THE EFFECT OF THERMAL PROCESSING SCHEDULES AND UNIT OPERATIONS ON THE QUALITY OF BLUE CRAB (*CALLINECTES SAPIDUS*) MEAT

by

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The Effect of Thermal Processing Schedules and Unit Operations on the Quality of Blue Crab (*Callinectes sapudis*) Meat

Jennifer Lynn Smith   ABSTRACT

The effects of initial thermal processing, plant sanitation, and employee habits on the microbiological quality of blue crab (*Callinectes sapidus*) meat were determined in a commercial crab processing facility. Thermal processing was evaluated at 5, 7, and 8 minutes at 250°F for the destruction of microorganisms, including *Listeria monocytogenes*. F-values calculated indicated a sufficient reduction of *L. monocytogenes* at each processing time. Fresh picked crab meat was evaluated for microbial levels when exposed to ambient temperatures over a four hour period. It was found that time and temperature did not influence the microbial populations significantly except in the fourth hour. Plant sanitation was evaluated based on levels of adenosine triphosphate (ATP) and microbial counts. Areas found to have high levels of ATP typically had low microbial counts, thus suggesting that crab meat residual was the problem. The presence of *Listeria* species in the plant was determined using a commercial polyclonal antibody test. *Listeria* species were found under picking tables, on cooler doors, employees' aprons, and on several employees' hands. In a laboratory setting, an automated hand wash was compared with a manual hand wash for the removal of *Listeria innocua*, as a model for *Listeria monocytogenes*. It was found that a manual hand wash of 15 seconds was superior to an equal time automated wash. The microbial quality of crab meat was found to be affected by daily plant procedures, and could be changed by modifying procedures.

DEDICATION

This work is dedicated to my parents, Julian and Sandra Smith. Thank you for all of the love and support you have shown me.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

J. Smith and D. Rawles

A. General characteristics of the *Listeria* genus

1. Description

Listeria monocytogenes is a Gram positive, non-sporeforming, facultatively anaerobic, short diphtheroid rod, which grows between -0.4°C and 50°C (Junttila et al., 1988; Walker and Stringer, 1987; Wilkins et al., 1972). It is oxidase negative and catalase positive and shows β-hemolysis, the production of clear zones on blood agar. The organism possesses peritrichous flagella, which in the temperature range up to 30°C, imparts tumbling motility. Flagellin is produced and assembled at the cell surface between 20°C and 25°C, but at 37°C, flagellin production is reduced (Peel et al., 1988). *L. monocytogenes* can multiply in high salt (Hudson et al., 1992; Hefnawy and Marth, 1993) or bile concentrations. Due to its ability to grow and reproduce at 1.1°C, *Listeria* is considered to be a psychrotrophic organism (NACMCF, 1991). The minimum pH required for growth initiation ranges from 5.0 to 5.7 at 4°C and 4.3 to 5.2 at 30°C, however, it is capable of growth in the pH range up to 9.6 (Wehr, 1987). When observed under obliquely transmitted light, *Listeria* colonies present a characteristic blue-green sheen. *L. monocytogenes* is widely found in plants, soil, and water samples (Weis and Seeliger, 1975), silage, sewage, slaughterhouse waste, milk of normal and mastitic cows, human and animal feces (McCarthy, 1990). As an intracellular parasite, it can grow inside leukocytes of human and animals (Farber, 1989; NACMCF, 1991).

Listeria species are taxonomically related to *lactobacilli* (Seeliger and Jones, 1986). Some bacteriocins produced by lactic acid bacteria will inhibit the growth of *Listeria* spp. (McKay and Baldwin, 1990; Jeppesen and Huss, 1993), and listeriocins or monocins produced by *Listeria* spp. have an antibiotic effect against *L. monocytogenes* and other organisms such as *staphylococci* (Ortel, 1989b).

Differentiation of *Listeria* species is achieved by utilizing carbohydrate fermentation reactions. Under anaerobic conditions, growth of *Listeria* spp. is supported only by hexoses and pentoses; whereas aerobically, by maltose and lactose. *L.*

monocytogenes and *L. innocua* use glucose, lactose and rhamnose under aerobic conditions; in addition *L. grayi* and *L. murrayi* utilize galactose. *L. ivanovii* and *L. seeligeri* are the only *Listeria* spp. to ferment xylose (Pine et al., 1989). Hemolytic *Listeria* spp. can be differentiated according to the Christie-Atkins-Munch-Peterson (CAMP) test (Dever et al., 1993; Farber and Peterkin, 1991). In the CAMP test, a β-hemolytic strain of *Staphyloccus aureus* and a strain of *Rodococcus equi* are streaked in parallel on a sheep blood agar plate and then test cultures are streaked parallel to one another between the *S. aureus* and *R. equi* streaks. The blood agar plates are incubated at 35°C for 24-48 h and then examined for hemolysis. *L. monocytogenes* and *L. seeliger*'s hemolysis are enhanced near the *S. aureus* streak, while that of *L. ivanovii* is enhanced near the *R. equi* streak.

Incidence of cryptic plasmids, responsible for antibiotic resistance in *L. monocytogenes* strains, has been reported to be between 0 to 20% (Perez-Diaz et al., 1982). However, according to Farber and Peterkin (1991), this low number could be the result of using acriflavin in the isolation media, which would act as a curing agent against plasmids. Poyart-Salmeron et al. (1990) isolated, from a clinical strain of *L. monocytogenes*, a self-transferrable 37-kbp plasmid carrying gene responsible for resistance to chloramphenicol, erythromycin, streptomycin, and tetracycline.

2. Taxonomy

The genus *Listeria* is found together with *Lactobacillus* in Bergey's Manual of Systematic Bacteriology in a section entitled "Regular, Nonsporing, Gram Positive Rods". Eight species for the genus *Listeria* are listed: *L. monocytogenes, L. seeligeri, L. ivanovii, L. innocua, L. welshimeri, L. grayi,* and *L. denitrificans* (Seeliger and Jones, 1986). In 1987, *L. denitrificans* was reclassified as *Jonesia denitrificans* (Rocourt et al., 1987). *L. murrayi* and *L. grayi* are being considered for reclassification into a single species named *L. grayi* (Rocourt et al., 1992), due to their existing similarities; both are non-pathogenic (Farber and Speirs, 1987) and are very rarely isolated from foods. Recently the genus *Listeria* has been reclassified as follows: *L. monocytogenes, L. innocua, L. ivanovii* subsp. *ivanovii, L. ivanovii* subsp. *londoniensis, L. welshimeri, L. seeliger,* and *L. grayi* (including *L. murrayi*) (Swaminathan et al., 1995). *L. ivanovii, L. seeligeri*, and *L.*

monocytogenes are the only species of *Listeria* pathogenic for both humans and animals. Although *L. ivanovii* has been linked with sporadic illness and *L. seeligeri* reportedly was the cause for one case of meningitis, *L. monocytogenes* remains the pathogen of major concern to man (Dever et al., 1993).

3. Pathogenesis and virulence of *Listeria monocytogenes*

Most *Listeria* species, with the exception of *L. monocytogenes*, can be regarded as harmless to man (Lovett, 1991). Not all strains of *L. monocytogenes* are pathogenic; rough variants possess reduced virulence and non-hemolytic mutants have completely lost their pathogenic potency (Hof and Rocourt, 1992). Differences in virulence between *Listeria* species and between different serovars of *L. monocytogenes* have been documented by Menudier et al. (1991). Thirteen serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab, and 7) (Bhunia, 1997) of *L. monocytogenes* have been described, three of which (4b, 1/2a, and 1/2b) have been implicated with 90% of all human listeriosis cases. The virulence mechanisms of *L. monocytogenes* are not fully understood; however, the single recognized factor proven to be crucial for virulence is listeriolysin O (Cossart, 1988); a chromosomal bound hemolytic extracellular 60 kDa protein (NACMCF, 1991; Kolstad et al., 1992). Listeriolysin, a heat-labile antigenic protein, is biochemically similar to streptolysin O and pneumolysin, and cross reacts antigenically with these hemolysins as well as with the hemolysins of *L. ivanovii* and *L. seeligeri* (Geoffroy et al., 1989). Listeriolysin's lytic activity is enhanced by reducing agents and suppressed by oxidation, exposure to cholesterol, or anti-streptolysin (NACMCF, 1991). Mutants of transposon-induced Hly (hemolysin deficient) *L. monocytogenes* are avirulent (Gaillard et al., 1986; Kathariou et al., 1987) and cannot grow in host tissue or in human enterocytes (Gaillard et al., 1987); however, their entry into macrophages is not affected (Gaillard et al., 1987; Kuhn et al., 1988). It has been concluded that once *L. monocytogenes* enters the macrophage, listeriolysin O is required to lyse the phagosomes, thus facilitating the release of the bacterium into the cytoplasm so that they can multiply (Datta et al., 1990). Internalin, an identified protein, appears to play an important role in the entrance of *L. monocytogenes* into epithelial cells (Gaillard et al., 1991) and a metalloprotease, encoded by a gene adjacent to the listeriolysin gene, may be part of its virulence as well (Domann et

