heated at 56°C for various times was substantially greater on tryptic soy agar than on tryptic soy agar supplemented with NaCl, suggesting that starvation protected heat-injured cells from the lethal effect of extended exposure to heat. In further investigations into the phenomenon known as "stress hardening" in *L. monocytogenes*, these same researchers stressed cells by starvation, heat shocking (45°C), or treating with ethanol, HCl, H_2O_2, or NaCl (Lou and Yousef, 1997). Adaptation of cells to pH 4.5 to 5.0 or 5% ethanol significantly increased resistance of *L. monocytogenes* to otherwise lethal doses of acid, ethanol, and H_2O_2. They also observed that adaptation to ethanol significantly increased the resistance to 25% NaCl. Adaptation to 500 ppm of H_2O_2, 7% NaCl, or heat caused increased resistance to 1% H_2O_2. Also, heat shock significantly increased resistance to ethanol and NaCl. Studies such as these have revealed that stress response in *L. monocytogenes* is complex and that some stresses can induce a resistance towards unrelated stresses. This phenomenon has been termed cross-protection. Cross-protection in *L. monocytogenes* has been studied, but there is a lack of knowledge about the phenomenon occurring in the pathogen as a result of exposure to high pH or to sublethal concentrations of sanitizers.

Alkaline stress

The acid stress phenomenon has been studied extensively in many species of bacteria of significance to human foodborne illness. The acid stress response of *L. monocytogenes* and its implication to food safety has also been investigated. Additionally, cross-protection of *L. monocytogenes* against various stresses has been observed after subjecting cells to stressful environments including starvation, heat, cold, acid, and chemicals (Lou and Yousef, 1996, 1997). Information on the behavior of *L.*

monocytogenes subjected to alkaline stress is limited. Some studies on alkaline stress in *L. monocytogenes* have been conducted despite greater interest being in acid stress. The organism survives well in alkaline conditions and has been known for some time to grow in media up to pH 9.6 (Gray and Killinger, 1966). Interestingly, a KOH enrichment step was included in an FDA protocol for enrichment of *L. monocytogenes* (Lovett and Hitchins, 1988).

Laird *et al*. (1991) investigated the viability of two strains of *L. monocytogenes* in synthetic egg washwater adjusted to pH values 8.0 – 10.5 by titration with a proprietary alkaline product containing a surfactant, sodium carbonate, alkaline phosphate, and sodium metasilicate with 0.6 – 0.8 % available chlorine. After a 4-h incubation at 33°C in alkaline detergent solutions, less than a 1-log decrease in two strains of *L. monocytogenes* was observed, while up to a 3-log decrease in populations occurred in neutral pH controls without added detergent. A third strain was less resistant than the other two strains. The organism was also isolated with frequency from the egg washing station environment and washwater.

L. monocytogenes survived in alkaline food process fluids after storage for two weeks (Rossmoore and Drenzek, 1990). Populations stored at 3.5°C in potable water containing 250 ppm of a proprietary corrosion inhibitor (pH 9.3) decreased by only 1.1 $\log_{10}$ CFU/ml, while populations decreased by 3.1 $\log_{10}$ CFU/ml in the same solution supplemented with 35% propylene glycol. Storage at 25°C in 1% conveyor lubricant solution (pH 9.5) resulted in only a 2.0 log reduction after two weeks.

In an investigation of the effects of pH stress on growth rate of *L. monocytogenes*, three strains tested were not affected by the presence of NaOH and NH_4^+ (Cheroutre-Vialette *et al.*, 1998). Cells adapted readily to the alkalinization of the medium to pH 9.0. Growth of a strain designated *L. monocytogenes* 14, which was obtained from a food-processing environment, was not affected by alkaline treatment whereas growth and lag phases were affected by acid.

Vasseur *et al*. (1999) performed similar experiments on five strains isolated from industrial food plants. In this study, growth rates were determined in meat bacto tryptone broth with 5 g/L glucose added and adjusted to pH 9.5, 10.0, 10.5 or 11.0 using NaOH. Increasing alkalinity resulted in increased lag phases for all five strains, but at pH 11, three of the five strains were more tolerant. Growth of *L. monocytogenes* at pH > 9.5 are unusual (Gray and Killinger, 1966), but the fermentation of the glucose present in the medium may have in fact allowed the organism to lower the pH prior to cell proliferation. Final pH values of growth media used in this study were not reported. Vasseur *et al.* (1999), however, did propose two hypotheses for the mechanism for damage of *L. monocytogenes* cells by NaOH: (i) at highly alkaline pH, the Na^+/H^+ antiporters might become saturated with excess Na^+ entering the cell, and thus, loss of ability of ion gradient maintenance would result as well as membrane protein inhibition, or (ii) high pH values could lead to saponification of membrane lipids and destabilization of the proteins responsible for lipid bilayer integrity.

In an attempt to determine the role alkaline pH plays in disruption of the cytoplasmic membrane, Mendonca *et al.* (1994) subjected several foodborne pathogens, including *L. monocytogenes*, to $NaHCO_3$-NaOH buffer solutions at pH 9, 10, 11, and 12 at 37 or 45°C for various times. They assessed viability by enumeration on selective and non-selective media and examined cell morphology using scanning electron microscopy and transmission electron microscopy. They observed substantially more resistance of *L. monocytogenes* to alkaline pH than *S.* Enteritidis and *E. coli*. At pH 9, populations of *L. monocytogenes* remained stable at either temperature. Exposure of the organism for 15 min at 37°C to pH 10 caused only slight decreases, while exposure to pH 11 and 12 resulted in a 1-log reduction. Microscopy revealed that contrary to the Gram-negative organisms tested, *L. monocytogenes* cells did not leak cell constituents following exposure to any of the pH values tested, nor was DNA detected in any of the filtrates

from *L. monocytogenes* cultures. While Gram-negative cells appeared collapsed and wrinkled, *L. monocytogenes* cells retained their shape. Interestingly, upon exposure of *L. monocytogenes* cells to alkaline pH the cytoplasmic membrane bulged against the cell wall. These researchers concluded that the presence of the thick rigid peptidoglycan layer in Gram-positive bacteria probably prevent the alkali-weakened cytoplasmic membrane from expanding and bursting.

The cross-protection phenomenon has even been studied in *L. monocytogenes* with respect to alkaline pH. Using a submerged vial heating technique, Palumbo *et al*. (1996) calculated $D_{56.6°C}$ value for *L. moncytogenes* in egg whites as affected by pH. The $D_{56.6°C}$ values for *L. moncytogenes* was 2.6 times greater at pH 9.3 than at pH 7.8. Conversely, in *Salmonella* spp. the D value was reduced with increasing pH. These researchers attributed the effect of high pH on increased heat resistance of *L. monocytogenes* to a lack of heat stability of the inherent, antilisterial enzyme, lysozyme, which is more heat-stable at pH 7 than at pH 9. While heat destruction of the enzyme may have played a role in thermotolerance, other studies suggest that alkaline pH alone has an effect *on L. monocytogenes* that results in tolerance to heating at 56 - 59°C.

Upon alkaline shock at 37°C for 35 min in a minimal medium at pH 10.0, repression of 67% of protein spots on two-dimensional polyacrylamide gel electrophoresis (PAGE) compared to non-stressed cells was observed (Phan-Thanh and Gormon, 1997). However, among 254 protein spots detected on PAGE, 11 were novel proteins and 16 others were up-regulated from two- to fourteen-fold. In a separate study of heat- and cold-shock proteins (Phan-Thanh and Gormon, 1995), two heat-shock proteins, nearly identical in molecular mass and isoelectric points to two constitutive proteins induced by alkaline stress (Phan-Thanh and Gormon, 1997), were induced at nearly the same ratios compared to non-stressed controls.

Starvation-induced chemical resistance.

Oftentimes chemical sanitizers are evaluated against microorganisms that were previously routinely or repetitively transferred in nutrient-rich, liquid growth media. Studies using cells cultured in such a way do not accurately resemble actual circumstances in food processing environments or food where cells are exposed to far more stressful conditions than those laboratory media. Lee and Frank (1991) observed that cells grown in a low nutrient medium were more resistant to inactivation with 1 ppm chlorine than were cells grown in TSB. Jacquet and Reynaud (1994) reported that the effectiveness of a chlorine-based commercial disinfectant was variable on several strains of *L. monocytogenes* isolated from human, animal, foodstuffs, or silage. In that study, 42% of the strains were resistant to the chemical. These findings indicate that the environmental conditions to which cells of *L. monocytogenes* are exposed play a role in the organism's ability to resist chemical destruction. This type of event should be confirmed with chlorine and examined using other food-grade sanitizers. The surfaces of many produce items may be lacking in nutritive substance, and hence serve as a starvation stress to *L. monocytogenes*. Cells surviving in food processing environments my also be nutritionally starved. Cells that have been subjected to starvation conditions enter an endospore-like state (Matin *et al*., 1989; Siegele and Kolter, 1992). This resistant state of bacteria induced by starvation stress may contribute to survival of cells during sanitization. There is a need to evaluate the effectiveness of sanitizers on *L. monocytogenes* subjected to conditions likely to exist during food production.

Justification and Objectives

In a colloquium organized by the American Academy of Microbiology, experts determined that future research in microbial ecology and adaptation is essential for increased understanding of factors implicated in the incidence of foodborne disease (Doores, 1999). This group suggested that the complex interactions between

microorganisms and their environment need to be better understood at every stage of food production. They went on to conclude that bacterial stress responses and their implications for food safety need to be studied in real world situations, and that such information has yet to be applied to food manufacturing practices to prevent processing from making foodborne bacteria more dangerous than when they arrived in the processing plant. As outlined above, *L. monocytogenes* is prevalent in most food processing environments and can persist despite utilization of rigorous cleaning and santitation programs. Furthermore, it has been documented that *L. monocytogenes* is unusually resistant to alkaline pH in a variety of different suspension media. Therefore, the following objectives were developed to address a void in information:

1. To determine the effects of alkaline pH stress and chlorine on the survival and subsequent thermotolerance of *L. monocytogenes* freshly isolated from a food processing environment.
2. To determine the ability of *L. monocytogenes* to survive stresses associated with cleaning and sanitizing food processing equipment.
3. To investigate the effect of exposing *L. monocytogenes* to solutions of alkaline pH cleaners commonly used in food processing environments on subsequent thermotolerance.
4. To investigate the behavior of cells of *L. monocytogenes* exposed to alkaline cleaners and alkali-adapted cells on frankfurters differing in fat and salt content during storage at frozen, refrigeration, and mildly abusive temperatures.
5. To determine the thermotolerance of cells of *L. monocytogenes* exposed to alkaline cleaners and alkali-adapted cells suspended in frankfurter exudates.

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CHAPTER 2

SURVIVAL AND HEAT RESISTANCE OF *LISTERIA MONOCYTOGENES* AFTER EXPOSURE TO ALKALI AND CHLORINE[1]

[1]Taormina, P. J., and L. R. Beuchat. 2001. Appl. Environ. Microbiol. 67:2555-2563. Reprinted here with permission of publisher.

ABSTRACT

A strain of *Listeria monocytogenes* isolated from a drain in a food processing plant was demonstrated, by determination of D values, to be more resistant to the lethal effect of heat at 56 or 59°C following incubation for 45 min in tryptose phosphate broth (TPB) at pH 12.0 compared to incubation for the same time in TPB at pH 7.3. Cells survived for at least 6 days when suspended in TPB at pH 9.0, 10.0, and 11.0 and stored at 4 or 21°C. Cells of *L. monocytogenes* incubated at 37°C for 45 min and then stored for 48 or 144 h in TPB at pH 10.0 were more resistant to heat treatment at 56°C than were cells stored in TPB at pH 7.3. The alkaline stress response in *L. monocytogenes* may induce resistance to otherwise lethal thermal processing conditions. Treatment of cells in 0.05 M potassium phosphate buffer (pH 7.00 ± 0.05) containing 2.0 or 2.4 mg/L free chlorine reduced populations by as much as 1.3 $\log_{10}$ CFU/ml, while treatment with 6.0 mg/L free chlorine reduced populations by as much as 4.02 $\log_{10}$ CFU/ml. Remaining subpopulations of chlorine treated cells exhibited some injury and cells treated with chlorine for 10 min were more sensitive to heating at 56°C than cells treated for 5 min. Contamination of foods by *L. monocytogenes* cells that have survived exposure to processing environments ineffectively cleaned or sanitized with alkaline detergents or disinfectants may have more severe implications than previously recognized. Alkaline pH-induced cross-protection of *L. monocytogenes* against heat has potential to enhance survival in minimally processed as well as in heat-and-serve foods, and in foods on holding tables, in foodservice facilities, and in the home. Cells surviving exposure to chlorine, in contrast, are more sensitive to heat, thus not compromising the effectiveness of thermal processing in achieving desired $\log_{10}$ reductions.

INTRODUCTION

Post-processing contamination of food with *Listeria monocytogenes* persists as a serious public health problem, particularly in the production of minimally processed and ready-to-eat foods. Recent outbreaks of listeriosis linked to smoked mussels (5), deli meats and hot dogs (8, 9), pork tongue jelly (16), and corn salad (1) have focussed attention on cross-contamination of processed foods from environmental sources. The ubiquity of *L. monocytogenes* in nature and its acknowledged presence in food processing environments (14, 26) explain the difficulty in producing minimally processed foods free of the pathogen. Consequently, food product recalls in the United States attributable to detection of *L. monocytogenes* by random sampling continue to rise (50), even as food processors attempt to comply with federally imposed zero tolerance policies.

The ability of microorganisms to adapt to acidic environments and subsequently become resistant to acid or other unrelated stresses has been demonstrated in several foodborne pathogenic bacteria, including *Salmonella* (21, 32, 33), *Escherichia coli* O157:H7 (2, 6, 23, 45), and *L. monocytogenes* (18, 30, 34, 35, 38, 40, 48). Alkaline stress in *E. coli* has been studied (3, 24, 44, 47), but observations on the ability of *L. monocytogenes* to survive exposure to highly alkaline environments are limited (11, 31, 35, 49). Information on alkali-induced cross-protection of *L. monocytogenes* against other environmental stresses, e.g., heat, is lacking. The influence of sanitizer-related stresses on the ability of bacteria to survive thermal treatment has also gained attention as another facet of the cross-protection phenomenon (20, 34, 51), but chlorine-induced cross-protection of *L. monocytogenes* has not been reported.

Cross-protection against heat as a result of alkaline stress has been documented in both Gram-positive and -negative bacteria. Heat resistance (55°C) of *Salmonella* Enteritidis PT4 in Lemco broth at pH 7.0 ± 0.2 was significantly increased by previous exposure to pH 9.2 ± 0.2 for 5 min or longer (28). Similarly, tolerance to heating at 62°C was induced by treating *Enterococcus faecalis* cells for 30 min at pH 10.5 (19).

Increased resistance of *L. monocytogenes* to heating at 56°C has been demonstrated following exposure of cells to starvation conditions, ethanol, acid, and H_2O_2 (34). Since induction of thermotolerance is known to occur in other bacteria exposed to various environmental stresses, including exposure to alkaline environments, the potential for development of thermotolerance in *L. monocytogenes* concurrent with alkaline shock would be likely.

There is a need to determine the effect of sanitizer-induced sublethal injury of *L. monocytogenes* on subsequent resistance to other stresses. Given the alkaline nature of detergents and some of the chemical sanitizers used to clean and sanitize equipment, floors, pipes, and drains in food and beverage processing plants where *L. monocytogenes* may reside, information on its response to alkaline stress would be useful when designing interventions to prevent post processing contamination of foods. Direct application of chlorine is relied upon for reducing microbial populations in foods (especially produce) or on food contact surfaces. However, concentrations of free (available) chlorine reaching microbial cells may not be lethal since the efficacy of chlorine as a disinfectant can be reduced by numerous factors. The objective of this study was to determine the effects of alkaline pH stress and chlorine on the survival and subsequent thermotolerance of *L. monocytogenes* freshly isolated from a drain in a food processing facility.

MATERIALS AND METHODS

Strain and culture conditions. An isolate of *L. monocytogenes*, serotype 4b, flagellar type ABC, from a drain in a food processing plant was used in this experiment. Stock cultures were prepared from a subculture of the initial isolate in tryptose phosphate broth (TPB, pH 7.3) (Difco Laboratories, Detroit, Mich.). Cultures were incubated at 37°C for 24 h, then supplemented with 15% glycerol and stored at −20°C until used.

Prior to each experiment, a stock culture was thawed and loop inocula were transferred to Erlenmeyer flasks (250 ml) containing 100 ml of sterile TPB. Flasks were incubated in an incubator shaker (New Brunswick Scientific, New Brunswick, N.J.) set at 37°C and 200 rpm for 13 h, a time at which the culture was at a state of transition between the late logarithmic or early stationary phase of growth, or for 48 h, well into the stationary phase of growth. The culture (25 ml) was dispensed into a 50-ml conical polystyrene centrifuge tube (Becton Dickinson Labware, Franklin Lakes, N.J.) and centrifuged (5,000 x *g*, 4°C) for 10 min (brake 3.5 min) in a pre-cooled Marathon 12KBR Benchtop Refrigerated Centrifuge (Fisher Scientific, Pittsburgh, Pa). Cell pellets were washed thrice in sterile, pre-cooled (4ºC) 0.05 M potassium phosphate buffer at pH 7.00 ± 0.05 prepared from filtered (0.45 μ) laboratory-grade water, (PB), then resuspended in treatment or control (PB, or TPB, pH 7.3) solutions.

Preparation of chemical treatment solutions. Alkaline treatment solutions were prepared by adding appropriate volumes of 1 or 2 N filter-sterilized (0.22 μ) NaOH to sterile TPB to achieve pH 9.0, 10.0, 11.0, 12.0, and 13.0 ± 0.1. Samples of alkali-adjusted or unadjusted (control, pH 7.3) TPB were measured with an Accumet pH meter (Denver Instrument Co., Arvada, Colo.) following standardization with pH 4.00, 7.01, and 10.00 buffers. TPB and NaOH solutions were prepared from filtered (0.45 μ) laboratory-grade water and used on the same day of pH adjustment.

Sodium hypochlorite (NaOCl) solution (minimum 4% available chlorine) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was used to prepare specific concentrations of "free" available chlorine by diluting with PB. Concentrations were verified using a Hach Digital Titrator (model 16900; Hach Company, Loveland, Colo.) fitted with a 0.0451 N phenylarsine oxide titration cartridge, an Amperometric Digital Titrator model 19300, and a TitraStir™ stir plate following the forward titration procedure for determining concentrations of free chlorine ranging from 0-10 mg/L. All solutions were prepared using chlorine demand-free glassware and sterile PB made from

filtered (0.45 μ), laboratory-grade, sterile water. Solutions were protected from light, held at 21 ± 2°C, and used within 1 h of preparation.

Alkaline treatment and heat inactivation. Washed cell pellets were resuspended in 25 ml of TPB (pH 7.3) or TPB adjusted to pH 9.0 – 13.0 ± 0.1 and incubated with agitation at 37°C for 15 or 45 min. Following incubation, unstressed (pH 7.3, control) and alkali-stressed cells of *L. monocytogenes* were centrifuged (5,000 x *g*, 10 min, 4°C) and resuspended in 25 ml of PB (pH 7.00 ± 0.05, 4°C). Cells suspensions (50 μl) were injected into Kimax-51 capillary tubes (0.8-1.0 mm I.D. x 90 mm long, no. 34507-99; Kimble, Vineland, N.J.), and the ends were flame sealed. Capillary tubes were brought to 21 ± 2°C before subjecting to heat treatment in a water bath at 56°C for 0, 1, 2, 5, 10, 20, or 25 min or at 59°C for 0, 0.5, 1, 1.5, 2, 4, 6, 10, and 15 min. Come-up times for tempered fluid-filled capillary tubes measured with a Microprocessor Thermometer (model HH23, Omega, Stamford, Conn.) connected with a type T thermocouple were 2 and 3 sec in the water bath at 56 or 59°C, respectively. Capillary tubes were immediately cooled and sanitized by immersing in an ice bath followed by 70% ethanol and then sterile water before they were aseptically transferred to screw-capped test tubes (16 mm I.D. x 125 mm long) containing 5 ml of sterile 0.1% peptone water. Within each test tube, the capillary tube containing the heated cell suspension was crushed using a sterile glass rod. The content of each test tube was thoroughly mixed using a vortex mixer, and undiluted and diluted suspensions were surface plated (0.25 ml in quadruplicate or 0.1 ml in duplicate) or serially diluted in 0.1 % peptone water and surface plated (0.1 ml in duplicate) on tryptose phosphate agar (Difco; TPA). All plates were incubated for 48 h prior to counting colonies using a manufacturer-recommended modification of the pcount01 file of the Countermat Automated Colony Counter (Cogent Technologies, Cincinnati, Ohio).

Based upon initial observations of heat resistance of alkaline-stressed cells, modifications were made to the stressing procedures of cells destined for heating trials.

Variations in stressing procedures included incubating cells for 15 or 45 min at 37°C in TPB adjusted with 2.0 N NaOH to pH 12.0, incubating stationary phase cells (48-h cultures) for 15 or 45 min at 37°C in TPB at pH 7.3, 10.0, or 12.0, and incubating cells for 45 min in TPB containing 20 mg/L cycloserine-D, 20 mg/L chloramphenicol, or 10 mg/L rifampicin at pH 7.3 or 12.0.

Cells subjected to alkaline stress for 45 min were also incubated at 4 or 21°C, with agitation, for up to 144 h. Populations of *L. monocytogenes* were determined after 48 and 144 h of incubation by surface plating undiluted and diluted suspensions on TPA and on TPA supplemented with 4% NaCl (TPAS) as described above. Heat (56°C) tolerance of cells incubated for 45 min at 37°C and then held at 4°C for 48 or 144 h in TPB at pH 7.3, 10.0, or 11.0 was also determined.

NaOCl treatment and heat inactivation. Flasks (250 ml) containing 50 ml of solutions of 0, 2.0, 2.4, or 6.0 mg/L available chlorine were placed on an Innova 2000 platform shaker (New Brunswick Scientific) set at 140 rpm. Cells of *L. monocytogenes* suspended in PB (10 ml) were added to treatment solutions. After 5 or 10 min, 10 ml were removed from the flask and the chlorine was neutralized by dispensing into a bottle (120 ml) containing 30 ml of sterile 0.01 N $Na_2S_2O_3$ (10) and vortexing for 10 sec. Samples were surface plated on TPA and TPAS as described above. Chlorine-stressed or unstressed (control) cells were also subjected to heat treatment at 56°C using capillary tubes as described above.

Statistical analyses. Three replicates were conducted for each trial. Population means, each representing six values (two duplicate plates from three replicate trials), were analyzed by the general linear model procedure and means separation analysis of SAS (SAS Institute, Inc., Cary, N.C.) using the Duncan's multiple range test (17).

The number of viable cells recovered by surface plating heated or unheated cell suspensions on TPA, expressed as $\log_{10}$ CFU/ml, was plotted against heating time. Normally, D values are calculated from the absolute value of the reciprocal of the slope

of the linear regression line of the plot of survivors versus time (survival curve). However, our data did not fit log-linear inactivation kinetics. Therefore, heat survivor data were analyzed using appropriate forms of the logistic equation (29, 43) applied by the nonlinear regression procedure of SAS. The log-transformed equations used to analyze data were as follows:

$$\log S = \log 2 - \log [1 + e(\beta t)] \quad (1)$$

where log S is the log (CFU/CFU_0) at any given time (t) and β is the maximum specific death rate.

For survival curves with no initial lag in killing but having two distinct killing phases (biphasic), data were fitted to the following two-term exponential form of equation 1:

$$\log S = \log (\{2f_1/[1 + e(\beta_1 t)]\} + \{2(1 - f_1) / [1 + e(\beta_2 t)]\}) \quad (2)$$

where f_1 and $(1 - f_1)$ represent two fractions of cells (differing with respect to heat resistance) and β_1 and β_2 are the specific killing rates for the two fractions, respectively. The assumption of this model is that two fractions (subpopulations) are killed exponentially but at different, independent rates.

Curves which included a lag in killing (shoulder) and biphasic inactivation were fitted to the following two-term exponential form of equation 1:

$$\log S = \log (f_1\{1 + e[-\beta_1 t_1]\} / \{1 + e[\beta_1(t - t_1)]\}) + \log ([1 - f_1] \{1 + e[-\beta_2 t_1]\} / \{1 + e[\beta_2(t - t_1)]\}) \quad (3)$$

where t_1 is the lag period.

For equation 1, logistic D values (43) were calculated as $D = 2.94/\beta$, and for equations 2 and 3, $D = \ln(19)/\beta_2$.

RESULTS

Survival of alkali-stressed *L. monocytogenes*. Cells from 13-h cultures of *L. monocytogenes* treated at 37°C for 45 min in TPB at pH 7.3 or 9.0 followed by holding

at 4°C in TPB at pH 7.3 or 9.0, respectively, were essentially unchanged after 144 h (Figure 2.1, top). A significant number of cells treated at pH 10.0 were injured as evidenced by their inability to form colonies on TPAS compared to TPA. Incubating cells in TPB at pH 11.0 was more stressful, as the initial population declined by 1 log and injury was evident after 48 h; populations further decreased after 144 h. Incubation at 37°C for 45 min in TPB at pH 12.0 reduced the population by almost 5 logs, while subsequent storage at 4°C caused further reductions. Incubation at 37°C for 45 min in TPB at pH 13.0 was lethal to all cells (data not shown).

Survival of *L. monocytogenes* in TPB (pH 7.3 – 12.0) at 21°C was similar to survival at 4°C (Figure 2.1, bottom). Populations in TPB at pH 7.3, however, declined slightly after 144 h at 21°C. A portion of *L. monocytogenes* cells incubated for 48 h in TPB at pH 10.0 were injured but resuscitated between 48 and 144 h.

Heat resistance of alkali-stressed *L. monocytogenes*. Logistic $D_{56°C}$ values and nonlinear regression parameter estimates for survivor curves of 13-h cultures *L. monocytogenes* cells, which were incubated at 37°C for 15 or 45 min in TPB at pH 7.3 or in TPB adjusted with 1.0 N NaOH to pH 9.0 – 13.0, are listed in Table 2.1. $D_{56°C}$ values of cells previously incubated in TPB at pH 7.3 for 15 or 45 min were 6.92 and 6.02 min, respectively, and, within treatment time, were not dissimilar from $D_{56°C}$ values of cells incubated at pH 9.0, 10.0, or 11.0. However, $D_{56°C}$ values of cells incubated in TPB at pH 12.0 for 15 or 45 min were 8.28 and 14.30 min, respectively, and were higher than that of cells exposed to pH 7.3 for 15 or 45 min. Approximately five log CFU/ml were killed during alkali shock in TPB (pH 12.0) incubated at 37°C for 45 min before suspensions were heated at 56°C. The $D_{56°C}$ value of cells subjected to that treatment was derived from the least heat-sensitive fraction of the population ($1 – f_1$), which was 20%. The other fraction of the population (f_1, 80%) had a death rate that appeared high, but was not accurately estimated by the model as evidenced by the standard deviation. Using 2.0 N NaOH rather than 1.0 N NaOH to adjust the pH of the TPB to pH 12.0 had

Figure 2.1. Populations of *L. monocytogenes* cells held at 4ºC (top) or 21°C (bottom) in TPB with pH adjusted or unadjusted with 1.0 N NaOH. Populations of alkali-injured and uninjured cells were recovered by plating onto TPA and TPA + 4% NaCl (TPAS), respectively. Cells were incubated in TPB at various pH values at 37ºC for 45 min prior to holding at 4 or 21ºC. Samples plated on TPA were taken from TPB at pH 7.3 (■), 9.0 (●), 10.0 (▲), 11.0 (◆), and 12.0 (■), and samples plated on TPAS were taken from TPB at pH 7.3 (□), 9.0 (○), 10.0 (△), 11.0 (◇), and 12.0 (●).

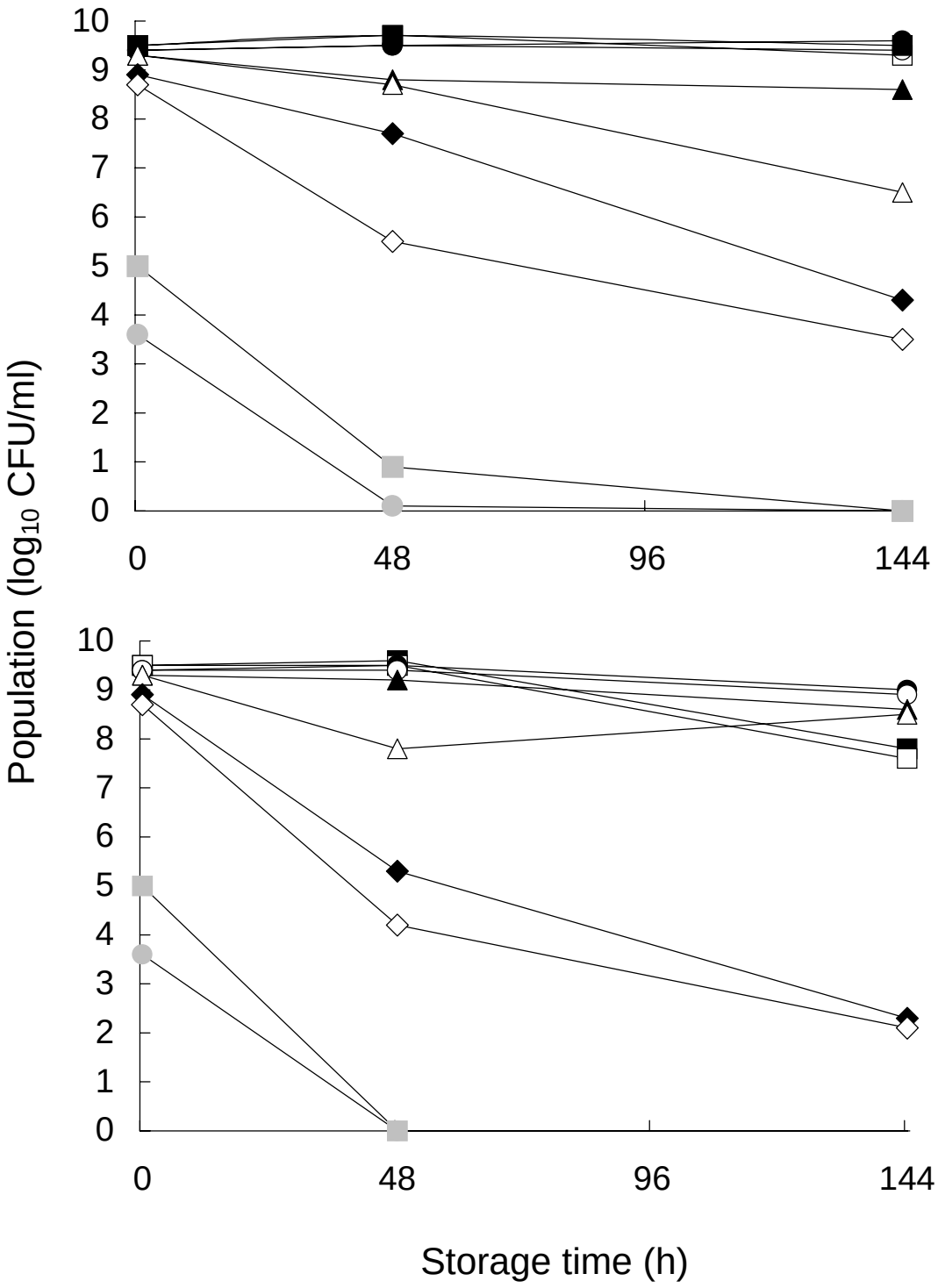
Population (log10 CFU/ml)
10
9
8
7
6
5
4
3
2
1
0
0
48
96
144
Storage time (h)

TABLE 2.1. Heat (56°C) survival parameter estimates for late logarithmic growth phase (13 h) *L. monocytogenes* cells incubated at 37°C for 15 or 45 min in TPB at pH 7.3 – 12.0[a].

Treatment time (min)	pH (± 0.1)	Eqn.[b]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [c]	Logistic D value (min)[d]
15	7.3	1	-	-	-	0.425 ± 0.021	0.973	6.92
	9.0	1	-	-	-	0.429 ± 0.014	0.987	6.85
	10.0	1	-	-	-	0.539 ± 0.029	0.968	5.45
	11.0	1	-	-	-	0.481 ± 0.004	0.999	6.11
	12.0	1	-	-	-	0.355 ± 0.020	0.958	8.28
	12.0*	1	-	-	-	0.350 ± 0.006	0.996	8.40
45	7.3	1	-	-	-	0.488 ± 0.013	0.991	6.02
	9.0	1	-	-	-	0.504 ± 0.014	0.991	5.83
	10.0	1	-	-	-	0.520 ± 0.014	0.991	5.65
	11.0	2	0.13	0.508 ± 0.011	0.789 ± 0.081	-	0.997	5.79
	12.0	2	0.2	0.205 ± 0.010	25.7 ± > 100	-	0.969	14.30
	12.0*	1	-	-	-	0.355 ± 0.009	0.993	8.28

[a] pH of TPB was adjusted using 1.0 N NaOH; pH was adjusted using 2.0 N NaOH if noted by *.

[b] Parameter estimates were obtained by fitting survival data to the appropriate logistic equations by nonlinear regression.

[c] Calculated as 1 – (residual sum of squares / corrected total).

[d] Calculated as $D = 2.94/\beta$ for equation 1, or $D = (\ln 19)/\beta_2$ for equation 2.

an effect on thermotolerance (Table 2.1). The $D_{56°C}$ value of cells previously treated in TPB for 15 min at pH 12.0 adjusted with 2.0 N NaOH was higher than the $D_{56°C}$ value of cells previously treated for 15 min at pH 7.3, but similar to the $D_{56°C}$ value of cells treated in TPB adjusted to pH 12.0 with 1.0 N NaOH. The $D_{56°C}$ value of *L. monocytogenes* cells incubated for 45 min in TPB adjusted to pH 12.0 with 2.0 N NaOH was lower than that of cells subjected to the same treatment in TPB adjusted with 1.0 N NaOH, although it was still higher than the $D_{56°C}$ value of the control.

The enhanced thermotolerance of 13-h cells of *L. monocytogenes* treated at pH 12.0 compared to pH 7.3 warranted further investigation. Table 2.2 lists $D_{59°C}$ values and nonlinear regression statistics for survivor curves of *L. monocytogenes* previously incubated at 37°C for 15 or 45 min in TPB at pH 7.3 or in TPB at pH 12.0. Each survival curve fit equation 2 the best. The $D_{59°C}$ value of cells that were incubated for 45 min in TPB at pH 12.0 was 10.10 min, which was much higher than that of cells incubated for the same time in TPB at pH 7.3.

The growth curve of *L. monocytogenes* in TPB at 37°C was determined following diluting and surface plating the culture on TPA and TPAS. The presence of 4% NaCl in TPA did not influence the number of cells detected at a given incubation time (data not shown). Nonetheless, alkaline-stressed *L. monocytogenes* cells in a late stationary phase (48 h) of growth responded differently (Table 2.3) than cells in late logarithmic growth (13 h) used in previous alkaline stress experiments. Stationary phase cells treated for 15 min at pH 12.0 had a $D_{56°C}$ value 3.19 times higher than the $D_{56°C}$ value of cells treated at pH 7.3 (control). However, $D_{56°C}$ values of cells treated for 45 min at pH 7.3, 10.0, or 12.0 were not dissimilar. Cells cultured for 48 h may have been entering death phase, making them more sensitive to treatment at pH 12.0. However, injured, i.e., NaCl-sensitive, 48-h cells were not observed.

Survival parameter estimates and logistic $D_{56°C}$ values of late logarithmic growth phase cells (13 h) incubated for 45 min in TPB pH 7.3, 10.0, or 11.0 at 37°C and then

Table 2.2. Heat (59°C) survival parameter estimates for late logarithmic growth phase (13 h) *L. monocytogenes* cells incubated at 37°C for 15 or 45 min in TPB at pH 7.3 or 12.0[a].

Treatment time (min)	pH (± 0.1)	Eqn.[b]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [c]	Logistic D value (min)[d]
15	7.3	2	0.01	1.400 ± 0.124	2.510 ± 0.424	-	0.964	2.10
	12.0	2	0.02	0.788 ± 0.072	1.410 ± 0.245	-	0.951	3.73
45	7.3	2	0.01	1.490 ± 0.177	2.920 ± 0.817	-	0.923	1.97
	12.0	2	0.01	0.292 ± 0.031	1.450 ± 0.172	-	0.967	10.10

[a] pH of TPB was adjusted using 1.0 N NaOH.

[b] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression.

[c] Calculated as 1 – (residual sum of squares / corrected total).

[d] Calculated as $D = (\ln 19)/\beta_2$.

Table 2.3. Heat (56°C) survival parameter estimates for late stationary phase (48 h) *L. monocytogenes* cells incubated at 37°C for 15 or 45 min in TPB at pH 7.3, 10.0, or 12.0[a].

Treatment time (min)	pH (± 0.1)	Eqn.[b]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [c]	Logistic D value (min)[d]
15	7.3	1	-	-	-	0.572 ± 0.025	0.979	5.14
	10.0	1	-	-	-	0.491 ± 0.007	0.998	5.99
	12.0	2	0.2	0.179 ± 0.007	0.542 ± 0.084	-	0.990	16.42
45	7.3	1	-	-	-	0.533 ± 0.028	0.968	5.52
	10.0	1	-	-	-	0.433 ± 0.011	0.993	6.79
	12.0	2	0.1	0.576 ± 0.021	1.850 ± 0.198	-	0.996	5.10

[a] pH of TPB was adjusted using 1.0 N NaOH.

[b] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression.

[c] Calculated as 1 – (residual sum of squares / corrected total).

[d] Calculated as $D = 2.94/\beta$ for equation 1, or $D = (\ln 19)/\beta_2$ for equation 2.