al., 1991). In a review by Bhunia (1997) the following were noted as essential for *L. monocytogenes* entry into mammalian cells, survival in the phagosome, release into the cytoplasm and cell to cell spread: internalin, listeriolysin, actin polymerization protein, phospholipase, metalloprotease, and p60 proteins. Schlech et al. (1993) developed a model for *L. monocytogenes* infection using the Sprague-Dawley rat. Using light and electron microscopy, they demonstrated the attachment to, and invasion of, gastrointestinal mucosa by the virulent organism, showing a dose-dependent invasive infection. They were also able to show the lowering effect, in infective dose, of a decrease in gastric acidity.

The cell wall of *L. monocytogenes* is typical of Gram positive bacteria; a thick homogenous structure surrounding the cytoplasmic membrane without the outer membrane characteristic of Gram negative bacteria. Isolated dry cell walls are composed of approximately 35% peptidoglycan (cross-linked meso-diamionopimelic acid), the remaining carbohydrate consists of cell wall teichoic acids (polymers covalently linked to a specific site on the peptidoglycan, usually glycerol or ribitol, neutral sugars, and phosphate) (Farber and Peterkin, 1991). Two types of cell wall teichoic acids exist among *Listeria* serotypes; in the first, ribitol residues are covalently linked by phosphodiester bonds between C-1 and C-5 and are sometimes found with N-acetylglucosamine substituted at C-2; this type is associated with serotypes 1/2a, b and c, 3a, b and c, and 7. In the second, N-acetylglucosamine is integrated into the chain; this type is associated with serotypes 4a, b and d. *Listeria* cell walls also consistently contain lipoteichoic acids, in which a gylcolipid moiety, such as galactosyl-glucosyl-diglyceride, is covalently linked to the terminal phosphomonoester of the teichoic acid. This lipid region anchors the polymer chain to the cytoplasmic membrane. These lipoteichoic acids resemble the lipopolysaccharidees of Gram-negative bacteria in both structure and function, being the only amphipathic polymers at the cell surface.

Pathogenicity of *L. monocytogenes* is associated with a combination of three responses: beta-hemolysis or positive CAMP reaction on sheep blood agar, acidification of rhamnose, and non-reactivity on xylose (NACMCF, 1991).

Del Corral et al. (1990), quantitatively comparing thirty food and clinical isolates of *Listeria*, found all *L. monocytogenes* to be hemolyitc, invasive, weakly cytotoxic and lethal to immunocompromised mice. No significant difference between the clinical and food isolates was found. Van der Kelen and Lindsay (1992) documented the production of a new extracellular cytotoxin, from both a virulent and an avirulent strain of *L. monocytogenes,* over a wide range of temperature (4-37°C).

4. Heat resistance

Composition of the food and the physiological characteristics of the microbial cell will affect the heat resistance the microorganism will exhibit in a food. The ability of *L. monocytogenes* to survive the minimum high-temperature short-time (HTST) pasteurization processing guidelines (71.7°C for 15 s) of the FDA in milk, has been reported by Knabel et al. (1990), Bunning et al. (1988), Doyle et al. (1987), and Fernandez-Garayzabal et al. (1987). Knabel et al. (1990), indicated that the organism could not be detected by direct plating immediately after pasteurization, but that it was detected after a period of liquid enrichment in a reduced media. However, evidence accumulated since the outbreak of listeriosis in 1983, which was reportedly due to consumption of pasteurized milk (Flemming et al., 1985), indicated that the organism could not survive a proper HTST pasteurization treatment (Mackey and Bratchell, 1989; Lovett et al., 1987; Farber et al., 1988). Mackey et al. (1990) did not find exceptional heat resistance in any of 27 strains of *L. monocytogenes* and two strains of *L. innocua* heated at 57°C in broth.

Three factors have been found to influence the thermoresistance of the cell (Farber et al., 1988): heat shock response, growth at elevated temperatures and anaerobic recovery of heat injured cells. Bunning et al. (1990) studied the effect of prior heat shock on the thermotolerance of *L. monocytogenes* in broth culture and reported that although induction of increased thermotolerance was observed, it was not significant. Nevertheless, acquired thermotolerance of *L. monocytogenes* after short pretreatment at sublethal temperatures in broth (Knabel et al., 1990) in cells grown at 43°C compared to those that had either been heat-shocked at 43°C or grown at temperatures below 43°C, was significant. Pagan et al. (1997) determined that thermotolerance increases with the

increase in the duration of heat-shock up to 120 min regardless of the heat-shock temperature. Kim et al. (1994) studied the relationship between heat shock and listeriolysin O (LLO) production. Although heat shocking *Listeria* cells resulted in total reduction in LLO activity; within 4 h the heat-shocked cells resumed production of LLO at $37^{O}C$, reaching an activity level 40 times that of the initial shocked cells. The non-heat-shocked cells only showed a 2-fold LLO increase over the same period of time. Kim et al. (1994) also showed that a prior heat-shock increased the tolerance of *Listeria* to a heat treatment at 62°C. Smith et al. (1991) reported an increase in the thermal resistance of *L. monocytogenes* with increasing growth temperatures, indicating that cells growing at low temperatures are more susceptible to heat induced death. Wang and Hitchins (1994) demonstrated that survivors of lethal heating were injured hetergeneously; showing that the minority of survivors are slightly injured while the majority of survivors were severely injured. Several studies show that *L. monocytogenes* can be recovered after a heat treatment if proper temperature and time are allowed for repair of injured cells, even at low temperatures. Meyer and Donnelly (1992) showed a recovery of *Listeria* cells in whole and 2% milk, after a 20 min heat treatment at 55°C, based on incubation temperature following the heat treatment. Incubation at 4°C, led to a lag period with repair initiation beginning between days 8 and 10 and complete repair by day 19. At 10°C, repair began immediately and all strains were repaired within 4 days. Increasing the incubation temperatures to 26°C and 37°C, lead to complete repair by 13 and 9 h respectively. Wang and Hitchins (1994) also recovered heat injured *Listeria* cells by increasing the incubation in enrichment media from 24 to 48 h. These cells were recovered even in the presence of inhibitory selective agents and inorganic salts; leading to the conclusion that a longer enrichment time for injured cells would overcome the inhibitory effects. According to Knabel et al. (1990), a larger number of heat-injured cells of *L. monocytogenes* can be recovered by strictly anaerobic procedures rather than by conventional aerobic recovery procedures. The physiological condition of the microorganism, the media used for enumeration and the growth environment have a significant impact on the heat resistance of log-phase cells of *L. monocytogenes* Scott A (Linton et al., 1992).

5. Resistance to disinfectants

Effective microbiological reduction in the environment is only possible when the disinfectant is capable of inactivating microorganisms on surfaces in the presence of organic material. It has been specified, by Chambers (1956) and Williams (1984), that an effective sanitizer should result in a 5-log cycle reduction in the viable cell count after 30 s of exposure of the microorganism to the sanitizer. Best et al. (1990) found microorganisms dried onto surfaces to be more resistant to disinfectants than those in suspension. From 14 disinfectants studied, only three (povidone-iodine, chlorhexidine gluconate, and glutaraldehyde) were effective against *Listeria* spp. in the carrier test in the presence of serum, but ineffective in the presence of milk (2% fat), for which only sodium dichloroisocyanurate was effective. Only four formulations (chloramine-T, phosphoric acid, an iodophor, and formaldehyde) were not effective in the suspension test, regardless of the content of organic matter. *L. monocytogenes* appeared to be slightly more resistant to disinfectants than *L. innocua*.