4°C for 48 h were determined (Table 2.4). Long-term exposure of *L. mononcytogenes* to pH 10.0 increased heat tolerance. After 48 h, the $D_{56°C}$ value of cells incubated at 4°C in TPB at pH 10.0 was 2.24 min more than that of cells incubated at pH 7.3. Similarly, after 144 h, the $D_{56°C}$ value of cells incubated at 4°C in TPB at pH 10.0 was 1.67 min more than cells incubated at pH 7.3. In addition to slower apparent rates of inactivation of alkaline-stressed cells compared to control cells, smaller numbers of alkaline-stressed cells were inactivated compared to control cells. The least heat-sensitive fraction of cells exposed to pH 11.0 used to derive logistic $D_{56°C}$ values was very small and, therefore, not representative of the original population.

Addition of antibiotics to the alkaline stress medium (TPB adjusted to pH 12.0) reduced the resistance of *L. monocytogenes* to heating at 56°C (Table 2.5). Survival parameter estimates of late logarithmic growth phase (13 h) cells previously treated in TPB at pH 7.3 (control) in the presence of antibiotics (Table 2.5) were similar to those of cells exposed to the same treatment without antibiotics (Table 2.1). However, 13-h cells treated in solutions of TPB at pH 12.0 containing cycloserine-D, chloramphenicol, or rifampicin each had reduced thermal tolerance (Table 2.5) compared to alkaline treated cells not exposed to antibiotics (Table 2.1) as evidenced by logistic $D_{56°C}$ values. Subjecting cells to alkaline stress in the presence of cycloserine-D had less effect on thermotolerance compared to the effects of other antibiotics.

Chlorine stress and effect on thermotolerance. Treatment of late logarithmic growth phase (13 h) *L. monocytogenes* cells for 5 or 10 min with 2.0, 2.4 and 6.0 mg/L chlorine resulted in injury of cells as evidenced by a significant difference ($P \leq 0.05$) in the ability to form colonies on TPAS compared to TPA (Table 2.6). Populations of cells treated with 2.0, 2.4, or 6.0 mg/L for 10 min were reduced by 0.62, 1.30, or 4.02 logs, respectively, compared to the control. At each chlorine concentration tested, treatment time did not have a significant effect on the number of cells recovered on TPA or TPAS.

Table 2.4. Heat (56°C) survival parameter estimates for *L. monocytogenes* cells previously incubated at 37°C for 45 min and then held at 4°C for 48 or 144 h in TPB at pH 7.3, 10.0, or 11.0[a].

Storage time (h)	pH (± 0.1)	Eqn.[b]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [c]	Logistic D value (min)[d]
48	7.3	1	-	-	-	0.739 ± 0.022	0.989	3.98
	10.0	1	-	-	-	0.473 ± 0.006	0.997	6.22
	11.0	2	0.0001	0.263 ± 0.034	4.170 ± 0.573	-	0.961	11.20
144	7.3	1	-	-	-	0.795 ± 0.040	0.968	3.70
	10.0	1	-	-	-	0.547 ± 0.021	0.981	5.37
	11.0	2	0.005	0.343 ± 0.036	4.840 ± 0.774	-	0.974	8.57

[a] pH of TPB was adjusted using 1.0 N NaOH.

[b] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression.

[c] Calculated as 1 – (residual sum of squares / corrected total).

[d] Calculated as $D = 2.94/\beta$ for equation 1, or $D = (\ln 19)/\beta_2$ for equation 2.

Table 2.5. Heat (56°C) survival parameter estimates for *L. monocytogenes* cells previously incubated at 37°C for 45 min in TPB at pH 7.3 or 12.0[a] in the presence of antibiotics.

Antibiotic / concentration	pH (± 0.1)	Eqn.[b]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [c]	Logistic D value (min)[d]
Cycloserine-D 20 μg/ml	7.3	2	0.01	0.587 ± 0.039	1.140 ± 0.231	-	0.978	5.01
	12.0	2	0.2	0.302 ± 0.011	0.609 ± 0.073	-	0.995	9.74
Chloramphenicol 20 μg/ml	7.3	3	0.08	0.510 ± 0.016	-0.335 ± 0.092	-	0.993	5.76
	12.0	1	-	-	-	0.371 ± 0.009	0.995	7.92
Rifampicin 10 μg/ml	7.3	3	0.1	0.574 ± 0.027	-0.192 ± 0.065	-	0.990	5.12
	12.0	3	0.2	0.346 ± 0.046	-0.037 ± 0.058	-	0.981	8.50

[a] pH of TPB was adjusted using 1.0 N NaOH.

[b] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression.

[c] Calculated as 1 – (residual sum of squares / corrected total).

[d] Calculated as $D = 2.94/\beta$ for equation 1, or $D = (\ln 19)/\beta_2$ for equations 2 and 3.

Table 2.6. Populations of late logarithmic growth phase (13 h) *L. monocytogenes* cells recovered on TPA and TPA + 4% NaCl (TPAS) following treatment with chlorine.

Chlorine conc. (mg/L)	Treatment time (min)	Population ± SE (log_{10} CFU/ml)[a]	
		TPA	TPAS
0	5	a 8.73 ± 0.0219	a 8.73 ± 0.1790
	10	a 8.77 ± 0.0133	a 8.70 ± 0.2190
2.0	5	a 8.21 ± 0.0556	b 7.56 ± 0.0760
	10	a 8.15 ± 0.0514	b 7.61 ± 0.0982
2.4	5	a 7.54 ± 0.1880	b 7.09 ± 0.0999
	10	a 7.47 ± 0.1270	b 6.78 ± 0.1540
6.0	5	a 4.90 ± 0.1610	b 4.03 ± 0.1590
	10	a 4.75 ± 0.1690	b 3.30 ± 0.2960

[a] Values in the same row that are not preceded by the same letter are significantly different ($P \leq 0.05$).

Heat survival parameter estimates for chlorine treated cells were variable (Table 2.7). Survivor curves of populations not exposed to chlorine (controls) were analyzed with equation 3 due to initial lag in death (shoulder) and biphasic inactivation. In these cases, the least heat sensitive fraction was rather large and estimated as 50%. Generally, logistic $D_{56°C}$ values of cells treated for 5 min with chlorine were higher than that of cells treated for 10 min. Logistic $D_{56°C}$ values of cells treated at 6.0 mg/L available chlorine for 5 or 10 min were 7.12 and 6.93, respectively, and were larger than controls.

DISCUSSION

Following incubation at 37°C for 45 min, *L. monocytogenes* survived well at 4°C in TPB at pH 9.0, 10.0, and 11.0. These high-nutrient, high-pH conditions represent conditions on floors, in drains, within conveyor belt rollers, and other areas within food processing facilities that may harbor food debris and alkaline detergent or sanitizer residues. Demonstration of injury to *L. monocytogenes* cells held for 144 h in TPB at pH 10.0, and after 48 h in TPB at pH 11.0, suggests that some cells were better at coping with alkaline environments than others. At 21°C, the slight decline in number of viable cells held in TPB at pH 7.3 may be due to mild acidification of TPB caused by metabolism of sugars by *L. monocytogenes*, thus killing weaker cells. Populations of cells stored at 21°C in TPB at pH 9.0, which is below the upper pH limit for growth of *L. monocytogenes* (13), were constant up to 144 h, perhaps because acid produced by the pathogen was neutralized by the alkaline environment to prevent the pH from decreasing to a stressful range. The pH of the stressing media generally decreased by ca. 1 pH unit during incubation.

Alkaline resistance of *L. monocytogenes* at higher temperatures has been documented by other researchers. The pathogen has been shown to be resistant to

Table 2.7. Heat (56°C) survival parameter estimates for late logarithmic growth phase (13 h) *L. monocytogenes* cells previously treated with chlorine.

Chlorine conc. (mg/L)	Treatment time (min)	Eqn.[a]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [c]	Logistic D value (min)[d]
0	5	3	0.5	0.709 ± 0.056	-0.604 ± 0.144	-	0.973	4.15
0	10	3	0.5	0.738 ± 0.047	-0.555 ± 0.121	-	0.983	3.98
2.0	5	1	-	-	-	0.593 ± 0.014	0.994	4.96
2.0	10		-	-	-	0.678 ± 0.011	0.997	4.34
2.4	5	2	0.2	0.512 ± 0.024	0.809 ± 0.160	-	0.990	5.74
2.4	10	1	-	-	-	0.962 ± 0.040	0.985	3.06
6.0	5	2	0.1	0.413 ± 0.053	2.090 ± 0.651	-	0.950	7.12
6.0	10	2	0.1	0.424 ± 0.008	3.950 ± 0.314	-	0.999	6.93

[a] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression.

[b] Calculated as 1 – (residual sum of squares / corrected total).

[d] Calculated as $D = 2.94/\beta$ for equation 1, or $D = (\ln 19)/\beta_2$ for equations 2 and 3.

storage in $NaHCO_3$-NaOH buffer at pH 9.0, 10.0, 11.0, and 12.0 at 37 or 45°C (35). Laird *et al*. (31) observed less than a 1-log decrease in viability of *L. monocytogenes* after a 4-h incubation at 33°C in synthetic egg washwater adjusted to pH values 8.0 – 10.5 by titration with an alkaline detergent product, while up to a 3-log decrease in the number of viable cells occurred in a neutral pH control.

Other researchers (11, 49) have reported the ability of food factory isolates of *L. monocytogenes* to grow in alkali-adjusted media. It is likely that growth would have been observed in our studies if smaller inocula had been used. Nonetheless, our data confirms the ability of *L. moncytogenes* to survive in alkaline media at refrigeration and ambient temperature, and gives insight into the extent of injury and death that occurs over time. Implications are that standing pools of detergent and possibly sanitizer residue in food-processing environments may permit survival of *L. monocytogenes* for extended periods, with potential development of cross-protection against other stress conditions.

The $D_{56°C}$ of cells previously exposed to pH 12.0 was larger than $D_{56°C}$ of cells exposed to lower pH environments (Table 2.1). The induction of cross-protection of bacteria against heat could, theoretically, increase gradually in proportion to increased levels of stress. A gradual increase in heat resistance of *L. monocytogenes* cells proportional to increasing alkalinity was not observed, although a gradual increase of thermotolerance of cells treated at pH 12.0 was observed with increasing length of exposure time. Results could indicate that after incubation at 37°C for 45 min at pH 12.0, the weaker, less resistant cells died, leaving only the more stable cells, perhaps those approaching stationary phase, to survive thermal treatment. Gilbert *et al.* (22) stated that populations of microorganisms will respond as collections of individuals from related backgrounds, rather than as heterogeneous mixtures of all possible biotypes. However, thermal inactivation studies using cells stressed for prolonged periods in alkaline TPB at 4°C (Table 2.4) verified enhanced thermotolerance due to alkaline stress. After prolonged storage at 4°C in TPB at pH 7.3 or 10.0 before heating,

populations of alkaline stressed cells were similar to unstressed populations (pH 7.3, control), so $D_{56°C}$ values were calculated using survival curves beginning with roughly the same number of cells. In this case, heating was still more lethal to control cells compared to alkaline-stressed cells treated and stored at pH 10.0, as evidenced by slower rates of death and less total inactivation of alkaline-stressed cells.

Achieving a pH of 12.0 in TPB by adding 1.0 N NaOH diluted the TPB by approximately 20%, thereby diluting the nutrient concentration. The procedure for adjusting pH was therefore modified by using 2.0 N NaOH to adjust TPB to pH 12.0. With this modification, cells stressed at pH 12.0 for 15 min appeared more thermotolerant than those stressed at pH 12.0 for 45 min. The increased nutrient availability in TPB adjusted to pH 12.0 with 2.0 N NaOH, compared to the original procedure, may have caused cells to use alternative mechanisms to cope with the high alkalinity. Cells may also have undergone cytoplasmic buffering due to synthesis of intracellular metabolites in an attempt to stabilize pH. Dilworth and Glenn (15) noted that the use of cytoplasmic buffering by bacteria to cope with continued ingress to OH^- might avert such stress temporarily, but would not be successful long term. This would be consistent with our findings since treatment for 45 min in TPB adjusted to pH 12.0 with 2.0 N NaOH tended to sensitize cells to heating compared to treatment for 15 min. This phenomenon did not occur in TPB adjusted to pH 12.0 with 1.0 N NaOH.

Another possible explanation for the differences in the heat sensitivity of cells based on increased normality of NaOH used to adjust the pH of TPB is that differences in concentration of Na^+ may have influenced heat resistance. Since cells treated for 15 min were more thermotolerant than populations treated for 45 min, Na^+ may take longer than 15 min to injure the cell. Small *et al*. (46) showed that the use of KOH rather than NaOH in broth used to stress *E. coli* resulted in increased survival from 0.06 to 50% at pH 10.2. Faster rates of entry of Na^+ occur at alkaline pH (4), and Na^+ may have damaged *L. monocytogenes* cells and sensitized them to heating.

Resistance of bacteria to stress is generally believed to increase in stationary-phase cells. Our observations on the alkaline stress-induced thermotolerance of stationary phase cells of *L. monocytogenes* were inconsistent with that generalization. Stationary-phase cells (48 h) stressed at pH 12.0 for 15 min were considerably more thermotolerant than cells exposed to other treatments, but exposure to the stress for 45 min was lethal and the majority of cells in the remaining population had decreased tolerance to heat. Being relatively inactive metabolically after treatment in alkali-adjusted TPB, stationary phase cells may have had limited or inoperable mechanisms to recover from alkaline stress and resorted to a short-term intracellular pH compensation mechanism to remain viable.

Information on the effects of alkalinity on thermotolerance of *L. monocytogenes* is limited. Palumbo *et al.* (39) used a submerged vial heating technique to determine $D_{56.6°C}$ values for *L. moncytogenes* in egg whites as affected by pH. In that study, $D_{56.6°C}$ values were calculated to be 2.6 times greater at pH 9.3 than at pH 7.8. Conversely, in *Salmonella,* the D values were reduced with increasing pH. These researchers attributed the effect of high pH on increased heat resistance of *L. monocytogenes* to a lack of heat stability of an antilisterial enzyme, lysozyme, which is more heat stable at pH 7 than at pH 9. While heat destruction of lysozyme in egg whites may have played a role in thermotolerance, our study indicates that alkaline pH itself enhances thermotolerance of *L. monocytogenes*.

Mendonca *et al.* (36) examined the morphology of alkali-stressed *L. monocytogenes* cells using scanning electron microscopy and transmission electron microscopy. Their study revealed that, contrary to the Gram-negative bacteria tested, *L. monocytogenes* cells did not leak cell constituents following exposure to pH 9.0, 10.0, 11.0, or 12.0, nor was DNA detected in any filtrates from suspensions of the organism. While Gram-negative cells appeared collapsed and wrinkled, *L. monocytogenes* cells retained their shape. Interestingly, exposure of *L. monocytogenes* cells to alkaline pH caused the cytoplasmic membrane to bulge against the cell wall. These researchers

concluded that the presence of a thick rigid peptidoglycan layer in Gram-positive bacteria probably prevents the cytoplasmic membrane from expanding and bursting.

Addition of antibiotics to alkali-stressing media resulted in reduction of thermotolerance of *L. monocytogenes* cells exposed to pH 12.0, implying possible mechanisms of injury repair. Phan-Thanh and Gormon (42) reported that alkaline stress (pH 10.0, 5 min) of *L. monocytogenes* repressed 67% of protein spots otherwise detected in control cells on two dimensional sodium dodecyl sulfate-polyacrylamide gels. However, among 254 protein spots that were resolved in alkaline-stressed cells, 11 novel proteins were revealed and 16 others were up-regulated from two- to fourteen-fold. Cycloserine inhibits peptidoglycan synthesis by inhibiting the enzymes involved in synthesis of the pentapeptide side chains, whereas chloramphenicol inhibits protein synthesis by combining with the 50S subunit ribosome and blocking the associated transpeptidation and translocation functions (41). Rifampicin blocks initiation, but not ongoing mRNA synthesis by binding to the β-subunit of DNA-dependent RNA polymerase (37). The presence of cycloserine-D or rifampicin in TPB during stress of *L. monocytogenes* at pH 12.0 resulted in less thermotolerant cells, but chloramphenicol reduced the thermotolerance even more noticeably. As with the acid tolerance response of *L. monocytogenes*, the synthesis of certain proteins probably associated with the cell wall or plasma membrane as a result of alkaline stress are necessary for survival and may be similar to heat shock proteins in that they confer thermotolerance to the cell.

Exposure of *L. monocytogenes* to alkaline stress may occur in a variety of situations that have implications to food safety. Goodson and Rowbury (25) stated that alkalinization of natural waters can be caused by run-off from naturally alkaline soils, but more likely occurs from the discharge of alkaline chemical wastes or highly ammoniacal agricultural slurries. Environmental exposure of *L. monocytogenes* to the pH levels shown to induce heat resistance in our study are more likely to occur as a result of the latter of the two mechanisms. Alkali-stressed *L. monocytogenes* could contaminate food by these vectors prior to even reaching the processing, production, and

distribution. Exposure of *L. monocytogenes* cells to alkaline stress in food processing facilities may occur repeatedly through the use of alkaline detergents and disinfectants routinely used to remove food residues from equipment and floors and for cleaning food contact surfaces. One of the most commonly used inorganic alkalies in the food industry is NaOH or caustic soda (12). Alkaline chemicals are also used to assist in lye peeling of fruits and vegetables and to clean and disinfect produce surfaces. Accumulation of these chemicals may occur in areas of food processing environments that also happen to favor survival and growth of *L. monocytogenes*. Routine sublethal exposure to alkaline chemicals may in fact induce stress responses that cross-protect the organism against heat and possibly other non-related stresses.

Cross contamination of foods with *L. monocytogenes* from environmental sources remains a critical issue in food product manufacture. It is reasonable to propose that alkali-stressed cells could survive otherwise lethal heat treatments and proliferate on foods during subsequent storage. Post-processing contamination of food with alkali-stressed *L. monocytogenes* cells may permit survival of the pathogen during reheating just prior to consumption. Similar scenarios could compromise the safety of minimally-processed, ready-to-eat, or heat-and-serve foods in retail, foodservice, or home settings where alkaline detergents and sanitizers are being used with increased frequency.

It is likely that the alkaline stress response of *L. monocytogenes* is transient, as has been shown in *E. coli* (47). When stressed cells are placed in a non-alkaline environment, they may revert to an original state of tolerance to secondary chemical or physical assault. Fortunately, no food is alkaline enough to induce the stress response and cross-protection to heat observed in our study. However, the duration of the alkaline stress response in *L. monocytogenes* upon exposure of near-neutral pH in situ and in food matrices needs to be determined.

The degree of chlorine injury of *L. monocytogenes* was assessed by plating cells onto non-selective (TPA) and selective agar media (TPAS). Demonstration of injury assured that subsequent heating studies would be performed on a range of stressed and

unstressed cells. Generally, within chlorine concentration, higher logistic $D_{56°C}$ values were calculated from populations of cells exposed to chlorine for 5 min as apposed to 10 min, indicating that longer exposure sensitizes cells to heat. However, chlorine treatment alone was also lethal to portions of the population, as shown in Table 6. Therefore, higher logistic $D_{56°C}$ values reported for cells previously exposed to 6.0 mg/L chlorine (Table 2.7) are probably not representative of the entire population. Other researchers' observations on *E. coli* O157:H7 subjected to chlorine stress revealed a decrease the heat tolerance (20). Our data suggest that oxidative stress from chlorine creates two subpopulations of *L. monocytogenes* differing in capacity to survive heating at 56°C. Bunduki *et al*. (7) proposed that in order for *L. monocytogenes* to repair from heat- or sanitizer-induced injury, cells require mRNA, protein synthesis, and oxidative phosphorylation. They further reported that the cell wall was not damaged by heat (56ºC for 20 min) or by a chlorine-based sanitizer (100 mg/L for 2 min). Whatever the site of injury, our data show that treating cells with chlorine for 10 min causes more rapid death during subsequent heating compared to the rate of inactivation of cells treated with chlorine for 5 min.