Four commonly used sanitizers, two quaternary ammonium compounds (QAC), an acid anionic sanitizer, and a chlorine based sanitizer, were evaluated for the efficacy against two strains of *L. monocytogenes* and one strain of *L. innocua* (Sallam and Donnelly, 1992). All sanitizers showed an efficacy of > 99.999% against all test organisms, regardless of the type of sanitizer, concentration, or exposure time. The ability of two enrichment broths, University of Vermont (UVM) and *Listeria* repair broth (LRB) to recover *Listeria* was also tested. In all cases, LRB recovered a greater number of cells, including repair and growth of sanitizer-injured *Listeria*.

Van de Weyer et al. (1993), studied the bactericidal activity of nine disinfectants, used by the food industry, on *Listeria* spp. (*L. innocua, L. welshimeri, L. monocytogenes* 1/2a, 1/2b, 1/2c, and 4b). The chemical agents evaluated included phenolic compounds, alcohols, quaternary ammonium compounds, surface active agents, aldehydes and discochlorine tablets. No particular resistance of *listerias* to the disinfectants was found, but a decrease in efficacy in some disinfectants due to organic matter was reported.

El-Kest and Marth (1988a), in a study of the effect of sodium hypochlorite (at 25°C and pH 7) on *L. monocytogenes* strain Scott A, reported higher bacterial resistance

to chlorine when the cells were harvested from a 24 rather than a 48 h old culture, grown in tryptose broth rather than on a tryptose agar slant, washed and suspended using 20 rather than 0.312 Mm phosphate buffer solution, and that reduction in the cell number occurred rapidly in the first 30 s of exposure. A larger number of *L. monocytogenes* Scott A survived at 25°C than 35°C, and more survivors were observed with increasing pH in the range of 5 to 9 (El-Kest and Marth, 1988b). Previous work from Brackett (1987) on reagent-grade sodium hypochlorite and household bleach showed that chlorine concentrations less than 50 ppm had no antimicrobial effect but exposure to 50 ppm or greater resulted in no recovery of viable cells.

Mustapha and Liewen (1989) studied the antimicrobial effect of sodium hypochlorite and quaternary ammonium sanitizers against *L. monocytogenes*, *in vitro* and on stainless steel chips inoculated with the organism. A larger decrease in the number of viable cells was observed for the *in vitro* experiment, and production of a fibrous like material, similar in appearance to acidic polysaccharide fibrils produced by *Pseudomonas* spp., was reportedly observed when evaluating the attachment characteristics of *L. monocytogenes*. Studies by Mosteller and Bishop (1989) and Herald and Zottola (1987), support the surface attachment and adherent microcolony formation potential of *L. monocytogenes*. Sashara and Zottola (1993) suggest, that under flowing conditions, the presence of an exopolymer-producing microorganism may be more important than hydrophobicity, surface charge, or flagellar movement in the attachment of *L. monocytogenes* to inert surfaces. Mosteller and Bishop (1989) reported on sanitizer resistance of microorganisms attached to gasket surfaces. The studies of Frank and Koffi (1990) and McCarthy (1992) confirmed the attachment of cells to surfaces could provide protection against chemical sanitizers.

Lee and Frank (1991) studied the resistance of adherent microcolonies of *L. monocytogenes*, attached to stainless steel, to hypochlorite and heat. They reported that cells incubated for 8 days were over 100 times more resistant to exposure to 200 ppm hypochlorite for 30 s, than the equivalent population incubated for 4 h. A surface dependent resistance of *L. monocytogenes*, towards sanitizers and cleaners, was documented by Krysinski et al. (1992). They found adherent cells to be more resistant to

sanitization and cleaning in polyester/polyurethane surfaces followed by polyester and stainless steel.

Ren and Frank (1993) studied the effect of starvation of *L. monocytogenes* in its resistance to a quaternary ammonium compound and found that for planktonic cells the effect of starvation on sanitizer susceptibility was less than the effect of growth in diluted media. They reported that the major effect influencing susceptibility to sanitizer is its growth as a biofilm, confirming previous findings reported by Frank and Koffi (1990).

A study by Tucan (1993) compared the efficacy of three sanitizers (quaternary ammonium compound, iodophor, and chlorine) at different exposure temperatures (2-25°C) and times (0.1-1.5 min). At 25°C, all three sanitizers were effective regardless of their concentrations. Quaternary ammonium compound (QAC) and chlorine, at 100-200 ppm and 25-200 ppm respectively, inactivated a comparable number of *Listeria* cells at 2°C as they inactivated at 25°C; showing that cold temperature did not have an effect on the sanitizers. At concentrations of 50 ppm and lower, the efficacy of the QAC and the iodophor decreased as the exposure temperature decreased, but this could be overcome by increasing the exposure time. Chlorine was effective throughout the temperature range tested.

The repair and growth of *Listeria*, after exposure to sanitizers, suggests that sanitizers may induce injury rather than death. Enrichment procedure, bacterial strain, exposure time, and type and concentration of sanitizer affect the extent of cell death, injury, and repair (Sallam and Donnelly, 1992). QACs lethal effect has been attributed to activities including reactions with cell membranes, denaturation of essential cell proteins, and enzyme inactivation. In the same article, Sallam and Donnelly (1992) suggest that chlorine sanitizers may damage the cell membrane of *Listeria* leading to leakage of cellular components, as well as, forming substitution products with amino acids and proteins. Bunduki et al. (1995) proved, through scanning supernantent fluids from sanitizer-injured *Listeria* cells, that any stress to the cell membrane was insufficient to allow leakage of cellular components. Using chloramphenicol, rifampin, CCCP, and cycloserine D which inhibit peptidyl transferase on ribosomes, ribonucleic acid (RNA) polymerase and hinders synthesis of messanger RNA, electron transport and oxidative phosphorlation,

incorporation of D-alanine into the peptide units of the cell wall, respectively, they showed that the sanitizer-injured cells were affected by all of the aforementioned chemicals except cycloserine D. Thus indicating that chlorine does not disrupt the cell wall, but causes internal damage to the *Listeria* cell.

B. Listeriosis

Due to the ubiquitousness of *L. monocytogenes*, it appears that most individuals frequently ingest the bacterium without experiencing ill effects. The organism is a transient constituent of the intestinal flora excreted by 1-10% of healthy humans (Farber, 1991).

In Cambridge in 1924, the bacterium *L. monocytogenes* and the disease listeriosis were first recognized in laboratory animals (Murry et al., 1926). The first evidence of listeriosis by food transmission was reported by Schlech et al. (1983) based on an outbreak, due to contaminated coleslaw, in the Maritime Provinces of Canada in 1981. It was recommended, in 1986, that listeriosis become a reportable disease (NACMCF, 1991).

Several predisposing, or risk, factors have been associated with listeriosis (NACMCF, 1991). These include age (those over 60 years of age and neonates), malignancy, immunosupression (corticosteroids are the immunosupressive agents most frequently associated), and pregnancy. All of the persons in these categories have a disruption in their T cell-mediated immunity. Other risk factors that have been associated with listeriosis include: liver disease, particularly cirrhosis, splenectomy or a dysfunctional spleen, antacid and/or cimetidine therapy which neutralizes gastric acidity. Intrinsic susceptibility to *Listeria monocytogenes* exists in certain inbred mice, this genetic susceptibility may also exist in humans (McLauchlin, 1996). Listeriosis may, in rare instances, develop in a healthy individual (McLauchlin, 1996).

Listeriosis can be classified by mode of transmission. There are three known modes of transmission: direct contact with infected animals; cross-infection during the neonatal period; and foodborne. Direct contact with infected animals, or animal material, leads to cutaneous lesions on the arms of those (typically farmers and veterinarians) who come in contact with infected animals (McLauchlin, 1996). During the neonatal period, cross-infection can occur between infants; this has been reported at least 51 times in literature (McLauchlin, 1996). When a healthy infant develops late onset listeriosis, it is often seen that delivery or nursing occurred in the same room as an infant born with congenital listeriosis (McLauchlin, 1996). The World Health Organization Working

11

Group (1988) agreed that the principle route of transmission of listeriosis was through contaminated foods.

Listeriosis is also classified based on onset. Schelch (1996) provides an outline of each of the three onset classes: early onset, late onset, and adult onset. Developing from septicemia in the mother, early onset listeriosis is a transplacental infection. Typically this is characterized as a flu-like illness in the mother and the delivery of an acutely ill infant. Once the infant is delivered, the mother spontaneously resolves and often does not require any therapy; while the infant shows severe sepsis characterized by wide spread granulomatous change in the viscera. Neonatal meningitis is the presentation of late onset listeriosis. It is presumed that the infant is colonized by *L. monocytogenes* during delivery though the birth canal. The mother shows no sign of illness, yet fecal and vaginal colinization may be demonstrated. An incubation period of 10-20 days, following birth, results in development of neonatal meningitis syndrome. Adult onset listeriosis frequently presents itself as meningoencephalitis, meningitis, and sepsis.

NACMCF (1991) reports the overall incidence of listeriosis to be 0.8 per 100,000 persons, with the incidences for those over the age of 70, pregnant women and persons with AIDS to be 2.1 per 100,000 population, 12.4 per 100,000 live births, and 200 per 100,000 population, respectively. In 1986 the FDA and CDC estimated that the annual incidence of listeriosis in the US to be 1600 cases with over 400 deaths; the study was repeated and in 1990 the annual incidence in the US was estimated to be 1850 cases with 425 deaths (Shank et al., 1996). The annual incidence of invasive listeriosis, between November 1988 and December 1990, was reported to be 7.4 cases per million population with 23% of the cases being fatal (Schuchat et al., 1992); this dropped to 4.4 per million population in 1993 (Tappero et al., 1995). The Dutch report an annual rate of 20-25 cases, resulting in 4-5 cases per million inhabitants (Qvist, 1996), while England and Wales have 2-3 cases per million population (McLauchlin, 1996).

C. Risk assessment and policy on *L. monocytogenes*

Due to the ubiquitousness, ability to grow at refrigeration temperatures, and unknown infective dose, *L. monocytogenes* is cause for concern to the food industry. This concern has lead to policies (Lahellec, 1996; Skinner, 1996; Qvist, 1996; Farber and Harwig, 1996; Shank et al., 1996) on the acceptable presence of *L. monocytogenes* in certain foods. Based on the fact that humans are frequently exposed to *L. monocytogenes* yet listeriosis is rare (McLauchlin, 1996; Notermans et al., 1998), one must first determine the risks before making policy.

Guidelines and regulatory programs for the control of foodborne microbial agents have existed in the U.S. for nearly 100 years. Currently, high-profile programs such as Hazard Analysis and Critical Control Points (HACCP) and International Operations Standard 9000 (ISO 9000) have been adopted to reduce the risk of pathogens (Jaykus, 1996).

1. Risk assessment

Jakus (1996) reviewed applying Quantitative Risk Assessment (QRA), the technical assessment of the magnitude and nature of a risk caused by a hazard, to microbial food safety risks. QRA is composed of four components: (1) hazard identification, (2) exposure assessment, (3) dose-response assessment, and (4) risk characterization. When evaluating *L. monocytogenes,* two populations must also be taken into account: those with a predisposing factor for listeriosis and those normally not affected (Van Schothorst, 1996).

Hof and Rocourt (1992) emphasize the importance of determining what strain of *L. monocytogenes* is present before deciding if the contaminated food is a health risk. They collected data that shows all recent human listeriosis outbreaks and sporadic cases were caused by serovar 4b, yet the percentage of food contaminated by strains of this serovar are low; with most food and environmental isolates belonging to serovar 1/2. They also state that risk cannot be determined based on hemolysis alone because hemolysis may or may not indicate pathogenicity, which is summarized as follows: all non-hemolytic *Listeria* strains are non-pathogenic; all pathogenic *Listeria* strains are hemolytic; not all hemolytic *Listeria* strains are pathogenic.

Notermans et al. (1998) studied the risk assessment of *L. monocytogenes* in respect to humans and mice. The risk assessment for humans was based on results from microbiological data obtained from various food products. Based on their analyses, they determined that each person was exposed 3.8 times to 10^5 organisms and 0.8 times to $> 10^6$ organisms per year. The mouse study produced several interesting results. The mouse study showed that there were clear thresholds in the dose-response curve; which may be attributed to the fact that inbred mice with nearly identical immune responses were utilized. The study also showed that the physical barrier offered by the intestines was finite and not clearly affected even when the immune system was depressed, and that there was a clear component of immunological protection from previous exposure. This showed two independent systems that contribute to protection: a non-adaptive response offered by the physical status of the intestine, and the adaptive response of the immune system. Applying this same idea to humans supports the epidemiological data that listeriosis is rare in humans.

Buchanan et al. (1997) developed a conservative dose-response relationship for incidence of listeriosis and levels of *L. monocytogenes* based on data, collected in Germany by Teufel and Bendzulla (1993), on the prevalence of *L. monocytogenes* in smoked fish. They assumed that *L. monocytogenes* fit the exponential dose-response model $P = 1 - e^{RN}$, where P is the probability of an adverse effect, N is the amount consumed (CFU), and R is a constant specific to each pathogen. Using their model, they showed the total probability of acquiring listeriosis to be 3.1×10^{-12}, 2.17×10^{-10}, 6.19×10^{-9}, and 6.19×10^{-7} for the levels of *L. monocytogenes* being 0.04 CFU/g, 1.00 CFU/g, 100 CFU/g, and 1000 CFU/g, respectively. Their overall total calculated risk of acquiring listeriosis from contaminated food was 6.25×10^{-7}. From this conservative model it can be seen that the probability of a high-risk individual acquiring listeriosis is extremely low unless the organism is present in high levels.

2. Policy

2.1 The French position and policy

Lahellec (1996) presented an overview of the French position on *L. monocytogenes* in foods. The Conseil Superieur D'hygiene Publique de France (Sept.

1992) set forth the following considerations for *L. monocytogenes*: it is impossible to guarantee *L. monocytogenes* free products with the exception of products heated in their final wrapping or aseptically conditioned after heating; strict prevention measures must be set up in order to remove *L. monocytogenes* from foods at each stage of food production, from raw materials to the final product in the possession of the consumer; when it seems impossible to avoid contamination, a tolerance level of 100 cells/g of product is acceptable. The Ministry of Agriculture, Fisheries and Food has set up controls as to what to do when *L. monocytogenes* is detected based on its past history. When a non-epidemic *L. monocytogenes* is isolated from a food, the food is classified into one of three groups: foods for 'at risk' populations; foods aseptically conditioned after heating or heated in their own wrapping; and raw foods or those susceptible to re-contamination after treatment. After classification, intervention measures are set up and guidelines for intervention are followed. If the strain of *L. monocytogenes* isolated belongs to an epidemic phagovar in a food implicated in one or more pathological cases in humans, then the following measures must be taken within 24 h of the isolation: corrective measures and recall of the foods still on the market that may be incriminated in listeriosis cases; official examination of official and auto control results; cessation of processing within 24-48 h for cleaning and disinfection; changes in thermal processing with respect to time and temperature; and three weeks after new cleaning and disinfection implementation, there must be no *L. monocytogenes* present before re-marketing of the products will be allowed. Similar measures, depending on the plant, will be taken if an epidemic phagovar strain of *L. monocytogenes* is isolated from a food that has not been implicated in human cases. France also stresses the importance of implementing HACCP.

2.2 The United Kingdom position and policy

The UK Department of Health has several initiatives in controlling the cases of listeriosis; these were outlined by Skinner (1996) at the International Food Safety Conference session on *L. monocytogenes* in 1995. The Department of Health has advised pregnant women and the immunocompromised to avoid eating soft ripened cheeses, pate, and to reheat cooked/chilled meals and ready-to-eat poultry. They have also provided advice to consumers on food hygiene. As far as a tolerance level of *L. monocytogenes* is

concerned, the UK does not favor devising an 'acceptable' level based on the fact that there is no scientific evidence for the infective dose and host susceptibility varies. Until such information can be obtained, the UK is channeling resources into good hygiene procedures and the implementation of HACCP.

2.3 The Danish position and policy

In an overview of the Danish policy on *L. monocytogenes* in foods, Qvist (1996) discusses the development of a realistic policy and technologies implemented to control growth of *L. monocytogenes* to high levels. Based on data collected in Denmark, the annual rate of listeriosis 20-25 cases or 4-5 cases per million inhabitants. Coupled with the information on the occurrence of *L. monocytogenes* in foods on the Danish market, it is concluded that millions of food products containing low levels of *L. monocytogenes* are sold on the market every year without evidence of listeriosis. With this information, the Danish government has declared that a concentration of *L. monocytogenes* not exceeding 10 cells/g of food at the point of consumption is considered acceptable for all populations. If the food supports the growth of *L. monocytogenes* and has a prolonged shelf life, or is specifically intended for high risk populations, then there should be no cells in a 25 g food sample. In foods with frequent occurrence of *L. monocytogenes*, the Danish government believes that satisfactory safety can be obtained by HACCP, hurdle technology (substances or processes which prevent or inhibit microbial, physical, or chemical deterioration of foods) and a reduced shelf life.