It should be noted that this study utilized planktonic cells of *L. monocytogenes* and that biofilm associated cells could have more closely resembled conditions in food processing environments. Also some of the pretreatments applied to cells, such as pH 12.0 for 45 min at 37°C, or 6.0 mg/L chlorine for 5 or 10 min, were in themselves lethal, and therefore, populations subsequently subjected to heating were not identical to controls. However, based upon heat survival parameter estimates of sublethal treatments, these data indicate that alkaline stress causes cells to become more tolerant to mild heating, and that chlorine sensitizes cells to heat.

Observations that chlorine stress creates two subpopulations of *L. monocytogenes* cells and that longer exposure time to chlorine sensitizes cells to subsequent heating warrants further investigations into chlorine-induced heat sensitivity and application to safe food production. Alkaline pH-induced cross protection of *L. monocytogenes* against

heat has potential to enhance survival in foods. Storage of food containing by alkali-adapted *L. monocytogenes* at temperatures as low as 4°C may permit growth and increased risk of illness. Further studies should be conducted to define the behavior of alkali- and chlorine-stressed *L. monocytogenes* on foods and in food processing environments.

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CHAPTER 3

SURVIVAL OF *LISTERIA MONOCYTOGENES* IN FOOD PROCESSING EQUIPMENT CLEANING SOLUTIONS AND SUBSEQUENT SENSITIVITY TO SANITIZERS AND HEAT[1]

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SUMMARY

Aims: To determine the ability of *Listeria monocytogenes* to survive exposure to commercial food processing equipment cleaning solutions and subsequent treatment with sanitizers or heat.

Methods and Results: Cells of five strains of *L. monocytogenes* were suspended in 1% solutions of eight commercial cleaners (pH 7.1 – 12.5) or in water (control), and incubated at 4ºC for 30 min or 48 h before populations were determined by plating on tryptose phosphate agar. After exposure of cells to cleaning solutions for 30 min, populations of the most resistant strain of *L. monocytogenes* were reduced by ≤ 1.63 $\log_{10}$ cfu ml^{-1}. In only three highly alkaline cleaning solutions (pH 11.6 – 12.4) were populations reduced significantly ($P \leq 0.05$) compared to reductions in water. After 48 h, populations were significantly higher in one cleaning solution (pH 10.4) than in water, while populations in six of the other seven cleaning solutions were reduced by ≥ 4.72 $\log_{10}$ cfu ml^{-1}. Cells exposed to cleaning solutions for 30 min became sensitive to 4.0 or 6.0 mg l^{-1} free chlorine, and to 50 or 100 ppm benzalkonium chloride and cetylpyridinium chloride, common components of quaternary ammonium sanitizers. Cells exposed to seven of the eight test cleaners had $D_{56°C}$ values less than or equal to those of the control cells.

Conclusions: *L. monocytogenes* tolerates exposure to a high concentration of alkaline pH cleaning solutions but, consequently, becomes sensitized to sanitizers.

Significance and Impact of the Study: Elimination of *L. monocytogenes* surviving exposure to alkaline cleaning solutions widely used for food processing equipment is essential, and the appropriate use of sanitizers for subsequent application to equipment is important in achieving this goal.

INTRODUCTION

Human listeriosis is characterized by encephalitis, meningitis, or sepsis. Pregnant women infected by *Listeria monocytogenes* may exhibit flulike symptoms, while unborn children may succumb to neonatal septicemia or meningitis and become spontaneously aborted, typically during or after the third trimester (Rocourt and Cossart 1997). Among neonates and immunocompromised adults, mortality rates often exceed 30%, but the mortality rate among the general population is closer to 20% (Gellin and Broome 1989). Among affected non-pregnant adults, most have some unrelated underlying condition that predisposes them to consequences of infection (Rocourt and Cossart 1997). It is probable that the general public is frequently exposed to low levels of *L. monocytogenes* by consumption of various foods (WHO Working Group 1988; Farber *et al*. 1989; Pinner *et al*. 1992; Beuchat 1996). Mead *et al*. (1999) estimated that *L. monocytogenes* was responsible for 3.8 % of the total cases of food-borne illness requiring hospitalization, and 27.6% of deaths related to food-borne illness in the United States.

Despite research showing lethality of sanitizers to *L. monocytogenes* (Mustapha and Liewen 1989; Best *et al*. 1990; Tuncan 1993), the pathogen is occasionally isolated from food processing environments, even following cleaning and sanitizing of equipment surfaces (Franco *et al*. 1999; Ojeniyi *et al*. 1996; Samelis and Metaxopoulos 1999). The frequency of food product recalls due to contamination with *L. monocytogenes* (Wong *et al*. 2000) attests to the ability of the organism to persist in food processing environments despite sanitation measures. Chemical cleaners used to remove food residues from these environments are not formulated to sanitize, but may sensitize *L. monocytogenes* to subsequent exposure to sanitizers.

Research has been done to evaluate sanitizers for their efficacy in killing planktonic and biofilm-associated cells of *L. monocytogenes* (Mustapha and Liewen 1989; Best *et al*. 1990; Sallam and Donnelly 1992; Ren and Frank 1993; Roy *et al*. 1993; Tuncan 1993). However, less work has been done to define the effects of alkaline cleaners and detergents on biofilm-associated *L. monocytogenes* (Krysinski *et al*. 1992).

Studies describing the ability of cleaners and sanitizers to remove cells of *L. monocytogenes* on surfaces typically found in food processing environments have been reported (Krysinski *et al*. 1992; Mosteller and Bishop 1993; Frank and Chmielewski 1997); however, studies describing the fate of cells dislodged from surfaces are lacking.

We undertook a study to determine the ability of *L. monocytogenes* to survive in 1% solutions of cleaners used in the food processing industry as well as its sensitivity to sanitizers and heat after exposure to cleaning solutions. The study was performed to assess the ability of *L. monocytogenes* isolated from food processing environments to survive at refrigeration temperature in cleaning solutions ranging from pH 7.1 to 12.6. Experiments were also performed to compare efficacy of sanitizers in killing *L. monocytogenes* after exposure to cleaning solutions. An additional study examined heat tolerance of cells that had been exposed to cleaning solutions. This research was designed to provide information to enable a greater understanding of the ability of *L. monocytogenes* to survive stresses associated with cleaning and sanitizing food processing environments and the effect of exposure to cleaners on subsequent thermotolerance of the pathogen.

MATERIALS AND METHODS

Strains and culture conditions

Five isolates of *L. monocytogenes* were used. Four were serotype 4b with flagellar antigen ABC (strains CFS 1, 3, 4, and 5) and one was serotype 1/2a with flagellar antigen AB (strain CFS 2). All were isolates from a food processing environment. Stock cultures were prepared from subcultures grown in tryptose phosphate broth (TPB, pH 7.3) (Difco Laboratories, Detroit, MI, USA). Cultures were incubated at 37°C for 24 h, then supplemented (15%, v/v) with glycerol and stored in cryogenic vials (Nalgene, Rochester, NY, USA) at −20°C until used.

Prior to each experiment, a stock culture was thawed and loop inocula were transferred to Erlenmeyer flasks (250 ml) containing 100 ml of sterile TPB. Flasks were

incubated for 18 h in an incubator shaker (New Brunswick Scientific, New Brunswick, NJ, USA) set at 37°C and 200 rpm. Cultures (10 ml) were dispensed into 15-ml conical polystyrene centrifuge tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and centrifuged (5,000 x *g*, 4°C) for 10 min (brake 3.5 min) in a pre-cooled Marathon 12KBR benchtop refrigerated centrifuge (Fisher Scientific, Pittsburgh, PA, USA). Pellets washed twice in sterile distilled water (water) were used as inocula for test cleaning solutions.

Exposure to cleaner solutions

Eight commercially available food processing equipment cleaners were selected for evaluation based on their range in pH, intended usage in food processing environments, differences in chemical composition, and variability of recommended application procedures (Table 3.1). Cleaners 1, 2, 3, and 4 were supplied by manufacturer A as 1% solutions and cleaners 5, 6, 7, and 8 were supplied by manufacturer B as concentrated liquids that were diluted with sterile deionized water to 1% (v/v) in our laboratory. Each product has a range of manufacturer-recommended use concentrations. The recommended use concentrations of cleaners 4 and 7 vary, depending on the manner of application. Some of the cleaning products were evaluated at concentrations higher or lower than those recommended by the manufacturer for the purpose of simulating misuse in a food processing environment.

Washed pellets were suspended in 10 ml of 1% solutions of cleaners or water (control) at 4°C to give a population of ca. 9.4 $\log_{10}$ cfu ml^{-1}, and suspensions were stored in tubes at 4°C. After 30 min or 48 h, the content of each test tube was thoroughly mixed using a vortex mixer, and undiluted suspensions (0.25 ml in quadruplicate or 0.1 ml in duplicate) or suspensions serially diluted in sterile 0.1 % peptone water (0.1 ml in duplicate) were surface plated on tryptose phosphate agar (TPA, pH 7.3) (Difco) and on TPA supplemented with 4% NaCl (TPAS) in order to assess total and uninjured populations of *L. monocytogenes*, respectively. All plates

Table 3.1 Properties of alkaline cleaners

Manufa -cturer	Cleaner No.	Product description	Properties*	Components†	Concentrations‡	
					Recommended (%)	At-use ratio
A	1	General purpose cleaner and foam additive	pH 7.8 at 100% pH 7.6 (7.1)* at 1%	5 - 20% anionic surfactants	0.13% in warm water	7.69
	2	Heavy-duty liquid alkaline detergent for CIP, bottlewashing, and boil out applications	pH 12.5 (12.4)* at 1% pH 11.7 at 0.1%	NaOH (caustic soda) 49%	Variable with application 0.25 – 1.5%	4.00 – 0.667
	3	Alkaline detergent for manual cleaning of bulk raw and pasteurized tanks, transfer lines, pumps, and associated equipment	pH 9.5 (8.4)* at 1%	Sodium tripolyphosphate 55%, sodium dodecylbenzenesulfonate 10%	0.19 - 0.75% in 21 - 88°C water	5.26 – 1.33
	4	Low alkaline foaming cleaner with solvents for manual or foam cleaning of floors and walls or maintenance areas	pH 10.8 at 2% pH 10.4 at 1%*	Nonionic surfactants 8%, sodium linear alkyl benezene sulfonate 3%, propylene glycol monomethyl ether 1 - 5%, dipropylene glycol methyl ether 1 - 5%, diethylene glycol methyl ether 3%, unspecified sequestering agent 1 - 5%	Foam cleaning: 2.34 - 4.68 % Manual cleaning: 0.39 – 1.56 % Spray cleaning: 0.78 – 2.34 %	Foam cleaning: 0.427 – 0.214 Manual cleaning: 2.56 – 0.641 Spray cleaning: 1.28 – 0.427

* pH of 1% solutions determined in laboratory

† Components listed are only those which are proprietarily insensitive or are disclosed as hazardous components on Material Safety Data Sheets.

‡ For consistency, concentrations are listed as percentages based on recommendations by manufacturers, which may refer to dilution of concentrated liquid or granulated powder in water. At-use ratio concentrations indicate test concentration (1%) divided by recommended concentrations.

Table 3.1 (cont.) Properties of alkaline cleaners

Manufa -cturer	Cleaner No.	Product description	Properties*	Components†	Concentrations‡	
					Recommended (%)	At-use ratio
B	5	Chlorinated, foaming, highly alkaline cleaner many surfaces in food processing environments	pH 13.4 concentrated pH 12.2 at 1%*	KOH, NaOH, NaOCl	2.34 – 3.14 %	0.427 – 0.318
	6	Non-butyl alkaline cleaner for many surfaces in food processing and preparation environments	pH 13.2 concentrated pH 11.6 at 1%*	KOH, sodium metasilicate	1.57 – 3.14%	0.637 – 0.318
	7	Non-butyl heavy-duty alkaline cleaner and degreaser	pH 13.2 concentrated pH 11.6 at 1%*	KOH, sodium metasilicate, NaOCl	General cleaning: 0.78 – 3.14% Foam cleaning: 1.57 – 3.14% Pressure cleaning: 1.57 – 3.14%	General cleaning: 1.28 – 0.318 Foam cleaning: 6.37 – 0.318 Pressure cleaning: 6.37 – 0.318
	8	Heavy-duty foaming alkaline cleaner	pH 13.4 concentrated pH 12.6 at 1%*	NaOH	5% - 10%	0.2 – 0.1

* pH of 1% solutions determined in laboratory

† Components listed are only those which are proprietarily insensitive or are disclosed as hazardous components on Material Safety Data Sheets.

‡ For consistency, concentrations are listed as percentages based on recommendations by manufacturers, which may refer to dilution of concentrated liquid or granulated powder in water. At-use ratio concentrations indicate test concentration (1%) divided by recommended concentrations.

were incubated at 37°C for 48 h prior to counting colony forming units (cfu) using a Countermat automated colony counter (Cogent Technologies, Cincinnati, OH, USA) and a manufacturer-recommended modification of the pcount01 file. Suspensions of the five isolates (strains CFS 1 – CFS 5) were combined to form an inoculum for further experiments.

Treatment with sanitizers

Selection of sanitizers for evaluation in this experiment was based upon their frequency of use in food processing environments and lack of published information on their effects on the viability of *L. monocytogenes* after exposure to cleaning solutions. Sodium hypochlorite (NaOCl) solution (minimum 4% available chlorine) (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) was used to prepare solutions with 4.0 and 6.0 mg l^{-1} available chlorine by diluting with 0.05 M potassium phosphate buffer at pH 7.00 ± 0.05 (PB). Concentrations were verified using a Hach Digital Titrator (model 16900; Hach Company, Loveland, CO, USA) fitted with a 0.0451 N phenylarsine oxide titration cartridge, an Amperometric Digital Titrator model 19300, and a TitraStir™ stir plate following a forward titration procedure for determining concentrations of free chlorine ranging from 0 to 10 mg l^{-1}.

Benzalkonium chloride (Sigma Chemical Co., St. Louis, MO, USA) and cetylpyridinium chloride (Sigma) were selected as chemicals to treat cells after exposure to cleaning solutions because they are major components of some quaternary ammonium sanitizers commonly applied to surfaces in food processing environments after cleaning. Stock solutions were prepared by dissolving 100 mg in 10 ml of water. Solutions containing 50 (pH 6.6) and 100 (pH 6.7) mg l^{-1} benzalkonium chloride and 50 (pH 6.6) and 100 (pH 6.4) mg l^{-1} cetylpyridinium chloride were used to treat *L. monocytogenes* cells. All chemical treatment solutions and PB used to prepare chlorine solutions were prepared using chlorine demand-free glassware and from filtered (0.45 μm), laboratory-

grade, sterile water with a total hardness < 10 mg l^{-1} $CaCO_3$. Solutions were protected from light, held at 21 ± 2°C, and used within 1 h of preparation.

Eighteen-hour TPB cultures (2 ml) of each of the five *L. monocytogenes* isolates were combined and centrifuged at 5,000 x *g* and 4°C for 10 min with a 3.5-min brake time. Each cell pellet was resuspended in 10 ml of water (control) or cleaner solution and incubated for 30 min at 4°C. After incubation, cells were centrifuged, washed, and resuspended in water prior to treatment with sanitizers. One milliliter of each washed cell suspension was dispensed into 99 ml of each sanitizer treatment solution at 25°C and thoroughly mixed by vortexing. After 30 s, 1 ml of treated cell suspension was dispensed into 9 ml of sterile Dey-Engley neutralizing broth (Difco) and mixed for 10 s. Undiluted, neutralized treatment suspensions (0.25 ml in quadruplicate or 0.1 ml in duplicate) or suspensions serially diluted in sterile 0.1 % peptone water (0.1 ml in duplicate) were surface plated on TPA. TPA plates were incubated at 37°C for 48 h before counting colonies as described above. Log populations of cells recovered on TPA after exposure to water (control) or cleaning solutions for 30 min and treatment with either PB or water (sanitizer treatment controls) for 30 s were subtracted from log populations recovered following exposure to water (control) or cleaning solutions and treatment with chlorine, benzalkonium chloride, or cetylpyridinium chloride to generate population reduction values.

Treatment with heat

Suspensions (50 µl) of cells previously exposed to cleaning solutions were injected into Kimax-51 capillary tubes (0.8 – 1.0 mm I.D. x 90 mm long, no. 34507-99; Kimble, Vineland, NJ, USA), and the ends were flame sealed. Capillary tubes were brought to 21 ± 2°C before subjecting to heat treatment in a water bath at 56°C for 0, 1, 2, 5, 10, 20, or 25 min. The come-up time for a tempered fluid-filled capillary tube in the water bath, as measured with a Microprocessor Thermometer (model HH23, Omega, Stamford, CT,

USA) connected with a type J thermocouple, was 2 sec. Capillary tubes were immediately cooled and sanitized by immersing in succession, in an ice bath, 70% ethanol, and sterile water before aseptically transferring to screw-capped test tubes (16 mm I.D. x 125 mm long) containing 5 ml of sterile 0.1% peptone water. The capillary tube containing the heated cell suspension was crushed using a sterile glass rod and thoroughly mixed with the peptone water. Undiluted suspensions (0.25 ml in quadruplicate or 0.1 ml in duplicate) or suspensions serially diluted in sterile 0.1 % peptone water (0.1 ml in duplicate) were surface plated on TPA. TPA plates were incubated at 37°C for 48 h before colonies were counted as described above.

Statistical analysis

Three replicates of each experiment were performed. Population means were calculated and analyzed using the general linear model procedure of SAS (SAS, Cary, NC, USA) and separated by significance ($P \leq 0.05$) using the Duncan's multiple range test. Populations of *L. monocytogenes* surviving heat treatment were analyzed using appropriate forms of the logistic equation (Pruitt and Kamau, 1993) applied by the nonlinear regression procedure of SAS. The log-transformed equations used to analyze data were as follows:

$$\log S = \log 2 - \log [1 + e(\beta t)] \quad (1)$$

where log S is the log (cfu/cfu_0) at any given time (t) and β is the maximum specific death rate.

For survival curves with no initial lag in killing but having two distinct killing phases (biphasic), data were fitted to the following two-term exponential form of equation 1:

$$\log S = \log (\{2f_1/[1 + e(\beta_1 t)]\} + \{2(1 - f_1) / [1 + e(\beta_2 t)]\}) \quad (2)$$

where f_1 and $(1 - f_1)$ represent two fractions of cells (differing with respect to heat resistance) and β_1 and β_2 are the specific killing rates for the two fractions, respectively.

The assumption of this model is that two fractions (subpopulations) are killed exponentially but at different, independent rates.

Curves which included a lag in killing (shoulder) and biphasic inactivation were fitted to the following two-term exponential form of equation 1:

$$\log S = \log (f_1\{1 + e[-\beta_1 t_1]\} / \{1 + e[\beta_1(t - t_1)]\}) + \log ([1 - f_1]\{1 + e[-\beta_2 t_1]\} / \{1 + e[\beta_2(t - t_1)]\}) \quad (3)$$

where t_1 is the lag period.

For equation 1, logistic D values (Pruitt and Kamau, 1993) were calculated as D = 2.94/β, and for equations 2 and 3, D = ln(19)/β_2.

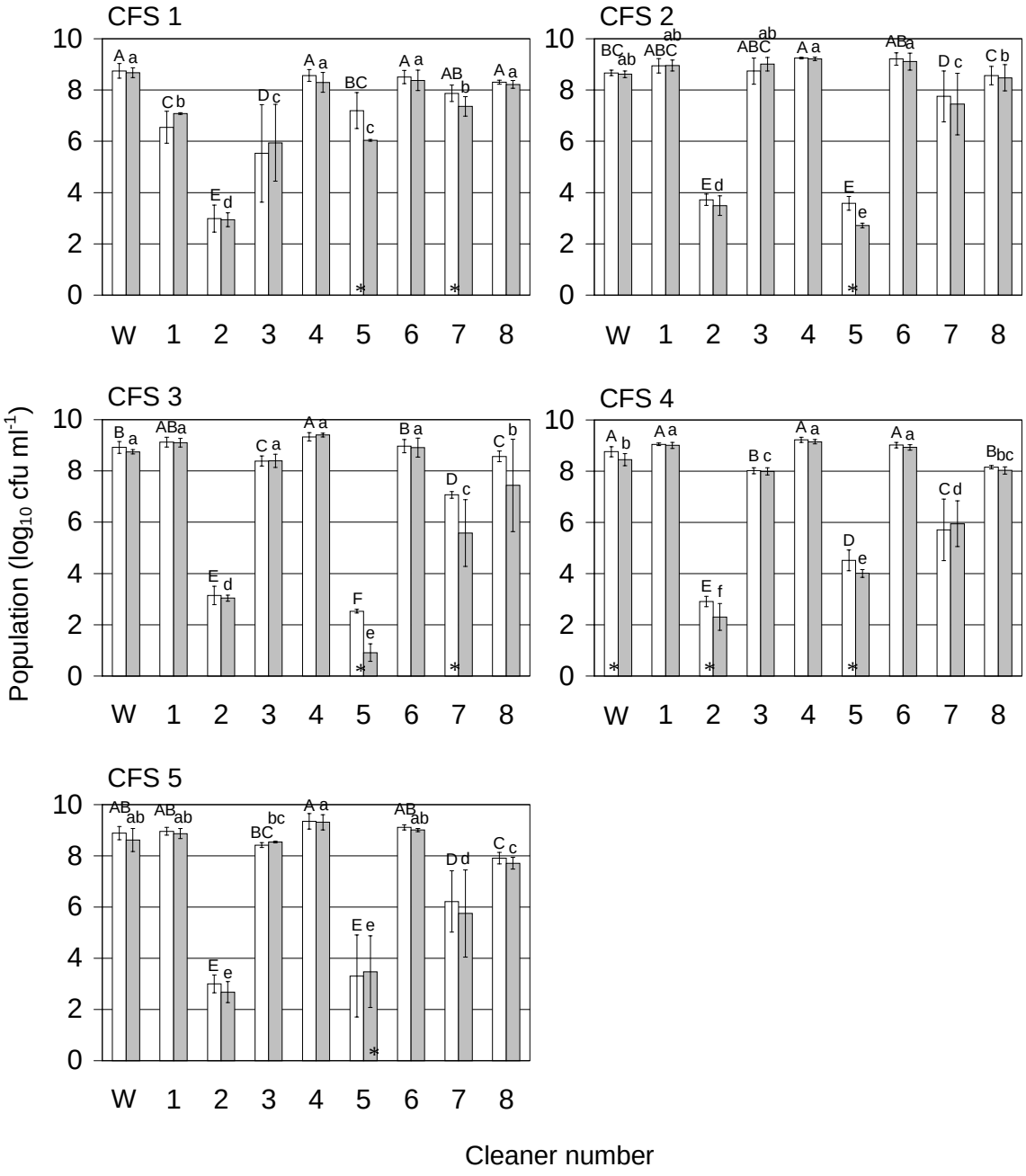
RESULTS

Populations of *L. monocytogenes* surviving exposure to cleaner solutions

Survival of *L. monocytogenes* isolates (CFS 1 – CFS 5) suspended in solutions of 1% cleaners or water (control) for 30 min at 4°C were compared (Figure 3.1). Cleaners 2 and 5 (pH 12.4 and 12.2 at 1%, respectively) were consistently more lethal than the other cleaners to isolates CFS 1 – CFS 4, although isolate CFS 1 survived better than other isolates after exposure to cleaner 5. With the exceptions of cleaners 2 and 5, exposure of isolate CFS 2 (the only 1/2a serotype) to cleaners reduced populations of *L. monocytogenes* by ≤ 1.63 $\log_{10}$ cfu ml^{-1}; in only three cleaning solutions (2, 5, and 7 at pH 11.6 – 12.4) were populations significantly ($P \leq 0.05$) less than those recovered from the control (water). Populations of isolates CFS 1 – CFS 5 surviving exposure to cleaning solutions 4 and 6 (pH 10.4 and 11.6, respectively) were not significantly less than those surviving exposure to water. Significantly lower numbers of *L. monocytogenes* were recovered on TPAS compared to TPA following exposure to cleaners 2, 5, and 7, indicating some degree of cell injury.

After 48 h at 4°C, populations of isolates CFS 2 – CFS 5 were significantly higher in a 1% solution of cleaner 4 (pH 10.4) than in water, while populations in 1%

Figure 3.1 Populations of isolates (CFS 1 – CFS 5) of *Listeria monocytogenes* from a food processing environment surviving incubation in 1% solutions of eight food processing equipment cleaners (described in Table 1) and water (W, control) at 4°C for 30 min as determined by plating inoculated solutions on TPA (open bars) and TPAS (shaded bars). Brackets on each bar denote standard deviations. Within strain and medium, bars not noted with the same letter are significantly ($P \leq 0.05$) different. Within strain and cleaner, bars noted by an asterisk are significantly greater.


CFS 1
CFS 2
CFS 3
CFS 4
CFS 5
Population (log10 cfu ml-1)
Cleaner number
W 1 2 3 4 5 6 7 8
0 2 4 6 8 10

solutions of all other cleaners except cleaner 6 (pH 11.6) were reduced by at least 4.72 $\log_{10}$ cfu ml^{-1} (Figure 3.2). A large percentage of cells of these isolates were injured following exposure to cleaner 6. Survival of isolate CFS 1 was slightly different than survival of isolates CFS 2 – CFS 5; populations of CFS 1 were significantly reduced by exposure to cleaner 4 and injury was not evident after exposure to cleaner 6. However, response of isolate CFS 1 to cleaners 2, 5, 7, and 8 was similar to that of the other isolates. The magnitude of reduction in population of *L. monocytogenes* was, overall, not correlated with at-use ratio concentrations relative to the concentrations recommended by the manufacturers (Table 3.1). Since 1% solutions of cleaners 2, 5, and 7 caused large reductions in populations of individual isolates of *L. monocytogenes* exposed at 4°C for 30 min, relative to other solutions of other cleaners, subsequent sensitivity to sanitizers or thermotolerance of those cells was not determined.

Effectiveness of sanitizers on cells previously exposed to cleaning solutions

Treatment of cells previously exposed to 1% solutions of cleaners 1, 3, 4, 6, and 8 with 4.0 or 6.0 mg l^{-1} free chlorine was lethal to all cells (Table 3.2). The numbers of cells previously exposed to water before treating with 4.0 or 6.0 mg l^{-1} chlorine were significantly reduced by 6.95 and 6.66 $\log_{10}$ cfu ml^{-1}, respectively. Treatment with buffer resulted in a 1.07 $\log_{10}$ cfu ml^{-1} reduction in population of cells previously exposed to a 1% solution of cleaner 8. Cells previously exposed to cleaner solutions were also highly sensitive to treatment with 50 and 100 mg l^{-1} benzalkonium chloride and cetylpyridinium chloride; treatment with these compounds caused significantly greater reductions than treatment with water. Populations of cells not previously exposed to cleaning solutions (control cells) were reduced by treatment with 50 and 100 mg l^{-1} benzalkonium chloride by 3.48 and 4.80 $\log_{10}$ cfu ml^{-1}, respectively, while treatment with 50 and 100 mg l^{-1} cetylpyridinium chloride reduced populations by 5.64 and 7.23 $\log_{10}$ cfu ml^{-1}, respectively. Performing this experiment using water of standard hardness (200 mg l^{-1} $CaCO_3$) to prepare sanitizer solutions gave similar results.

Figure 3.2 Populations of isolates (CFS 1 – CFS 5) of *Listeria monocytogenes* from a food processing environment surviving incubation in 1% solutions of eight food processing equipment cleaners (described in Table 1) and water (W, control) at 4°C for 48 h as determined by plating inoculated solutions on TPA (open bars) and TPAS (shaded bars). Brackets on each bar denote standard deviations. Within strain and medium, bars not noted with the same letters are significantly ($P \leq 0.05$) different. Within strain and cleaner, bars noted by an asterisk are significantly greater.

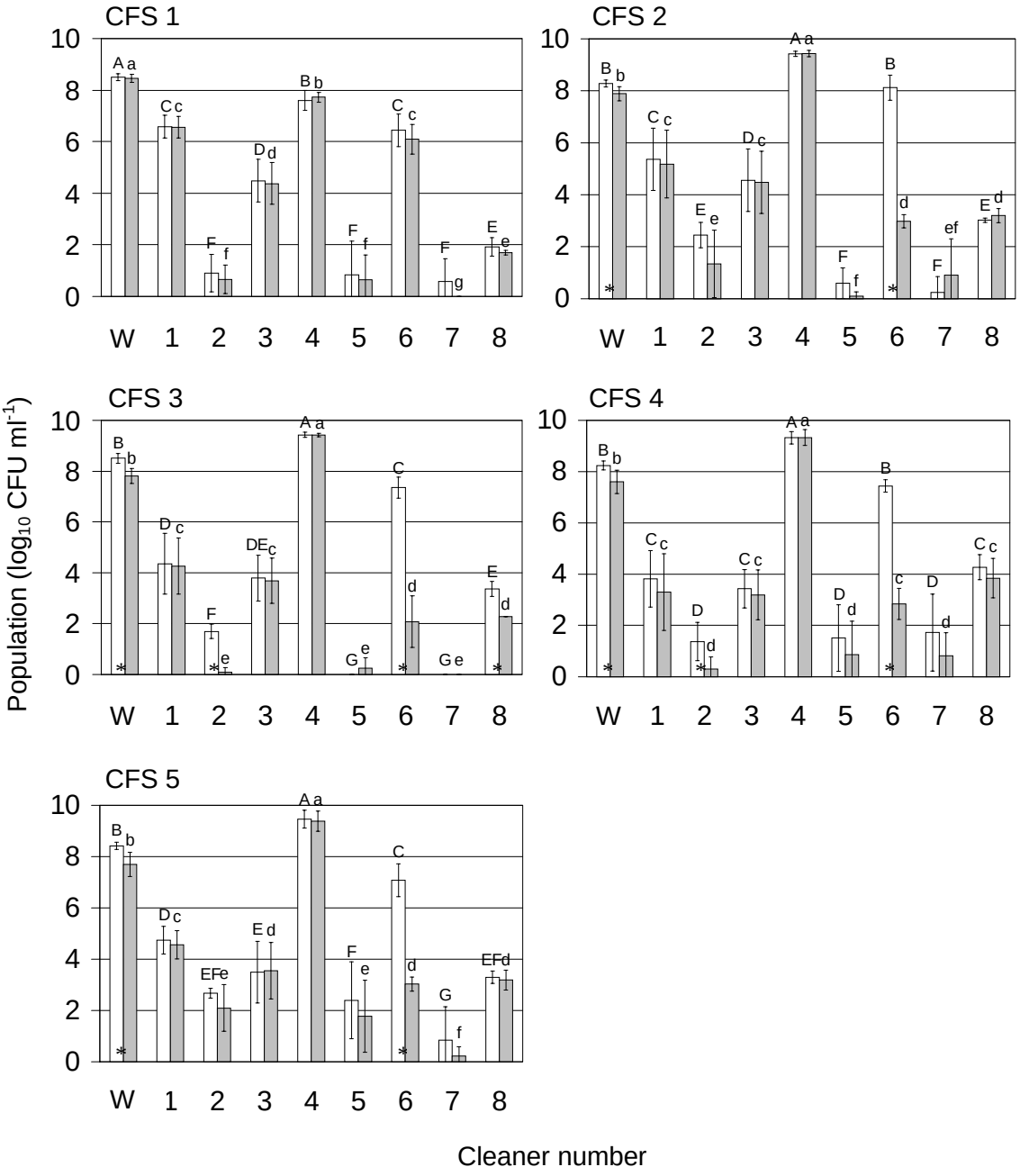

CFS 1
CFS 2
CFS 3
CFS 4
CFS 5
Population (log10 CFU ml-1)
Cleaner number
W 1 2 3 4 5 6 7 8
0 2 4 6 8 10

Table 3.2 Effectiveness of sanitizer treatments on *L. monocytogenes* cells surviving exposure to 1% solutions of various commercial cleaning solutions∗

Treatment†		Population reduction (log_{10} cfu ml^{-1})‡					
			Cleaner number				
Solution	Conc. (mg l^{-1})	Water (control)	1	3	4	6	8
Phosphate buffer (control)		e 0.00 e	b 0.33 c	b 0.82 b	b 0.10 de	c 0.24 cd	b 1.07 a
Chlorine	4.0	a 6.95 b	a 7.28 a	a 7.28 a	a 7.28 a	a 7.28 a	a 7.28 a
	6.0	a 6.66 b	a 7.28 a	a 7.28 a	a 7.28 a	a 7.28 a	a 7.28 a
Water (control)		e 0.00 d	b 0.53 c	b 0.96 b	b 0.33 c	b 0.45 c	b 1.26 a
Benzalkonium chloride	50	d 3.48 b	a 7.56 a	a 7.56 a	a 7.56 a	a 7.56 a	a 7.56 a
	100	c 4.80 b	a 7.23 a	a 7.56 a	a 7.56 a	a 7.56 a	a 7.56 a
Cetylpyridinium chloride	50	b 5.64 b	a 7.56 a	a 7.56 a	a 7.56 a	a 7.56 a	a 7.56 a
	100	a 7.23 b	a 7.23 b	a 7.56 a	a 7.56 a	a 7.56 a	a 7.56 a

∗ Cells were suspended in water (control) or 1% solutions of cleaners (described in Table 1) and incubated for 30 min at 4°C prior to exposure to treatments.

† Treatment consisted of dispensing 1 ml of a cell suspension previously exposed to cleaning solutions into 99 ml of treatment solution at 25°C, incubating 30 s, transferring 1 ml to 9 ml of neutralizing broth, and then surface plating on TPA.

‡ Values in the same column not preceded by the same letter are significantly ($P \leq 0.05$) different. Values in the same row not followed by the same letter are significantly different.

Thermotolerance of cells previously exposed to cleaning solutions

Logistic $D_{56°C}$ values indicate that previous exposure of cells of *L. monocytogenes* to cleaning solutions altered tolerance to heat treatment at 56°C (Table 3.3). $D_{56°C}$ values of cells previously exposed to cleaners 3 and 4 were 6.67 and 6.97 min, respectively, and were significantly lower than the $D_{56°C}$ value of control cells or cells previously exposed to cleaners 6 or 8. $D_{56°C}$ values of cells exposed to cleaners 1 and 6 were not significantly different from the $D_{56°C}$ value of control cells. Although the $D_{56°C}$ value of cells exposed to cleaner 8 was significantly higher than that of the control, the r^2 indicates a less than optimal fit of the equation to the survival curve.

DISCUSSION

Typical cleaning processes in food processing environments involve a series of steps, each with a specific purpose (Tamplin 1980). A pre-rinse is performed using water to remove gross, loose soil, followed by a cleaning step using suitable detergents (often alkaline) for removal of residual soils. An inter-rinse then removes all detergent and soil, and sanitizing is performed by application of antimicrobial chemicals, either with or without heat. Finally, a post rinse using potable water removes the antimicrobial chemicals. The effectiveness of sanitizers may be influenced by numerous factors, including the presence of residuals from cleaners not properly removed before application of antimicrobials.

The ability of *L. monocytogenes* to survive exposure to alkaline pH cleaner solutions for 30 min at 4°C, and to a lesser extent for 48 h, was demonstrated. Other studies have also revealed that *L. monocytogenes* can survive rather well in high pH environments (Rossmoore and Drenzek 1990; Laird *et al*. 1991; Cheroutre-Vialette *et al*. 1998; Taormina and Beuchat 2001). These survival capabilities may help explain why the pathogen is difficult to eradicate from food processing environments treated with

Table 3.3 Heat (56°C) survival parameter estimates for *L. monocytogenes* cells previously incubated at 4°C for 30 min in 1% cleaner solutions.

Cleaner∗	Eqn.†	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 ‡	Logistic $D_{56°C}$ value (min)§
Water (control)	1	-	-	-	0.278 ± 0.0145	0.970	10.58 b
1	2	0.13	0.322 ± 0.0115	4.24 ± 2.13	-	0.990	9.13 bc
3	2	0.13	0.441 ± 0.0147	2.74 ± 1.042	-	0.992	6.67 c
4	3	0.40	0.422 ± 0.0461	- 1.19 ± 0.375	-	0.956	6.97 c
6	1	-	-	-	0.282 ± 0.0120	0.978	10.43 b
8	2	0.50	0.191 ± 0.130	0.0939 ± 0.0235	-	0.884	15.40 a

∗ See Table 1 for description of cleaners

† Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression. See text for equations.

‡ Calculated from 1 – (residual sum of squares / corrected total).

§ Calculated per replicate from $D = 2.94/\beta$ for equation A, or $D = (\ln 19)/\beta_2$ for equation B. Means not followed by the same letter are significantly ($P \leq 0.05$) different.

alkaline pH cleaning products. Although commercial food processing equipment cleaners are not intended to be lethal to *L. monocytogenes* cells, they may, inadvertently, through detergency, relocate cells from equipment to other areas within the processing environment that do not receive sanitization. These cells may survive exposure to high-pH cleaners and be transferred to sanitized surfaces where they may contaminate food.