2.4 The Canadian position and policy

The Canadian policy on *L. monocytogenes* as explained by Farber and Harwig (1996) was based on HACCP and the use of a health risk assessment. They have adapted their policy based on the knowledge that the risk of contamination can be reduced, but eradication of the organism is not always possible. All ready-to-eat foods (RTE) (those not requiring further heating by the consumer) are classified into three categories: (1) RTE foods casually linked to listeriosis (soft cheese, pate, coleslaw mix with a shelf-life > 10 days, jellied pork tongue); (2) RTE foods that support the growth of *L. monocytogenes* with a refrigerated shelf-life > 10 days, and are not included in the previous category; (3) RTE foods that support the growth of *L. monocytogenes* with a

refrigerated shelf-life ≤ 10 days, as well as all RTE foods that do not support growth. Action levels and consequences are as follows: category 1, if > 0 CFU/50 g sample is present a Class I recall at the retail level is issued, as well as, consideration of a public alert, and a follow-up at the plant are included in the immediate action; category 2, if > 0 CFU/50 g sample is present a Class II recall at the retail level is issued, as well as, consideration of a public alert, and a follow-up at the plant are included in the immediate action; category 3, if ≤ 100 CFU/g and adequate Good Manufacturing Practices (GMPs) are in place then the product may be sold, but if ≤ 100 CFU/g and inadequate or no GMPs are in place then a Class II recall or stop of sale will be considered, or in the case of > 100 CFU/g a Class II recall will be issued or a stop of sell. An exception to this policy is for RTE foods specifically marketed to susceptible individuals (pregnant women, elderly, infants, immunocommprommised). These foods would automatically be placed in category 1 or 2 irrespective of the product.

2.5 The United States position and policy

Shank et al. (1996) describe the U.S. policy on the presence of *L. monocytogenes* in foods. The US federal agencies (FDA and USDA) have established a zero-tolerance for *L. monocytogenes* in cooked, RTE foods. The FDA policy for *L. monocytogenes* was established in 1985 and states that the detection of *L. monocytogenes,* by the FDA method, in a RTE food is a violation of the Federal Food, Drug and Cosmetics Act, section 402(a) (1) and (4); thus if *L. monocytogenes* is isolated from a RTE food, the food is considered to be adulterated. The USDA-FSIS policy is also based on the definition of adulteration; stating that *L. monocytogenes* in a RTE food is considered an added agent (Definitions, 9 CFR 301.2). Additional U.S. protective measures include implementation of GMPs and HACCP.

The U.S. zero-tolerance policy has been vigorously challenged by many, based on facts such as that in the large population of the US there are only a few cases of listeriosis per year and that most of these cases involve only certain foods, mainly soft cheeses, undercooked chicken and poorly reheated hotdogs. The World Health Organization's Informal Working Group on Foodborne Listeriosis (WHO, 1988) concluded that 'the total elimination of *L. monocytogenes* from all food is impractical and may be impossible'.

The Danish government has criticized the zero-tolerance policy based on its negative results: needless withdrawal of wholesome foods, unnecessary loss of consumer confidence, and unjustified losses for the food industry (Qvist, 1996).

D. Isolation and Culture Protocols

Most methods will allow isolation of all *Listeria* species, therefore, specific tests to identify *L. monocytogenes* must be performed. Lovett (1987; 1988) reported on the difficulty of using direct plating for the isolation of *L. monocytogenes*. The organism is present in most foods at low concentrations (less than 100 CFU/g) making it necessary to use an enrichment procedure prior to its successful isolation. The presence of naturally occurring microflora in foods, also makes the isolation of *L. monocytogenes* difficult (Cassiday et al., 1989).

1. Enrichment

The use of cold enrichment (Gray and Killinger, 1966; Gray et al., 1984; Hayes et al., 1986), as both a pre-enrichment and as the only enrichment step, has been the most popular technique applied to increase population numbers of *Listeria* spp. while inhibiting microflora unable to grow at refrigeration temperatures of 4°C. This procedure involves long incubation periods since the generation time for *Listeria* spp. at this temperature is 1.5 days (Lovett, 1988).

More recent is the preparation of enrichment formulations using antibiotics as selective agents to suppress the growth of competitors to *Listeria* spp. (Cassiday et al., 1989). Since the incubation temperature used can be the optimum growth temperature for *Listeria* spp., a more rapid growth of the organism is obtained (Lovett, 1988). Two procedures, the Food and Drug Administration (FDA) method (Lovett and Hitchins, 1988) and the U.S. Department of Agriculture - Food Safety and Inspection Service (USDA-FSIS) method (McClain and Lee, 1988) are the cultural methods most used in the U.S. today. Both procedures use enrichments with selective agents to isolate *L. monocytogenes* in a sample. Selective agars, based on a formula by Curtis et al. (1989), are used in both procedures for detection of *Listeria* species. Both agars rely on the ability of *Listeria* spp. to hydrolyze esculin to esculetin (6,7-dihydroxycoumarin) which reacts with ferric ions in the medium to produce a black color (McFaddin, 1980).

Following the concerns of not detecting injured cells, the FDA now recommends using 0.1% sodium pyruvate in the pre-enrichment broth (Hitchins, 1992). Studies by Knabel and Thielen (1995) and Patel and Beuchat (1995) showed that the removal of

oxygen from both the medium and the headspace greatly increased recovery of heat injured *Listeria*. In addition to removing oxygen, Knabel and Thielen (1995) identified lithium as a selective agent; it inhibits the growth of background microflora without inhibiting the resuscitation and growth of heat injured *L. monocytogenes*.

Bailey and Cox (1992) developed a universal pre-enrichment (UP) broth that allows the simultaneous recovery and detection of *Salmonella* and *Listeria* in foods. They reported that as few as 10 heat-injured *L. monocytogenes* multiplied to at least 10^5 CFU/ml. Viable *L. monocytogenes* can be enumerated from pure cultures by a direct microscopic observation method. Enumeration of starved cells can be achieved following the procedure by Frank et al. (1992). Cells grown in a tryptic soy broth, containing yeast extract and novobiocin, are stained with acridine orange. The cells become elongated and can be counted with an epifluorescent microscope.

2. Isolation procedures

2.1 Cultural methods

2.1.1 FDA method

The Food and Drug Administration (FDA) method developed by Lovett et al. (1987) and revised by Lovett and Hitchins (1988) and Hitchins (1991, 1992) has been used for isolating *L. monocytogenes* from milk and dairy products in both the U.S. and Europe. In the current method (Hitchins, 1992), samples are mixed with an enrichment broth (EB), which is based on a modification of the medium of Ralovich et al. (1970, 1971) and consists of trypticase soy broth supplemented with 0.6% yeast extract and with acriflavin-HCl (15 g/ml), nalidixic acid (40 g/ml), and cycloheximide (50 g/ml) as selective agents. EB is incubated for 24 to 48 h at 30°C and then streaked onto two selective agars, Lithium Chloride-Phenylethanol-Moxalactam (LPM) and Oxford agar (OAX) at each time. These agars are incubated at 30°C and 35°C, respectively, for 24 to 48 h. Ferric iron salt and esculin may be added to LPM to avoid the use of Henry's illumination. Suspect colonies from either agar must be streaked onto non-selective agar and confirmed using biochemical and hemolytic reactions. For samples suspected of containing stressed *Listeria* cells (heat or freeze-damaged cells), the sample may be cultured in enrichment broth with 0.1% (w/v) sodium pyruvate and no selective agents and incubated for 6 h at

30 °C (Hitchins, 1992). After 6 h, the prescribed levels of selective agents are added and incubation is continued at 30°C for 48 h and the isolation procedure is continued as described above. Once all suspect colonies are isolated, they are identified and confirmed with biochemical tests.