The alkaline cleaning solutions used in this study exhibited various degrees of lethality against *L. monocytogenes* within 30 min of treatment. This observation is useful when seeking to add yet another hurdle to control *L. monocytogenes* in food processing environments. Cleaners 4 and 6 permitted survival of significantly higher populations of individual isolates of *L. monocytogenes* after 48 h compared to the other six cleaners. This indicates that any potentially lethal component that may be present in solutions prepared from these cleaners was at an ineffective concentration in solutions of cleaners 4 and 6. All cleaners were evaluated at a concentration of 1% because some of the products were provided to our laboratory in liquid form at that concentration. While some of the cleaners are recommended for use at higher or lower concentrations, a uniform at-use concentration of 1% was selected regardless of concentration of active component (Table 3.1). Cleaners 1 and 3 are recommended for use by the manufacturer at concentrations lower than 1%, but exposure of *L. monocytogenes* to these cleaners at 1% in practice could result from evaporation of water after application, egregious mixing, or dispensing errors. Cleaners 5, 6, and 8, are recommended for use by the manufacturer at concentrations higher than 1%, and are more likely to occur in food processing environments at 1% as a result of dilution in the sanitizing process. The at-use concentration (1%) of cleaners 2, 4, and 7 was within the range of manufacturer recommended concentrations for use for at least one intended application, and was otherwise lower than the recommended concentration for other applications. The effectiveness of treatment with 1% cleaning solutions must be assessed relative to concentrations recommended by manufacturers. If all cleaners had been tested at recommended concentrations, results may have been different. Nevertheless, no overall

relationship was evident between higher or lower at-use ratio concentrations and reduction in populations of *L. monocytogenes* exposed to the test cleaners. Cleaning solutions not lethal or having minimal lethality to most of the isolates of *L. monocytogenes* within 30 min of exposure were used to further assess the behavior of cleaner-exposed cells upon treatment with sanitizers or challenge with heat.

Cells exposed to cleaning solutions that had minimal effects on viability were sensitive to subsequent treatment with sanitizers. Similar observations on sanitizer sensitivity of chemically shocked *L. monocytogenes* cells were reported by Pickett and Murano (1996). Since treatment with 4.0 or 6.0 mg l^{-1} chlorine and 50 or 100 mg l^{-1} cetylpyridinium chloride reduced populations of control cells by ≥ 6.66 $\log_{10}$ cfu ml^{-1} and ≥ 5.64 $\log_{10}$ cfu ml^{-1}, respectively, the independent influence of prior exposure of cells to cleaning solutions on viability when subsequently treated with those particular sanitizers has not been determined. Although cells exposed to cleaning solutions were at least as sensitive as control cells to chlorine and cetylpyridinium chloride, the degree to which they were sensitized is masked by the high lethality of these sanitizers. Populations of control cells were so greatly reduced by treatment with these sanitizers that a comparison of sensitivity of control and cleaner-exposed cells upon subsequent exposure to the sanitizers is confounded. Increased sensitivity of *L. monocytogenes* to benzalkonium chloride after exposure to cleaning solutions is clearer since reduction in population of control cells (treated with water) was less pronounced than that observed in cells treated with chlorine or the same concentrations of cetylpyridinium chloride.

Previous studies in our laboratory revealed that *L. monocytogenes* can survive for 6 days at 4°C in TPB adjusted to pH 9.0, 10.0 or 11.0 with NaOH, while cells stored for 2 or 6 days at 4°C in TPB at pH 10 or shocked for 45 min at 37°C in TPB at pH 12.0 had increased tolerance to mild heating at 56°C and 56 or 59°C, respectively (Taormina and Beuchat, 2001). In the present study, heating of *L. monocytogenes* cells previously exposed for 30 min to commercial cleaning solutions (pH 7.1 – 12.6) resulted in significant differences among logistic $D_{56°C}$ values. $D_{56°C}$ values of cells exposed to

cleaning solutions 3 and 4 were significantly lower than the $D_{56°C}$ value of control cells, while cells previously exposed to a 1% solution of cleaner 8 had a significantly higher $D_{56°C}$ value. Interestingly, cleaner 8 is the only test cleaner containing only NaOH listed as a hazardous component.

Future research to determine survival characteristics of *L. monocytogenes* in manufacturer-recommended concentrations of cleaners at a range of temperatures and upon subsequent exposure to sanitizers in the presence and absence of organic matter at refrigeration temperatures would be of interest. Also, studies on *L. monocytogenes* in biofilms, preferably in mixed-species biofilms, would more accurately reflect actual conditions in food processing environments. Observations reported here provide important information on the potential effects of sanitation regimens on *L. monocytogenes* cells isolated from a food processing environment and underscores the need for strict adherence to cleaning and standard sanitation operating procedures to ensure foods do not become contaminated in post processing environments. Further investigations to characterize the effects of exposure of *L. monocytogenes* to alkaline cleaners and other alkaline environments are warranted. Of particular interest are the survival and growth characteristics of alkaline-stressed *L. monocytogenes* in ready-to-eat foods during refrigerated storage.

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CHAPTER 4

SURVIVAL AND GROWTH OF ALKALI-STRESSED *LISTERIA MONOCYTOGENES* ON BEEF FRANKFURTERS AND THERMOTOLERANCE IN FRANKFURTER EXUDATES[1]

[1]Taormina, P. J., and L. R. Beuchat. 2001. To be submitted to J. Food Prot.

ABSTRACT

Cells of *Listeria monocytogenes* exposed at 4°C to 1% solutions of two alkaline cleaners or alkali-adapted in tryptose phosphate broth (pH 10.0) at 37°C for 45 min, then 4°C for 48 h, were inoculated onto beef frankfurters containing high fat (16 g) and high sodium (550 mg) or low fat (8 g) and low sodium (250 mg) per 57-g serving. Frankfurters were surface-inoculated (2.0 $\log_{10}$ CFU/g), vacuum packaged, stored at –20, 4, or 12°C, and analyzed for populations at 2-day to 2-week intervals. No significant differences in populations of *L. monocytogenes* were observed on frankfurters stored at -20°C for up to 12 weeks. After storage at 4°C for 6 weeks (1 week before the end of shelf life), populations of control cells and cells exposed to alkaline cleaners were ca. 6.0 $\log_{10}$ CFU/g of low fat, low salt (LFLS) frankfurters and ca. 3.5 $\log_{10}$ CFU/g of high fat, high salt (HFHS) frankfurters, but growth of alkali-adapted cells on both types of frankfurters was retarded. Overall, growth on frankfurters stored at 12°C was more rapid but a delay in growth of alkali-adapted cells on HFHS frankfurters was evident after storage for 9 days and on LFLS frankfurters after 6 days. $D_{59°C}$ and $D_{62°C}$ values of control and alkali-stressed cells heated in exudates from the two frankfurter formulations were not significantly different. Growth characteristics of *L. monocytogenes* inoculated onto the surface of frankfurters may be altered by previous exposure to alkaline environments. Differences in growth characteristics of *L. monocytogenes* on HFHS versus LFLS beef frankfurters stored at refrigeration temperature indicate that composition influences the behavior of both alkaline stressed and control cells.

The psychrotolerant, Gram-positive bacterium, *Listeria monocytogenes*, is considered an opportunistic pathogen, yet human listeriosis is not infrequent nor does it lack severity. A 1999 study conducted by the Centers for Disease Control and Prevention estimated that 2,518 total cases of listeriosis occur each year in the U.S. (*12*). It was also estimated that 90% of the infected individuals require hospitalization. Contamination of processed, ready-to-eat foods as a result of exposure to processing environments is a concern. Understanding the behavior of *L. monocytogenes* in these environments and on foods, and using that information to predict the risk of listeriosis has become a public health priority.

Isolation of *L. monocytogenes* from floors, drains, cleaning aids, walls and ceilings, and critical control point areas in food processing facilities has been documented (*6, 13, 14, 20*). Moreover, the pathogen has been reported to persist in meat processing environments, even following rigorous cleaning and sanitization (*6, 16*). Such persistence, despite control measures, raises the need to investigate the influence of environmental stresses such as those that may result from chemical cleaners on subsequent survival and growth characteristics of *L. monocytogenes*. The presence of *L. monocytogenes* in processing environments renders post-processed foods at risk for contamination sometime prior to or during packaging. The influence, if any, exposure of *L. monocytogenes* to chemical cleaners has on subsequent behavior of the pathogen in foods has not been described.

Outbreaks of listeriosis have been associated with the consumption of frankfurters (*2, 3, 17*) even though a 3-log reduction of *L. monocytogenes* per gram of frankfurter should be achieved by thermal processing (*23*). Exposure of heat-processed frankfurters to the environment during peeling has been proposed as point of contamination with *L. monocytogenes* (*22*). Wang and Muriana (*21*) isolated *L. monocytogenes* from 7 out of 93 (7.5%) retail frankfurter samples and the pathogen has been reported to occur in other vacuum-packaged processes meats (*7*).

Survival of *L. monocytogenes* in food processing environments indicates that some cells may survive exposure to chemical cleaners and sanitizers. Surviving cells may be injured as a result of this exposure but subsequently resuscitate, contaminate processed meat products, and grow. Routine washing food processing equipment, floors, walls, or drains with water and application of cleaning chemicals, often with alkaline pH, is followed by application of sanitizers. The influence of alkaline pH on viability of *L. monocytogenes* and development of cross protection against other environmental stresses imposed by vacuum packaged frankfurters has not been reported. An objective of this research was to investigate the behavior of *L. monocytogenes* on frankfurters after exposure to two commercial alkaline cleaners used in meat processing environments. Survival and growth of these cells and alkali-adapted *L. monocytogenes* inoculated onto two types of frankfurters differing in fat and salt content, followed by storage at freezing, refrigeration, and mildly abusive temperatures, were studied. A second objective was to determine the thermotolerance of alkaline stressed *L. monocytogenes* cells suspended in frankfurter exudates.

MATERIALS AND METHODS

Strains and culture conditions. Five isolates of *L. monocytogenes* were used. Four were serotype 4b with flagellar antigen ABC and one was serotype 1/2a with flagellar antigen AB. All isolates were from a food processing environment. Stock cultures were prepared from subcultures grown in tryptose phosphate broth (TPB, pH 7.3) (Difco Laboratories, Detroit, Mich.). Cultures were incubated at 37°C for 24 h, then supplemented (15%, v/v) with glycerol and stored in cryogenic vials (Nalgene, Rochester, N.Y.) at −20°C until used.

Prior to each experiment, a stock culture of each isolate was thawed and loop inocula were transferred to individual Erlenmeyer flasks (250 ml) containing 100 ml of sterile TPB. Flasks were incubated for 18 h in an incubator shaker (New Brunswick Scientific, New Brunswick, N.J.) at 37°C and 200 rpm. Cultures of each isolate were

combined, dispensed (10 ml) into 15-ml conical polystyrene centrifuge tubes (Becton Dickinson Labware, Franklin Lakes, N.J.), and centrifuged (5,000 x *g*, 4°C) for 10 min (brake 3.5 min) in a pre-cooled Marathon benchtop refrigerated centrifuge (12KBR; HERMLE Labortechnik GmbH, Wehingen, Germany). Pellets washed twice in sterile distilled water (water) were subjected to alkaline cleaners or alkali-adaptation procedures.

Alkaline stressing and adapting of cells. Alkaline stress procedures were selected based on a previous study in our laboratory demonstrating survival of each of the five *L. mononcytogenes* isolates after exposure to alkaline solutions of commercial food processing equipment cleaners (*19*). The procedure for producing alkali-adapted cells was the same as that observed to result in cross protection of *L. monocytogenes* to heat inactivation described in another study (*18*). Pellets were resuspended in 10 ml of sterile distilled water (control), a 1% solution of a low foaming alkaline cleaner containing solvents (pH 10.4), or a 1% solution of a non-butyl alkaline cleaner (pH 11.6). These treatments are referred to as treatments A, B, and C, respectively in this paper. Cell suspensions were incubated at 4°C for 30 min. To produce alkali-adapted cells, referred to as cell treatment D, pellets were resuspended in 10 ml of TPB adjusted to pH 10.0 with 1 N NaOH and incubated at 37°C for 45 min and then 4°C for 48 h. Populations of treated cells were either adjusted to 3.36 $\log_{10}$ CFU/ml using sterile 0.1% peptone water and inoculated onto frankfurters, or centrifuged (5,000 x *g*, 4°C) for 10 min and resuspended in frankfurter exudates before being subjected to heat treatment.

Inoculation, storage, and sampling of frankfurters. Two types of all beef frankfurters (22 mm diameter x 120 mm long), one containing 16 g of fat and 550 mg of Na per 57-g serving and the other containing 8 g of fat and 250 mg of Na per 57-g serving were purchased from a retail grocer. These two formulations are referred to as HFHF and LFLS frankfurters, respectively, in the following text. Shelf life (use by) dates were ca. 7 weeks from the date of initiation of the experiment. Although frankfurters were manufactured by different companies, ingredient listings for both

products included, in order listed on the label, beef, water, dextrose, flavorings, salt, corn syrup (listed before salt on LFLS frankfurter label), sodium phosphates, sodium erythorbates, and sodium nitrite. Ingredients in LFLS frankfurters also included modified corn starch (listed before dextrose) and sodium citrate (listed before sodium nitrite).

The surface of packages was swabbed with 70% ethanol prior to opening. Frankfurters (4°C) were removed from packages with sterile tongs and placed (two each) into preformed packages (20 x 20 cm) made from film (T4035B, 3.5 mils, OTR 4 $cc/cm^2/24$ h; Cryovac, Duncan, S.C.) commonly used to package frankfurters. Inocula (200 μl) consisting of 0.1% peptone water suspensions of control cells, cells exposed to a alkaline cleaner containing solvents, cells exposed to a non-butyl alkaline cleaner, and alkali-adapted cells of *L. monocytogenes* (treatments A, B, C, and D, respectively) were applied to the surfaces of both types of frankfurters to achieve 2.0 $\log_{10}$ CFU/g. Frankfurters in each package were rotated three times to distribute the inocula over the surfaces. Inocluated frankfurters placed side by side in packages were sealed under vacuum (negative pressure of 101,310 Pa in package) using a Koch vacuum packaging machine (model X200; Koch, Kansas City, Mo.).

Sealed packages of inoculated frankfurters were stored at –20, 4, or 12°C, and samples were removed from storage and analyzed for populations or presence of *L. monocytogenes* at 2-day to 2-week intervals, depending on the storage temperature. Maximum storage times for frankfurters stored at –20, 4, and 12°C were 12, 10, and 4 weeks, respectively. Additionally, inoculated frankfurters that had been stored at -20°C for 48 h and then stored at 12°C after the packages had been opened were monitored for populations of *L. monocytogenes* for up to 10 days. In either case, the surfaces of packages were swabbed with 70% ethanol prior to opening with a sterile scissors.

At each sampling time, one of the two frankfurters in each package was analyzed for the presence and populations of *L. monocytogenes*. The frankfurter was combined

with 228 ml of sterile 0.1% peptone water and homogenized for 60 s at medium speed in a stomacher (model 400; Seward, London, U.K.). Packages of frankfurters stored at -20°C were immersed in a water bath at 37°C for 5 min to thaw before opening. Undiluted homogenate (0.25 ml in quadruplicate and 0.1 ml in duplicate) was surface plated on the Oxford formulation of *Listeria* Selective Agar (Oxoid, Ogdensburg, N.Y.) with selective supplement SR0140E (Oxoid) (MOX agar) and on tryptic soy agar (Difco) (TSA). Samples (0.1 ml in duplicate) of homogenate serially diluted in sterile 0.1% peptone water were also plated on MOX agar and TSA. *Listeria* selective enrichment broth base with supplement SR0141E (Oxoid) (2X, 285 ml) was added to the homogenate after samples were removed for plating on MOX agar and TSA. Presumptive colonies of *L. monocytogenes* formed on plates incubated at 37°C for 48 h were counted, and colonies were randomly selected for confirmation using the API *Listeria* biochemical test kit (bioMérieux Vitek, Inc., Hazelwood, Mo.). The enrichment slurry was incubated at 37°C for 48 h, streaked on MOX agar, and incubated as described above. Selected presumptive colonies were confirmed.

Heating cells in exudates. Sterile pipettes were used to remove and pool fluid exudate separately from packages of HFHS and LFLS frankfurters. Exudates were analyzed for fat content as described by the Association of Official Analytical Chemists, Inc. method 989.05 (*1*). After centrifugation of alkali stressed and control cells, pellets were resuspended (10 ml) in the two types of exudates from packages of each frankfurter type. Cell suspensions were injected (50 μl) into Kimax-51 capillary tubes (0.8 – 1.0 mm I.D. x 90 mm long, no. 34507-99; Kimble, Vineland, N.J.) and the ends were flame sealed. Capillary tubes were brought to 21 ± 1°C before subjecting to heat treatment by submerging in a water bath at 59°C for 0, 1, 2, 4, 6, 8, or 10 min or at 62°C for 0, 1, 2, 3, or 4 min. The come-up times for exudate-filled capillary tubes in the water bath at 59 or 62°C were measured with a Microprocessor Thermometer (model HH23; Omega, Stamford, Conn.) connected with a type T thermocouple. After heat treatment, capillary

tubes were immediately cooled and sanitized by immersing, in succession, in an ice bath, 70% ethanol, and sterile water (21°C) before aseptically transferring to screw-capped test tubes (16 mm I.D. x 125 mm long) containing 5 ml of sterile 0.1% peptone water. Capillary tubes containing the heated cell suspension were crushed using a sterile glass rod and thoroughly mixed with the peptone water. Undiluted suspensions (0.25 ml in quadruplicate or 0.1 ml in duplicate) and suspensions serially diluted in sterile 0.1 % peptone water were surface plated (0.1 ml in duplicate) on MOX agar. Plates were incubated at 37°C for 48 h before presumptive colonies were counted and confirmed as described above.

Statistical analysis. Three replicates of each experiment were performed. Population means were calculated using SAS (SAS, Cary, N.C.). Populations of *L. monocytogenes* surviving heat treatment and forming colonies on MOX agar were analyzed using appropriate forms of the logistic equation (*10*) applied by the nonlinear regression procedure of SAS. The log-transformed equations used to analyze data were as follows:

$$\log S = \log 2 - \log [1 + e(\beta t)] \qquad (1)$$

where log S is the log (CFU/CFU_0) at any given time (t) and β is the maximum specific death rate.

Data resulting in survival curves with an initial lag in population reduction (shoulder) followed by a distinct one-phase inactivation were modeled to the following form of equation 1:

$$\log S = \log [1 + e(-\beta t_{1/2})] - \log \{1 + e[\beta(t - t_{1/2})]\} \qquad (2)$$

where $t_{1/2}$ is the time at which the number of CFU is $CFU_0/2$ and is a measure of lag in killing, and other terms are as defined in equation 1.

For survival curves with no initial lag in killing but having two distinct killing phases (biphasic), data were fitted to the following two-term exponential form of equation 1:

$$\log S = \log (\{2f_1/[1 + e(\beta_1 t)]\} + \{2(1 - f_1) / [1 + e(\beta_2 t)]\}) \qquad (3)$$

where f_1 and $(1 - f_1)$ represent two fractions of cells (differing with respect to heat resistance) and β_1 and β_2 are the specific killing rates for the two fractions, respectively. The assumption of this model is that two fractions (subpopulations) are killed exponentially but at different, independent rates.

For equations 1 and 2, logistic D values (*15*) were calculated as $D = 2.94/\beta$ and for equation 3, $D = \ln(19)/\beta_2$.

RESULTS

Survival and growth of *L. monocytogenes* on frankfurters. No marked changes in populations of control or alkali-stressed *L. monocytogenes* occurred on frankfurters stored at -20°C for up to 12 weeks (Figure 4.1). Populations remained constant on both types of frankfurters, regardless of previous exposure to alkaline treatments. Populations of aerobic microorganisms were generally higher than populations of *L. monocytogenes*, although the number of aerobic microorganisms recovered on TSA may also have included some of the pathogen applied in the inoculum.

Storage of frankfurters at 4°C enabled good growth of three of the four cell types (Figure 4.2). Treatment of cells with alkaline cleaners (treatments B and C) did not affect the rate of growth compared to that of controls cells (treatment A) on either HFHS or LFLS frankfurters. Alkali adaptation (treatment D), however, rendered cells less capable of growth compared to control cells. LFLS frankfurters, overall, supported better growth of *L. monocytogenes* than did HFHS frankfurters. On frankfurters inoculated with cells from treatments A, B, and C and stored for 6 weeks (1 week before the end of shelf life), populations were ca. 6 $\log_{10}$ CFU/g of LFLS frankfurters and only ca. 3.5 $\log_{10}$ CFU/g of HFHS frankfurters. Although growth of alkali-adapted cells (treatment D) was retarded compared to treatments A, B, and C, faster growth rates of cells from treatment D were evident on LFLS compared to HFHS frankfurters. Populations of total aerobic microorganism were similar to populations of *L.*

Figure 4.1. *Populations of* L. monocytogenes (■) *and aerobic microorganisms* (●) *on HFHS frankfurters and* L. monocytogenes (□) *and aerobic microorganisms* (○) *on LFLS frankfurters stored at -20°C for up to 12 weeks. Quadrants A, B, C, and D show survivor curves for control cells, cells treated with low-foam alkaline cleaner, cells treated with non-butyl alkaline cleaner, and alkali-adapted cells, respectively. Dashed lines indicate product "use by" date.*

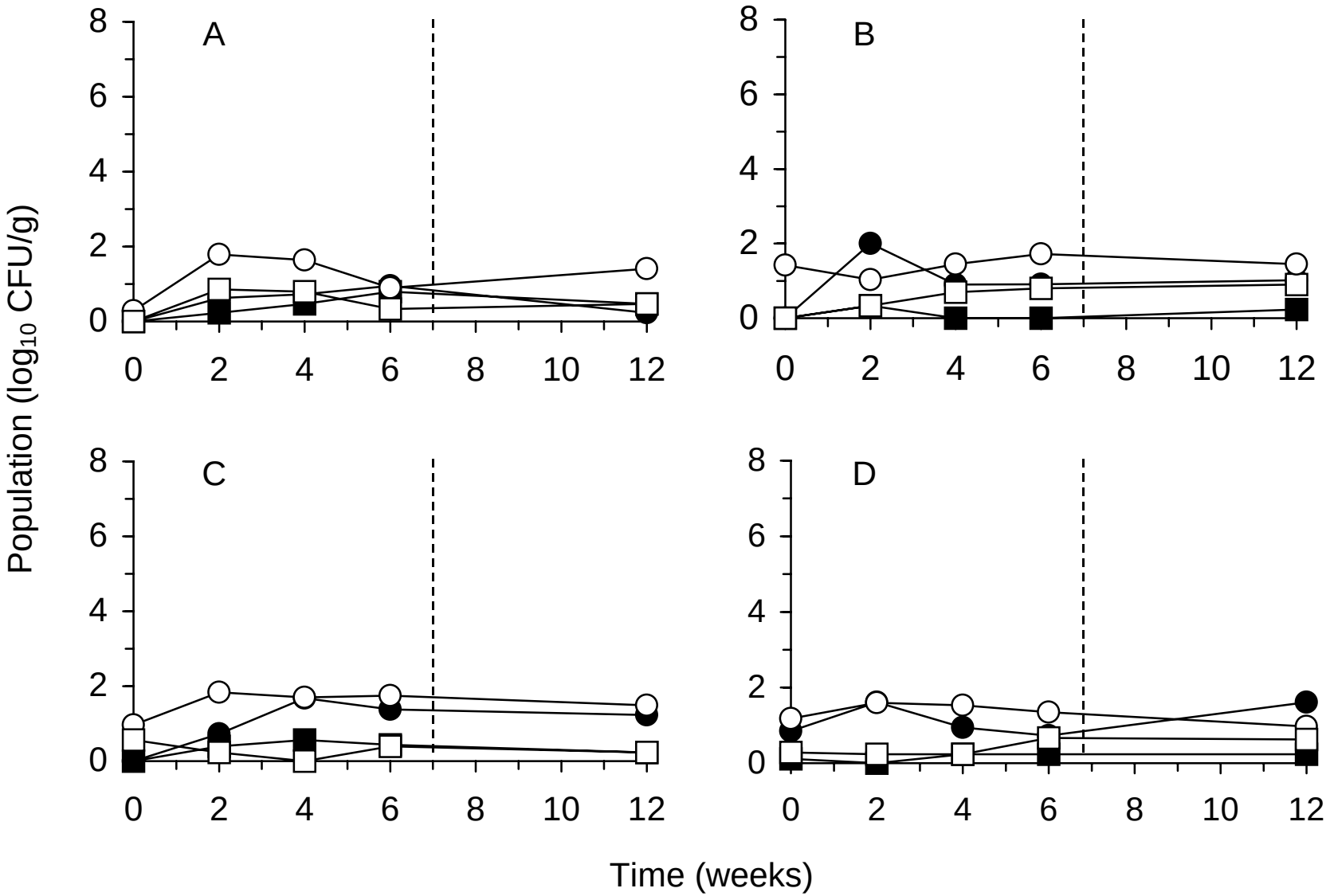
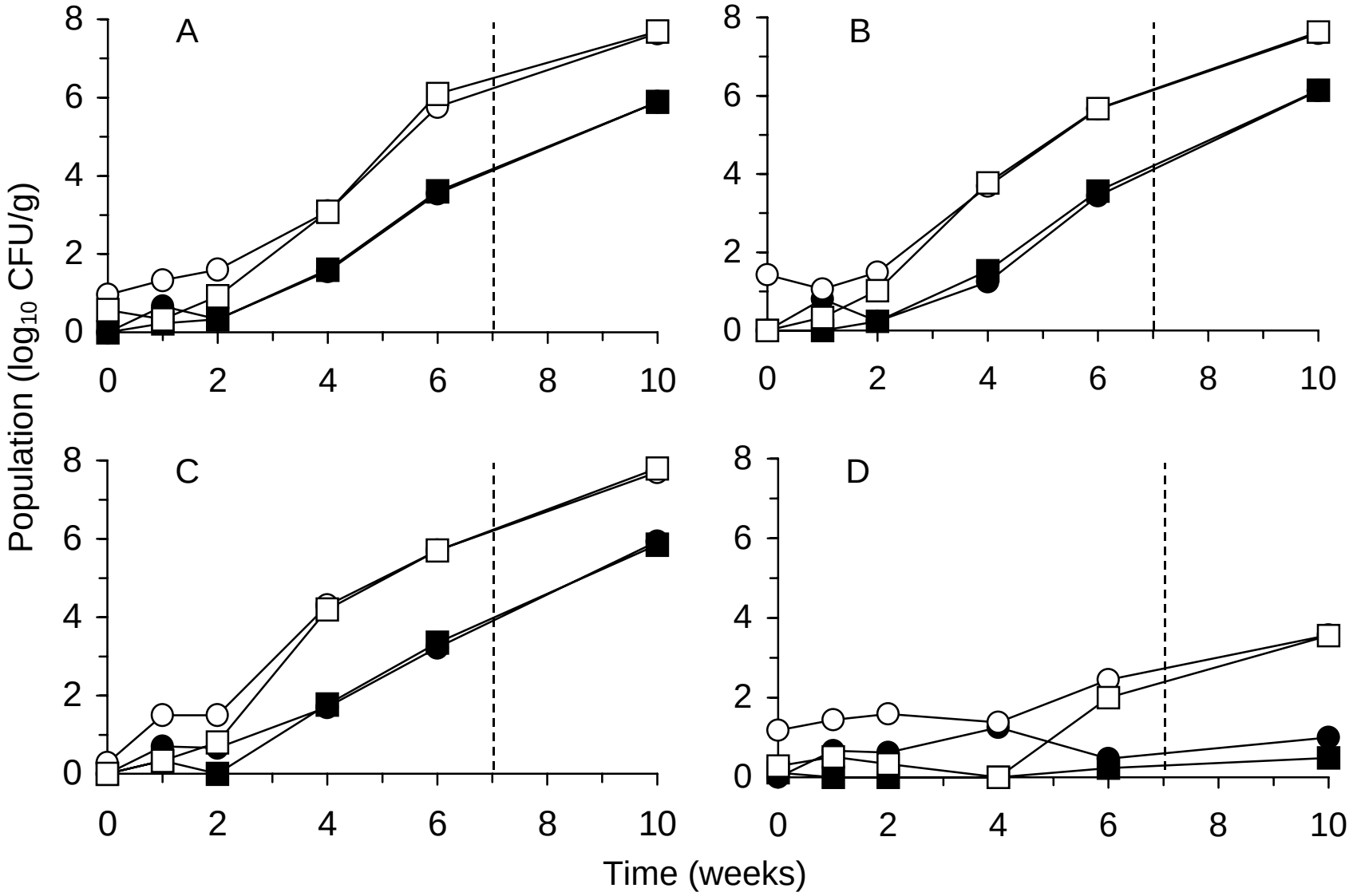

A
B
C
D
Population (log10 CFU/g)
Time (weeks)
0
2
4
6
8
10
12

Figure 4.2. *Growth of* L. monocytogenes (■) *and aerobic microorganisms* (●) *on HFHS frankfurters and* L. monocytogenes (□) *and aerobic microorganisms* (○) *on LFLS frankfurters stored at 4°C for up to 10 weeks. Quadrants A, B, C, and D show survivor curves for control cells, cells treated with low-foam alkaline cleaner, cells treated with non-butyl alkaline cleaner, and alkali-adapted cells, respectively. Dashed lines indicate product "use by" date.*


A
B
C
D
Population (log10 CFU/g)
Time (weeks)
0
2
4
6
8
10

monocytogenes, regardless of treatment of *L. monocytogenes* before inoculating onto frankfurters.

Growth of *L. monocytogenes* was more rapid on frankfurters stored at 12°C for up to 4 weeks (Figure 4.3) compared to growth at 4°C. Differences in population of *L monocytogenes* on the two types of frankfurters stored for 1 and 2 weeks at 12°C were greater than those on frankfurters stored 1 and 2 weeks at 4°C. After storage for 4 weeks at 12°C, populations of *L. monocytogenes* on the two types of frankfurters were similar, regardless of cell treatment, and appeared to reach a maximum. Populations of *L. monocytogenes* on both types of frankfurters stored at 12°C were largely unaffected by treatments A, B, or C before inoculation. Delayed growth of alkali-adapted *L. monocytogenes* (treatment D) on HFHS frankfurters was evident up to 9 days, but populations steadily increased thereafter. Only a slight delay in growth of alkali-adapted *L. monocytogenes* occurred on LFLS frankfurters during the first 6 days, while populations were similar to those on frankfurters inoculated with cells subjected to other treatments after 9 days. Minimal differences between populations of *L. monocytogenes* and aerobic microorganisms were observed after a given storage time at 12°C.

Growth of control or alkaline stressed *L. monocytogenes* on vacuum-packaged frankfurters stored at -20°C for two days, thawed, and stored in ambient atmosphere at 12°C for up to 10 days was monitored (Figure 4.4). HFHS frankfurters supported similar growth patterns of control cells and cells treated with alkaline cleaners. Growth of cells treated in alkaline TSB (alkali-adapted cells) was inhibited. Populations did not exceed 1 $\log_{10}$ CFU/g during the 10-day storage period. LFLS frankfurters supported similar increases in populations originating from cells in inocula of control and alkaline cleaner treatments, but growth of alkali-adapted cells was retarded. Overall, growth on HFHS frankfurters was slower than growth on LFLS frankfurters. Interestingly, inoculated HFHS frankfurters were spoiled, as determined by subjective evaluation, after storage for 6 days at 12°C. These frankfurters, inoculated with alkali-adapted cells

Figure 4.3. *Growth of* L. monocytogenes (■) *and aerobic microorganisms* (●) *on HFHS frankfurters and* L. monocytogenes (□) *and aerobic microorganisms* (○) *on LFLS frankfurters stored at 12°C for up to 4 weeks. Quadrants A, B, C, and D show survivor curves for control cells, cells treated with low-foam alkaline cleaner, cells treated with non-butyl alkaline cleaner, and alkali-adapted cells, respectively.*

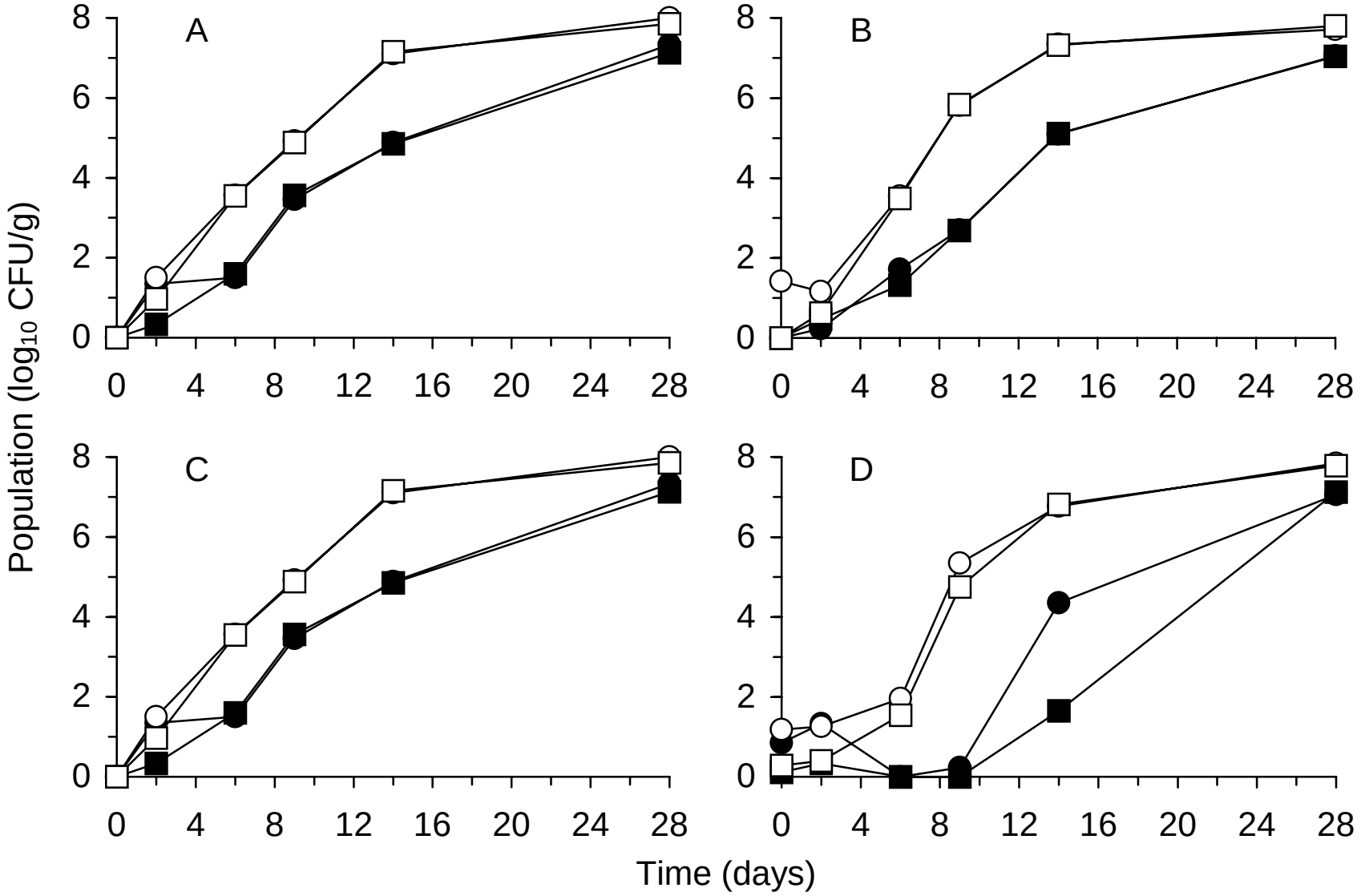
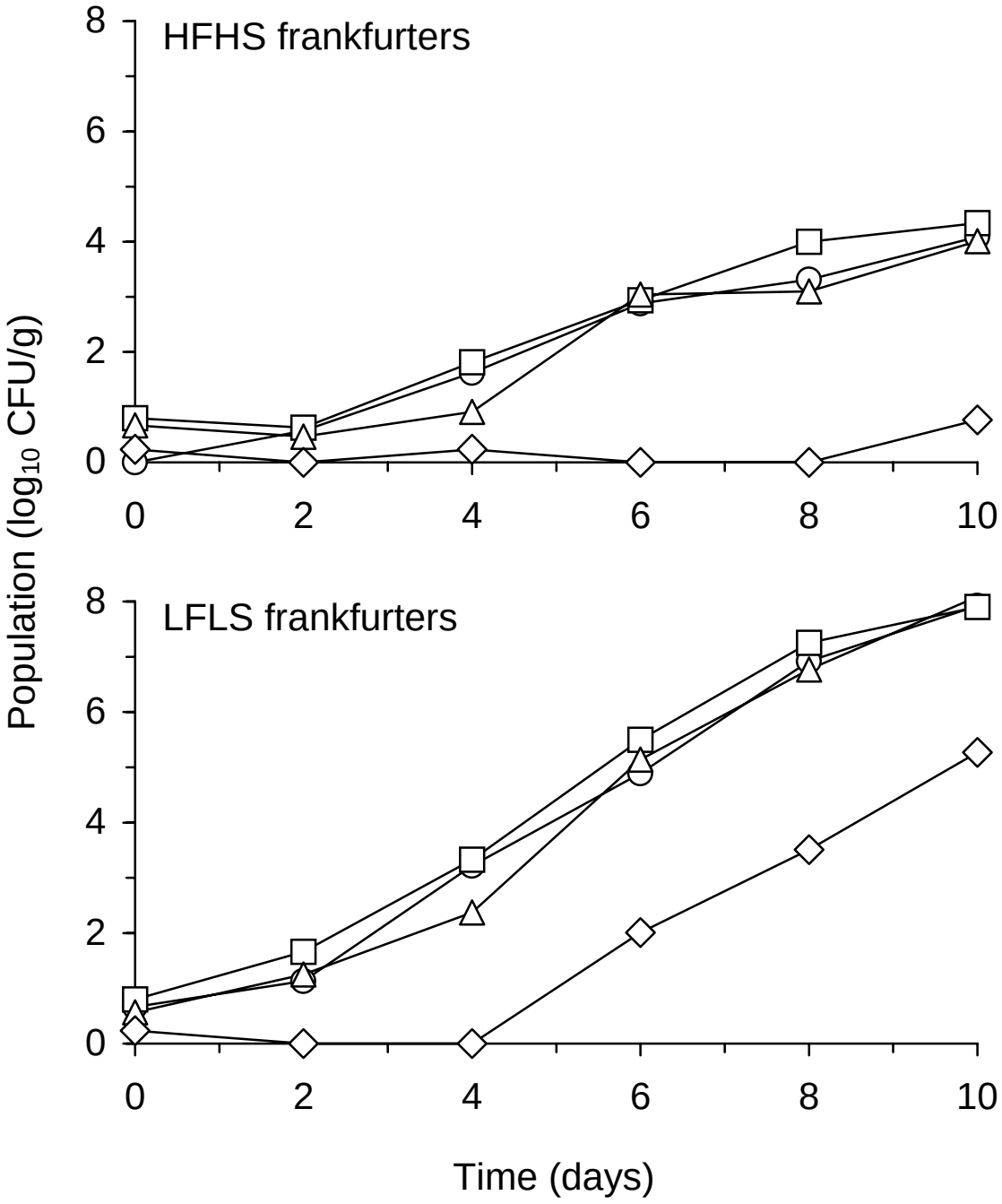

A
B
C
D
Population (log10 CFU/g)
Time (days)
0
2
4
6
8
0
4
8
12
16
20
24
28

Figure 4.4. *Growth of control* (○) *cells of* L. monocytogenes *and cells exposed to a low-foam alkaline cleaner* (□)*, a non-butyl alkaline cleaner*(△)*, or TSB at pH 10.0 (alkali-adapted)* (◇) *on HFHS frankfurters (top) and on LFLS frankfurters (bottom) after packages stored at -20℃ for 48 h were opened and stored at 12℃ for up to 10 days.*


HFHS frankfurters
LFLS frankfurters
Population (log10 CFU/g)
Time (days)
0
2
4
6
8
10

(treatment D), contained less than 1 $\log_{10}$ CFU of *L. monocytogenes*/g, compared to 3 $\log_{10}$ CFU/g on HFHS frankfurters inoculated with cells from treatments A, B, and C.