2.1.2 USDA method

The USDA method for isolation of *L. monocytogenes* from meats and poultry products was developed by McClain and Lee (1987, 1988, 1989). This procedure (Pusch, 1989) uses a two step *Listeria* enrichment broth to reduce the interference of competing microflora. The current method revised and issued in 1989 uses a 25 g sample. The first enrichment broth (LEB) consists of protease peptone, tryptone, Lab Lemco powder (Oxoid, Unipath, Ogdensburg, NY), yeast extract, sodium chloride, buffers, esculin, nalidixic acid (20 g/ml) and acriflavin (12 g/ml). The primary enrichment is transferred to a secondary broth, Fraser Medium (FM) (Fraser and Sperber, 1988), which uses LEB as a base with the addition of increased acriflavin-HCl, lithium chloride, and ferric ammonium citrate. This indicator broth relies on the ability of *Listeria* spp. to hydrolyze esculin, producing a black color. Lithium chloride is added to FM to inhibit enterococci, since these organisms also hydrolyze esculin. A negative result for *Listeria* spp. is indicated by no change in the color of the medium. Fraser medium is known to have a false positive rate as high as 18% and a false negative rate of less than 1% (Fraser and Sperber, 1988). A presumptive positive is streaked onto modified Oxford agar (MOX), a selective agar modified from Oxford *Listeria* selective medium, developed by Curtis et al. (1989), which replaces the use of Lithium Chloride-Phenylethanol-Moxalactam agar (LPM). This plating medium, with the esculin hydrolysis system, provides rapid visual observation of presumptive *Listeria* colonies, eliminating the use of Henry's illumination technique required with LPM agar. All suspect colonies are identified and confirmed using blood agar and biochemical tests.

2.1.3 Comparison of cultural methods

Lee and McClain (1986) reported the usefulness of LPM agar in recovering *L. monocytogenes* from mixed cultures due to the inhibitory control upon the growth of other bacteria. Buchanan et al. (1989) compared Modified Vogel Johnson (MVJ) agar

and LPM agar in their efficiency to detect *Listeria* spp. in foods, including seafood, when using both direct plating and direct plating with a three-tube most-probable-number (MPN) enrichment. They reported the adequacy of direct plating methods when analyzing samples with high levels of *Listeria* spp. and in cases where background microflora is minimal. However, levels of *Listeria* spp. of less than 2 CFU/g are usually found in seafood samples (Buchanan et al. 1989; Budu-Amoako et al., 1992b), making it impossible to detect them by direct plating. MVJ agar presents an advantage in the enumeration of *Listeria* spp. from foods due to its capacity to differentiate *Listeria* spp. from other microorganisms; based on the results obtained by these investigators, the Centers for Disease Control and Prevention adopted the simultaneous use of the USDA and the NGFIS method to isolate *L. monocytogenes* from foods. Farber (1993) also suggests the use of two conventional methods, believing that 17% of naturally contaminated samples may be falsely reported as negative when a single method is used.

2.2 Rapid methods

Among methods developed for the rapid detection and identification of *Listeria* are the use of antibody-based assays (enzyme-linked immunosorbant assay: ELISA, EIA) (Mattingly et al., 1988; Kerr et al., 1990; Vanderlinde and Grau, 1991; Knight et al., 1996; Feldsine et al., 1997), nucleic acid probe-based assays (DNA hybridization) (Pusch, 1989; Datta and Wentz, 1989; Chenevert et al., 1989; Edmond et al., 1993) and a modified microtiter plate procedure (MMP) for characterization of *Listeria* spp. (Siregusa and Nielsen, 1991). Newer kits such as Accuprobe by Gen-Probe, Inc. (San Diego, CA), have been developed to specifically detect *L. monocyotgenes* in foods (Dever et al., 1993; Farber, 1993; Okwumabua et al., 1992.)

Listeria-Tek, an ELISA manufactured by Oreganon Teknika Corp. (Durham, NC), can detect *Listeria* spp. within three days. Monoclonal antibodies, which react specifically with an antigen found in all *Listeria* spp., were developed and characterized by Butman et al. (1988). The *Listeria*-Tek assay (Mattingly et al., 1988) was developed using two of these specific monoclonal antibodies directed against the *Listeria* antigen. Samples were enriched in broth (FDA or USDA), heated, and added to polystyrene microtiter plate wells coated with antibodies. Immune complexes form between the *Listeria*-specific antigen

portion of the immune complex when it is added. After incubation, aspiration, and thorough washing, the sample is incubated with tetramethylbenzidine (TMB). TMB is a substrate for horseradish peroxidase. A stop solution (H_2SO_4) is added and a yellow color develops if the sample is positive. Color intensity is determined spectrophotometrically. All *Listeria* species give a positive result, so samples must be streaked to selective media and tested for hemolytic and biochemical reactions for final identification of the species (Dever et al., 1993). The detection limit of the ELISA for *L. monocyotgenes* in pure culture is approximately 10^6 CFU/ml (Norrung et al., 1991). Recently an ELISA based test, the TECRA *Listeria* Visual Immunoassay (TLVIA), was accepted by the Association of Official Analytical Chemists (AOAC) International (Knight et al., 1996).

Feldsine et al (1997) describes the Assurance Polyclonal Enzyme Assay (EIA) which uses highly purified antigens against *Listeria*. This assay utilizes a microwell format with a 96-well test plate. The purified antibodies to *L. monocytogenes* and related *Listeria* spp. are bound to the microwell plates. Samples are enriched in modified Fraser Broth with lithium chloride (28 h) and are transferred to buffered *Listeria* enrichment broth (24 h). Enriched samples and positive controls are added to the microwell plate. *Listeria* antigens, present in the samples, bound to the microwells forming antibody-antigen complexes. Unbound sample is washed away. *Listeria* specific antibody is added, followed by an incubation and wash. Conjugate alkaline phosphatate is added to bind enzyme to the *Listeria* antigens. Following an additional incubation and washing, the substrate p-nitrophenylphosphate is added. The enzyme-substrate reaction yields a product with an absorbance reading which is read spectrophotometrically at 405-410 nm. Presumptive positive samples are indicated as those reading above the calculated cutoff value, and are confirmed by cultural methods. This method has been adopted first action by AOAC International (Feldsine et al., 1997).

Development of kits that do not require enrichment steps, which confirm the identity of Listeria spp. within 24 h, includes the Listertest® (VICAM, Watertown, MA). Listertest consists of two separate tests that can be run simultaneously. Listertest Lift® identifies all *L. monocyotgenes* and several non-*monocytogenes Listeria* species, while Listertest Mac® identifies pathogenic strains. The test employs immunomagnetic beads

coated with antibodies directed against *Listeria*. These beads capture *Listeria* in a food sample after 2 h of exposure. Then, by using a magnet, the beads are extracted and subsequently analyzed by either the Listertest Lift® or the Listertest Mac®. Sensitivity of both tests can be < 1 CFU/g depending on the type of sample analyzed (Dever et al., 1993). A comparison between immunomagnetic capture and standard cultural methods for detection of *Listeria* in several spiked and naturally contaminated food and environmental samples was done by Jackson et al. (1993a). They reported that the immunomagnetic capture was at least as sensitive as cultural methods for detection of *Listeria* in seafood, meats, dairy foods, and environmental samples; quantitative results were obtained within 24 h. Further testing of the Listertest® method, conducted by Mitchell et al. (1994), showed that the USDA method to detect *Listeria* was less efficient than the Listertest® at detecting low levels based on statistical significance. The Listertest® has also been compared to the FDA Bacteriological Analytical Manual MPN method (McCarthy, 1997a). In this study, the Listertest was found to be statistically more sensitive for detecting and/or enumerating *L. monocytogenes* at low levels (<10 CFU/g) in crab meat and smoked salmon. In addition, results are obtained, both positive and negative, within 48 and 72 hours respectively, whereas the MPN method requires 4 (positive) and 6 (negative) days. A similar test kit, ListerScreen™ (AES Laboratoire, Combourg, France), using immunomagnetic beads and selective plating on PALCAM agar, was approved by the French Association of Normalization, based on the work by Avoyne et al. (1997).

McLauchin and Pini (1989) reported on the use of two monoclonal antibodies in a direct immunofluorescent test to detect *L. monocyotgenes* in foods. Two monoclonal antibodies were conjugated to fluorescein isothiocyanate (FITC) and successfully used in a direct immunofluorescence test to demonstrate *Listeria* in seven samples of soft cheese previously determined positive for *L. monocyotgenes* through cultural methods.

The Gene-Trak *Listeria* Assay by Gene-Trak Systems (Framingham, MA) is a commercial DNA hybridization method used to detect *Listeria* species in food and environmental samples (King et al., 1989). A more recent version detects *Listeria* species using *Listeria* specific DNA probes, and a colorimetric, non-radioactive, detection system.