Survival of alkaline stressed cells in heated frankfurter exudates. The exudate (pH 5.8) from HFHS frankfurters contained 0.279% fat while the exudate (pH 5.8) from LFLS frankfurters contained 0.104% fat. Come-up times in exudates in capillary tubes immersed in a water bath at 59 or 62°C were 5 and 6 sec, respectively. Logistic $D_{59°C}$ values revealed significant differences between the heat tolerance of alkali-stressed and control *L. monocytogenes* cells (Table 4.1). Heating cells previously exposed to treatment B in the exudate from HFHS frankfurters resulted in a significantly lower logistic $D_{59°C}$ value compared to that of control cells (treatment A) and cells exposed to other treatments. Cells previously alkali-adapted (treatment D) had significantly lower $D_{59°C}$ values than cells exposed to alkaline cleaners although they were not significantly different from that of control cells. Cells previously exposed to one alkaline cleaner (treatment C) had a significantly lower $D_{62°C}$ value when heated in LFLS frankfurter exudates compared to some other treatments (Table 4.2).

DISCUSSION

Treating of *L. monocytogenes* cells with alkali by exposure to a 1% solution of a low-foam alkaline cleaner or a 1% solution of a non-butyl alkaline cleaner did not change their growth characteristics on frankfurters stored at 4 or 12°C. Alkali-adaptation of cells did, however, greatly extend the lag phase of growth of the pathogen. Cells weakened by adaptation (i.e., suspended in TPB at pH 10.0, 4°C for 48 h) may have required more time to resuscitate and overcome inhibiting factors such as pH ≤ 5.5, salt, and antimicrobials imposed by frankfurters. This suggests that cells exposed to alkaline cleaners or detergents in food processing environments that escape subsequent exposure to sanitizers would not be inhibited on vacuum packaged processed meats

Table 4.1. Heat (59°C) survival parameter estimates for control or alkaline-stressed *L. monocytogenes* cells suspended in frankfurter exudates

Cell treatment[a]	Exudate[b]	Eqn.[c]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [d]	Logistic $D_{59°C}$ value (min)[e]
A	HFHS	2	–	–	–	1.12 ± 0.160	0.945	2.63 abc
	LFLS	2	–	–	–	1.11 ± 0.080	0.969	2.65 abc
B	HFHS	3	0.5	1.60 ± 0.87	- 0.165 ± 0.124	–	0.978	1.94 d
	LFLS	2	–	–	–	1.00 ± 0.040	0.996	2.95 a
C	HFHS	2	–	–	–	0.987 ± 0.063	0.977	2.98 a
	LFLS	2	–	–	–	1.04 ± 0.160	0.898	2.79 ab
D	HFHS	2	–	–	–	1.20 ± 0.180	0.918	2.45 bc
	LFLS	2	–	–	–	1.25 ± 0.180	0.941	2.35 c

[a] Treatments A, B, C, and D represent exposure of cells to water (control), a 1% solution of low foam alkaline cleaner, a 1% solution of non-butyl alkaline cleaner, and TPB at pH 10.0, respectively.

[b] Pooled exudates from high fat, high salt (HFHS) frankfurters and low fat, low salt (LFLS) frankfurters.

[c] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression. See text for equations.

[d] Calculated from 1 – (residual sum of squares / corrected total).

[e] Calculated per replicate from $D = 2.94/\beta$ for equation 2, or $D = (\ln 19)/\beta_2$ for equation 3. Means not followed by the same letter are significantly ($P \leq 0.05$) different.

Table 4.2. Heat (62°C) survival parameter estimates for alkaline-stressed or control *L. monocytogenes* cells suspended in frankfurter exudates

Cell treatment[a]	Exudate[b]	Eqn.[c]	(1-f) least heat-sensitive fraction	β_2 – least heat-sensitive fraction (min^{-1})	β_1 – most heat sensitive fraction (min^{-1})	β (min^{-1})	Pseudo r^2 [d]	Logistic $D_{62°C}$ value (min)[e]
A	HFHS	2	–	–	–	2.42 ± 0.770	0.918	0.972 a
	LFLS	2	–	–	–	3.25 ± 0.860	0.904	0.904 ab
B	HFHS	2	–	–	–	3.24 ± 0.820	0.903	0.914 ab
	LFLS	2	–	–	–	3.04 ± 0.600	0.918	0.972 a
C	HFHS	2	–	–	–	3.44 ± 0.560	0.954	0.857 ab
	LFLS	2	–	–	–	3.50 ± 0.950	0.918	0.844 b
D	HFHS	1	–	–	–	3.21 ± 0.550	0.936	0.917 ab
	LFLS	3	0.15	3.07 ± 0.420	32.3 ± 0.00	–	0.973	0.958 ab

[a] Treatments A, B, C, and D represent exposure of cells to water (control), a 1% solution of low foam alkaline cleaner, a 1% solution of non-butyl alkaline cleaner, and TPB at pH 10.0, respectively.

[b] Pooled exudates from high fat, high salt (HFHS) frankfurters and low fat, low salt (LFLS) frankfurters.

[c] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression. See text for equations.

[d] Calculated from 1 – (residual sum of squares / corrected total).

[e] Calculated per replicate from $D = 2.94/\beta$ for equations 1 and 2, or $D = (\ln 19)/\beta_2$ for equation 3. Means not followed by the same letter are significantly ($P \leq 0.05$) different..

stored at refrigeration temperature, while cells exposed to a high-nutrient alkaline environment for longer periods, such as in standing pools of diluted caustic soda, water, and food residue, may ultimately have less potential for rapid growth on such products. Inhibition of growth of *L. moncytogenes* on HFHS frankfurters compared to LFLS frankfurters, regardless of previous exposure to an alkaline environment, may be due, in part, to differences in salt concentration. The label on packages of HFHS frankfurters indicated that they contained 1% salt whereas the label on LFLS frankfurters indicated they contained 0.5% salt. In previous studies, we observed that colony formation by *L. monocytogenes* that had been exposed to solutions of alkaline cleaners (*19*) or adapted to a high-pH environment (*18*) was not inhibited on tryptose phosphate agar (TPA) supplemented with 4% NaCl. The rate of colony formation on TPA containing an additional 4 % NaCl was much slower than on TPA. Thus, while *L. monocytogenes* may not die when inoculated onto HFHS frankfurters, the rate of growth is markedly reduced compared to that on LFLS frankfurters. Synergism between the salt and other preservatives (e.g., erythorbates and phosphates) in HFHS frankfurters may also have contributed to slower rates of growth at refrigeration temperature. Another factor that may have influenced growth is the probable higher concentration of unsaturated fatty acids in HFHS frankfurters. Unsaturated fatty acids are known to inhibit the growth of Gram-positive bacteria (*9*). Animal fats contain unsaturated acids, mostly oleic and linoleic (*11*). The HFHS frankfurters contained ca. 9 g of unsaturated fat per 57-g serving, while LFLS frankfurters contained ca. 5 g per serving. Since, with the exception of salt, other ingredients were present in both frankfurter formulations in similar quantities, different amounts of unsaturated fats, in conjunction with the different concentrations of salt, most likely played a major role in controlling the growth of *L. monocytogenes* on HFHS and LFLS frankfurters.

Predictably, the viability of *L. monocytogenes* was preserved on frankfurters stored at -20°C for up to 12 weeks. This is consistent with other observations on the survival of *L. monocytogenes* in frozen foods (*4*). Johnson et al. (*8*) suggested that

research is needed to determine the hazards of low numbers of *L. monocytogenes* on ready-to-eat meat products and behavior during simulated consumer abuse situations. We were interested in knowing the behavior of *L. monocytogenes* that survived storage on frankfurters at -20°C, once the frankfurters were thawed and stored at 12°C, as might simulate practices used in food service or home settings. This experiment was intended to represent a scenario involving the use or misuse of frankfurters. The behavior of *L. monocytogenes* subjected to freeze/thaw conditions, followed by storage at 12°C, was similar to that observed on frankfurters not frozen before storing at 12°C. The LFLS frankfurters supported more rapid growth than did the HFHS frankfurters, and growth of alkali-adapted cells was retarded compared to growth of cells subjected to other treatments. Freezing and thawing of treated cells of *L. monocytogenes* on vacuum packaged frankfurters does not alter their growth characteristics under atmospheric pressure conditions in opened packages subsequently stored at 12°C. Processed refrigerated sausage products, such as frankfurters, purchased and then frozen by the consumer may be thawed some time later, rendering use-by dates on packages irrelevant. Some consumers may believe that refrigerated frankfurters are safe for longer periods if previously frozen, when in fact populations of *L. monocytogenes* can rapidly increase to high populations upon thawing and refrigeration. Appropriate measures should be taken to ensure that food service personnel and consumers are fully informed of the safety risks associated with storage of frankfurters, whether they had been frozen or not frozen before refrigerated storage.

Modeling the tolerance of *L. monocytogenes* to heat treatment in frankfurter exudates revealed that, regardless of previous exposure of cells to an alkaline environment, cells remain viable for several minutes, depending on temperature, and then have similar rapid inactivation rates. The only notable difference in thermotolerance of cells that had been exposed to various pretreatments, albeit possibly due to dissimilarity in models, was the reduced rate of inactivation of cells exposed to treatment B and heated in HFHS frankfurter exudate at 59°C. These data indicate that

exposure of *L. monocytogenes* to high pH either for several minutes in 1% solutions of alkaline cleaners or for 48 h in the high-nutrient environment of TPB, does not alter heat resistance. Even though growth of treatment D cells on frankfurters was inhibited, thermotolerance was not different than that of control cells (treatment A). Thermotolerance of *L. monocytogenes* was likewise largely unaffected by the source of the exudate used as a heating menstrum, whether from HFHS or LFLS frankfurters.

This study shows that the rate of growth of *L. monocytogenes* inoculated on beef frankfurters may be altered by previous exposure of cells to alkaline environments. Survival and growth of cells previously exposed to 1% solutions of alkaline cleaners commonly used in food processing environments was not different than that of control cells inoculated onto frankfurters and stored frozen or at refrigeration temperatures. However, cells exposed to a high-nutrient, high-pH environment for 48 h prior to inoculation of frankfurters had markedly reduced growth rates at refrigeration temperatures. Cells of *L. monocytogenes* subjected to similar stress conditions in frankfurter processing environments where alkaline cleaners and/or caustic soda are used to clean or partially clean equipment remain as a public health concern.

Differences in growth characteristics of *L. monocytogenes* on HFHS and LFLS frankfurters subjected to various storage conditions were clearly evident. In a related study, differences in growth characteristics of *L. monocytogenes* on vacuum packaged processed meat products and frankfurters were attributed to phenols present in liquid smoke preparations applied to frankfurters (*5*). The phenol content of frankfurters in our study was not determined but cannot be ruled out as possibly affecting the growth of *L. monocytogenes*. To our knowledge, studies on the survival and growth of *L. monocytogenes* on vacuum packaged beef frankfurters containing different fat and salt contents and stored at freezing and refrigeration temperatures have not been reported. Enhanced growth of *L. monocytogenes* on refrigerated LFLS frankfurters compared to HFHS frankfurters demonstrates the effect of replacing fat and salt with other

ingredients on risks of higher numbers of the pathogen being reached before the use-by date.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

A strain of *Listeria monocytogenes* (serotype 4b) freshly isolated from a food processing environmnent was more resistant to heating at 56 or 59°C following incubation for 45 min in tryptose phosphate broth (TPB) at pH 12.0 compared to treatment for the same time in TPB at pH 7.3. Cells of *L. monocytogenes* survived at least 6 days in TPB at pH 9.0, 10.0, and 11.0 stored at 4 or 21°C, and cells stored in TPB at pH 10.0 for 48 h were more resistant to heating at 56°C than were cells stored in TPB at pH 7.3 for the same time.

Five food processing environmental isolates of *L. monocytogenes* (one with serotype 1/2a and four with serotype 4b) were suspended in 1% solutions of eight commercial cleaners (pH 7.1 – 12.5) or in water (control), and incubated at 4ºC for 30 min or 48 h. In only three alkaline cleaning solutions (pH 11.6 – 12.4) were populations reduced significantly ($P \leq 0.05$) after 30 min compared to reductions in water. After 48 h, populations were significantly higher in one cleaning solution (pH 10.4) than in water, while populations in six other cleaning solutions were reduced by ≥ 4.72 $\log_{10}$ CFU/ml. Cells exposed to cleaning solutions for 30 min became sensitive to 4.0 or 6.0 ppm free chlorine, and to 50 or 100 ppm benzalkonium chloride and cetylpyridinium chloride, common components of quaternary ammonium sanitizers. Following 30 min of exposure of *L. monocytogenes* to 1% solutions of a non-butyl alkaline cleaner (pH 11.6) and a heavy duty foaming alkaline cleaner (pH 12.6) at 4°C, cells became more tolerant to heating at 56°C than were control cells.

Cells of *L. monocytogenes* exposed at 4°C to 1% solutions of two alkaline cleaners or alkali-adapted in TPB (pH 10.0) at 37°C for 45 min, then 4°C for 48 h, were

inoculated onto beef frankfurters containing high fat (16 g) and high sodium (550 mg) (HFHS) or low fat (8 g) and low sodium (250 mg) (LFLS) per 57-g serving. Frankfurters were surface-inoculated (2.0 $\log_{10}$ CFU/g), vacuum packaged, stored at –20, 4, or 12°C, and analyzed for populations over time. Populations *of L. monocytogenes* remained stable on frankfurters stored at -20°C for up to 12 weeks. After storage at 4°C for 6 weeks, populations of control cells and cells exposed to alkaline cleaners were ca. 6.0 $\log_{10}$ CFU/g of LFLS frankfurters and ca. 3.5 $\log_{10}$ CFU/g of HFHS frankfurters, but growth of alkali-adapted cells on both types of frankfurters was retarded. Growth on frankfurters stored at 12°C was more rapid, but there was also delayed growth of alkali-adapted cells on HFHS and LFLS frankfurters after storage for 9 and 6 days, respectively. Control, alkaline cleaner exposed, and alkali-adapted cells were resuspended in exudates from both types of frankfurters and subjected to heat treatment. There were significant ($P > 0.05$) but unrevealing differences in $D_{59°C}$ and $D_{62°C}$ values between cell treatments and between types of exudate.

Based upon these results, summarized from three separate studies, the following conclusions have been drawn:

1. *Listeria monocytogenes* is capable of survival for at least 6 days in high-nutrient alkaline environments, and becomes more heat resistant as a consequence.
2. Alkaline pH-induced cross-protection of *L. monocytogenes* against mild heat (e.g. 56 or 59°C) has potential to enhance survival in minimally processed as well as heat-and-serve foods, and in foods on holding tables, in foodservice facilities, and in the home.
3. Cells surviving exposure to chlorine, in contrast, are more sensitive to heat, thus not compromising the effectiveness of thermal processing in achieving desired $\log_{10}$ reductions.

4. Cells of *L. monocytogenes* can survive for as much as 48 h when stored at 4°C in 1 % solutions of alkaline cleaners, but become sensitized to sanitizers as a result.
5. Cells of *L. monocytogenes* suriving exposure to some types of alkaline cleaners become more resistant to heating.
6. Exposure of *L. monocytogenes* to some alkaline cleaning solutions does not alter survival and growth characteristics on frankfurters stored at freezing and refrigeration temperatures.
7. Growth of alkali-adapted *L. monocytogenes* is retarded on frankfurters stored at refrigeration temperatures.
8. Growth rates of *L. monocytogenes* on frankfurters formulated with reduced fat and salt are more rapid compared to growth rates on frankfurters formulated with normal fat and salt levels.

The mechanism causing increased heat resistance of *L. monocytogenes* following alkali-adaptation should be further studied. Research to determine if certain genes are associated with alkaline stress is warranted. The role of the cell wall and plasma membrane in response to alkaline stress should also be determined. Future work should also incorporate experiments to determine the duration of alkali-induced heat resistance after restoring cells to neutral pH and the precise level of protection or sensitivity of cells against sodium. A logical advancement of this research should involve biofilm-associated cells rather than planktonic cells.

While the ability of *L. monocytogenes* to survive in 1% solutions of alkaline cleaners was demonstrated, further work is needed to evaluate the orgnanism's survival in manufacturer-recommended concentrations of alkaline cleaners in the absence and in the presence of nutrients. Inclusion of these parameters might simulate some types of food processing environments more closely than work reported here. Interestingly, alkali-adaptation of *L.* monocytogenes in alkaline TPB and its prolonged storage in

alkali-adjusted TPB rendered cells more tolerant to heating. Conversely, short or long term exposure of *L. monocytogenes* to alkaline cleaning solutions did not consistently cause cells to become more resistant to heat. It was not determined whether or not this difference was due to lack of nutrient, type of alkali, or presence or absence of solvents in cleaners.

The differences in growth characteristics of control cells and cells exposed to alkaline cleaners, compared to growth charactersistics of alkali-adapted cells on frankfurters, should be further investigated. It is not clear why alkali-adapted cells would become more resistant to heating, but less capable of growth on frankfurters at refrigeration temperatures. Also, the enhanced growth of *L. monocytogenes* on low fat, low salt frankfurters compared to high fat, high salt frankfurters should be investigated further and verified using other processed meat products with various fat and salt contents. Finally, the observation that alkali-stressed cells of *L. monocytogenes* were not more heat resistant than control cells when heated in frankfurter exudates was not consistent with previous data collected using non-food heating menstra. Although these inconsistencies were possibly due to differences in experimental procedures (e.g., enumeration media), further work is needed to determine if alkali-stressed *L. monocytogenes* exhibit changes in resistance to heat inactivation in a wide range of food products.