After a two day broth and agar enrichment of the food sample, the assay (2.5-3 h) begins by lysis of the target organisms to release ribosomal RNA (rRNA). Two synthetic oligodeoxyribo-nucleotide probes (capture and detection probes) are directed against rRNA of the target organism. The advantage of using a probe directed against rRNA instead of chromosomal DNA is that multiple copies of rRNA (1,000-10,000) are present in each cell. The capture probe hybridizes to a unique sequence of target rRNA. A poly-dA (polydeoxyadenylic) tail attached to one end of the capture probe binds the target probe hybrids to a solid support. A detection probe labeled with fluorescein hybridizes to a unique sequence of the same target rRNA molecule. The solid support in this assay is a plastic "dipstick" coated with poly-dT (polydeoxythymidylic acid). Hybridization between poly-dA and poly-dT molecules binds the target probe hybrids to the solid support. Colorimetric detection is accomplished spectrophotometrically. In the Gene-Trak *Listeria* Assay, growth is removed from the entire LPM plate and resuspended in 1 ml of phosphate-buffered solution, which would provide a detection limit of approximately 10^6 CFU/ml (Dever et al., 1993).

Matar et al. (1997) developed a method based on listeriolysin O (LLO), using an anti-listeriolysin O monoclonal antibody. The listeriolysin O latex agglutination assay (LLOLAT) is specific for *L. monocytogenes* and can detect LLO without the isolation of pure cultures. In this assay, polystyrene amidine-modified latex beads were sensitized with purified monoclonal antibody against LLO and then mixed by inversions for 1 h at room temperature. Following an overnight incubation at 4°C, sonication and washing prepared the cells for testing. The LLOLAT was carried out on clear glass slides with 13 mm rings. Fifty microliters of each sample to be tested were mixed on the slides with 10μl of latex reagent. After a 5 min rotation at 100 rpm, slides were observed under a high-intensity lamp; scoring results as either positive or negative based on the presence or absence of agglutination. Using the same principles of the latex agglutination test, Serobact™ *Listeria* (Medvet Science Pty Ltd, Adelaide, Australia) has been released. This assay utilizes latex particles, coated with polyvalent antisera against the A, B, C, and E flagellar antigens of *Listeria* spp., and their ability to agglutinate with all motile strains of *Listeria* (Murtiningsih and Cox, 1997). In order to determine the species present, the Microbact™

Listeria test (Medvet Science Pty Ltd, Adelaide, Australia) is paired with the Serobact™ test. Microbact™ *Listeria* is a miniturized biochemical test kit designed to identify all species of *Listeria*. This system utilizes fermentation of several of carbohydrates, esculin hydrolysis and hemolysis (Murtiningsih and Cox, 1997).

With the advances in molecular biology, the polymerase chain reaction (PCR) has been tested as a method for detecting *L. monocytogenes* (Herman, 1997). PCR is a very sensitive method, which amplifies DNA or RNA sequences and allows for the reduction or elimination of enrichment procedures. One of the advantages of PCR over other methods is that it can detect culturable, viable but non-culturalable (VBNC), and dead cells. Culture methods (FDA and USDA) can not detect cells unless they multiply during enrichment. A VBNC bacterium, which would not be detected by culture methods, may be able to resuscitate to a normal culturable state under proper conditions leading to concern in the case of pathogens (Oliver, 1995). Direct PCR, from the DNA extracted from the bacteria, shows all *L. monocytogenes* (culturable, VBNC, and dead). By using mRNA as the target for PCR, all culturable and VBNC can be detected based on the principle that most bacterial mRNA half lives are < 2 min (Belasco and Higgins, 1988).

2.2.1 Comparison of rapid methods

Both ELISA and DNA hybridization assays have been evaluated in studies comparing rapid methods to cultural methods. Hesick et al. (1989) compared four methods: *Listeria*-Tek, Gene-Trak *Listeria* Assay, FDA culture method and the FDA *Listeria* DNA Probe (Hill, 1987) by screening 309 food samples including raw milk, and ten different supermarket vegetables. More positive milk samples (98-100%) were detected using the four procedures than vegetable samples (45-86%). No one procedure detected all positive samples. The FDA probe procedure detected 85% of the 44 positive vegetable samples, the FDA culture procedure, 75%, the *Listeria*-Tek ELISA, 68%, and the Gene-Trak DNA probe, 45%. The improved version of the Gene-Trak *Listeria* Assay was compared in parallel to the FDA and USDA culture methods by detecting *Listeria* in 1200 food and 100 environmental samples. The Gene-Trak *Listeria* Assay was comparable, and in all occasions more sensitive than the conventional procedures for detecting *Listeria* (Dever et al., 1993).

Knight et al. (1996) compared the TLVIA with the standard culture methods of both the FDA and USDA. Two foods (cooked chicken and cooked ground turkey) under the USDA jurisdiction, and three foods (pre-cooked fish fillets, vanilla ice cream, and shredded lettuce) under the FDA jurisdiction were chosen for the study. Out of the 900 samples tested, 300 were inoculated with high levels of *Listeria* spp. (10-50 cells/25g) and 300 were inoculated with low levels (1-5 cells/25 g) of *Listeria* spp. The agreement between the conventional culture methods and the TLVIA were 94.7% with visual observation and 93.6% when a TLVIA reader was used. Based on this data, collected by 26 laboratories, AOAC International has adopted the TLVIA first action. Kerdahi and Istafanos (1997) compared the TLVIA (a visual ELISA, detecting through colorimetry) to the Vitek immunodiagnostic assay system for *Listeria* (VIDAS LIS) (a fully automated system, detecting through immunofluorescence). Both systems take 48 h, with 2 h and 45 min for determination of positive and negative samples for TLVIA and VIDAS LIS, respectively. Out of the 52 food samples tested at both high (11-42 CFU/25 g sample) and low (2-8 CFU/25 g sample) counts, both ELISA methods detected all positive samples. Although these two methods do not confirm *L. monocytogenes* in foods, they can be used to rapidly screen foods for the presence of *Listeria* spp., ruling out a large number of negative samples analyzed for *L. monocytogenes*. The VIDAS LIS system has not yet been AOAC International approved, as it awaits further testing with different strains of *Listeria* (Kerdahi and Istafanos, 1997).

Feldsine et al. (1997) conducted a collaborative study comparing the Assurance EIA and the FDA or USDA method, respective of each food tested. Six foods (nonfat dry milk, ice cream, raw poultry, raw shrimp, cooked roast beef, and green beans) were tested by 19 participating laboratories. A total of 1764 samples were tested, of these, 492 were confirmed positive and 947 were negative by both the Assurance EIA and culture methods; 188 samples were negative by the culture method yet confirmed positive by Assurance EIA; and 159 samples were confirmed positive by culture method but negative by Assurance EIA. Two food items, raw shrimp and green beans, produced results that suggested the methods were not comparable. Out of two raw shrimp runs, one resulted in the culture method detecting more confirmed positives at low levels (0.003 CFU/g) and

run two resulted in the EIA detecting more confirmed positives at both the low (0.003 CFU/g) and high (0.043 CFU/g) levels. It was suggested that the EIA method might be more permissive to the growth of *Listeria* in raw shrimp. The green beans were naturally contaminated with *L. monocytogenes* and *L. innocua* at levels of 0.009 CFU/g, 0.075 CFU/g, and 0.01 CFU/g, for lots 1, 2, and 3, respectively. For all lots, the EIA method detected more confirmed positives than the culture method. This may be attributed to the improved selective properties (suppressing the growth of non-*Listeria* miroflora) of the EIA primary enrichment medium. Both the raw shrimp and green bean data were found to be statistically significant. All other food item results were found to be comparable for both methods, thus the AOAC International has recommended that the Assurance EIA be adopted first action for selected food excluding raw shrimp and green beans.

Several research groups have compared imunomagnetic capture (ListerScreen™, Listertest®) to culture methods (Mitchell et al., 1994; Avoyne et al., 1997; and McCarthy, 1997a). Mitchell et al. (1994), using the Listertest® method, showed that immunomagnetic capture detected *Listeria* in 100% of samples, at contamination levels where culture methods only detected *Listeria* in 36% of the samples. The Listertest® is also quantitative, more than 10^8 immunomagnetic beads are used per sample, thus ensuring that a single bead does not bind more than one *Listeria* cell. Once the beads are spread on a media plate, it can be assumed that each *Listeria* colony has arisen from a single cell (Mitchell et al., 1994). Due to the fact that immunomagnetic capture does not rely on enrichment, the number of *Listeria* colonies obtained is directly related to the original level of contamination of the product or surface. Avoyne et al. (1997) tested the ListerScreen™ assay on 52 strains of *Listeria*, resulting in all strains testing positive. When tested in food samples, the ListerScreen™ method resulted in 99% agreement with cultural methods, and could detect levels of *Listeria* as low as 4 CFU/25 g. Running tests in 10 laboratories with 144 spiked milk samples (12-117 CFU/25 g) further expanded the study. One hundred and forty-three of the 144 samples produced identical results, thus showing the reproducibility of the test. McCarthy (1997a) evaluated the Listertest™ method (similar to the ListerScreen™ method) versus the FDA MPN test for the enumeration of *L. monocytogenes* on laboratory inoculated (<1.0-4.0 log CFU/g analyzed)

cooked crabmeat and cold-smoked salmon. The MPN method yielded counts that were significantly lower than those obtained from the Listertest™ for cooked crabmeat, but there was no significant difference for the cold-smoked salmon. This study also showed, for both products, that the Listertest™ was more sensitive than the FDA MPN test for the detection and/or enumeration of low (<10 CFU/g) levels of *L. monocytogenes* cells in both products.

Matar et al. (1997) compared LLOLAT to the USDA and the Netherlands (Hayes et al., 1992) culture methods on 208 food samples (primarily meats and cheeses). The LLOLAT test showed 100% specificity and 95% sensitivity, when compared to the isolation of *L. monocytogenes* using the USDA method. This test is also more rapid because LLO can be detected within 48 h after laboratory processing, whereas culture methods require at least 4 days to identify *L. monocytogenes*. It is also noted that the LLOLAT does not require expensive reagents or sophisticated equipment that many rapid methods require.

Murtiningsih and Cox (1997) compared another latex agglutination test, Serobact™, to the conventional USDA method. Six food groups were utilized: dairy; seafood; meat; poultry; salad; and vegetable. Foods selected either had natural contamination or were inoculated in the laboratory with *L. monocytogenes* or *L. innocua*. For detection of low levels (1-10 cells/25 g) of both *L. monocytogenes* and *L. innocua*, the Serobact™ reagent was shown to be effective when compared to culture methods. This reagent does require a primary and secondary enrichment, as do the culture method. Extending the enrichment time, primary or secondary, increased the number of samples positive by both methods, as well as increasing the number of false positive reactions. Compared to the culture method, Serobact™ reagent yielded 100% sensitivity and 93.9% specificity.

3.   Confirmation of *Listeria* species

3.1 Conventional biochemical methods

Isolated colonies of small rods, which are motile with tumbling action and give a positive catalase reaction with typical Gram stain, should be further tested for biochemical reactions. Three *Listeria* spp. have the ability to lyse red blood cells (Datta and Wentz,

1989): *L. monocyotgenes* is weakly hemolytic, *L. seeligeri*'s hemolytic activity is even harder to determine, while *L. ivanovii* produces pronounced hemolytic zones after 24 h on blood agar plates. Stabbing, instead of streaking sheep blood agar, increases the ability to detect the hemolytic zone around a *L. monocytogenes* stab (Lovett, 1988; Schonberg, 1989). Blanco et al. (1989) described a technique to directly detect the hemolytic activity of *Listeria*. A top layer of red cells was added to selective plating medium after *Listeria* growth had occurred. Colonies were more easily detected by this method compared to using red blood cells incorporated into the medium.

The CAMP test is used to detect or confirm hemolysis, being useful in differentiating species. The test is based on the hemolysis enhancement by the metabolites of *Staphylococcus aureus* and *Rhodococcus equi* onto a blood agar a blood agar plate. *L. monocytogenes* and *L. seeligeri* would have enhanced hemolysis in the vicinity of the *S. aureus* streak, whereas *L. ivanovii* would show enhanced hemolysis in the vicinity of the *R. equi* streak (Lovett, 1988). However, there is some controversy regarding the ability of *L. monocytogenes* to react synergistically with *R. equi*, whereas, the *Bergey's Manual of Systematic Bacteriology* (Seeliger and Jones, 1986) indicates that *L. monocytogenes* gives a positive CAMP reaction with *S. aureus* but not with *R. equi*, some authors (Skalka et al., 1982; Smola, 1989; McKellar, 1993) have demonstrated a positive reaction between *L. monocytogenes* and *R. equi*. McKellar (1993) demonstrated a synergistic lysis of erythrocytes with *L. monocytogenes* and cholesterol oxidase, which is proposed, could replace the CAMP reaction with *R. equi*.

Poyski et al. (1993) developed a selective and differential plating medium, Hemolytic Ceftazidime Lithium Chloride Agar (HCLA), for the isolation of *L. monocytogenes* from fishery products. Selectivity is based upon lithium chloride, colistin methane sulfonate, and ceftazidime and differentiation is obtained by the incorporation of horse blood which allows for hemolysis.

3.2 Rapid identification

Identification of *L. monocytogenes* can take 2-5 days using conventional biochemical test procedures, therefore, the use of biochemical kits for rapid identification has increased. Kerr et al. (1990) found good correlation when comparing the API 50CH

(BioMerieux, La Balmeles-Grotoes, France) and the MAST ID system (Mast Laboratories, Bootle, U.K.) to conventional biochemical procedures. Identification of *Listeria* to species can be achieved with the MICRO-ID *Listeria* system developed by Organon Teknika (Durham, NC). Evaluation of this kit was done by Robison et al. (1990) in 170 *Listeria* cultures, including confirmed stock cultures and food isolates. Correct identification of 96.1% of the isolates to the species level was obtained. Comparison between the MICRO-ID system with conventional biochemical procedures (Bailey et al., 1990b; Bannerman et al., 1992) showed good correlation between these methods. Bille et al. (1992) evaluated the API *Listeria* system (BioMerieux, La Balmeles-Grottes, France) to identify *Listeria* isolates at the genus, species and subspecies level, within 24 h. Correct identification of 85% of the 646 *Listeria* strains at the species and subspecies level was obtained by the 10-test strip system. This system uses arylamidase (DIM) to differentiate *L. monocytogenes* and *L. innocua* strains. Arylamidase is present in *L. innocua* and many other *Listeria* strains but not in *L. monocytogenes*. API *Listeria* correctly identified 97.7% (252 of 258) of *L. monocytogenes* strains and 99.4% of *L. innocua* strains tested. Murtiningsih and Cox (1997) compared the Microbact™ *Listeria* 12L (Medvet Science Pty Ltd., Adelaid, Australia) system to the conventional biochemical and physiological tests and found that all but two, out of 81, isolates could be identified by Microbact™ in 24h. The Microbact™ *Listeria* system consists of 12 microtiter plate-sized cupules containing dehydrated media. The system utilizes esculin hydrolysis (well 1), fermentation of selected carbohydrates (wells 2-10), and hemolysis (well 12).

Wiedmann et al. (1992) described a ligase chain reaction assay based on a single base-pair in the V9 region of the 16S rRNA gene that allows for the differentiation of *L. monocytogenes* from other *Listeria* species. This proved to be a highly sensitive and specific diagnostic technique for the detection of *L. monocytogenes*.

A rapid PCR method was developed by Wang et al. (1992) for the detection of *L. monocytogenes* in foods. In 4 h they were able to detect as few as 2 to 20 CFU of *L. monocytogenes* in pure cultures and as few as 4 to 40 CFU of *L. monocytogenes* in inoculated (108 CFU), diluted samples, using a pair of primer based on a region in the 16S rRNA sequence of *L. monocytogenes*. Datta et al. (1990) developed a DNA probe, based

on the listeriolysin O gene, for a colony hybridization assay that showed a high degree of specificity and could be used for routine examination of contaminated foods. A highly sensitive and specific colony blot assay, using a digoxigenin-labeled probe, was developed by Kim et al. (1991) for the rapid confirmation of *L. monocytogenes* on *Listeria* selective agars streaked with food enrichment cultures.

4.   Recovery of injured cells

Listeria monocytogenes, like other non-spore forming bacteria, is injured under exposure to heating, drying, freezing or low pH (Mossel, 1989). According to Lovett (1988) most *Listeria* found in food products may be sublethally injured. As a result of the sublethal injury, structural and physiological deficiencies, including inability to grow in a selective media that otherwise would support growth, occurs; nevertheless, the pathogenic properties of the cells are maintained (Mossel, 1989). Freezing cells results in a removal of water with consequent concentration of cell solutes which can lead to disruption of cellular lipoproteins. Freeze-thaw injury of *L. monocytogenes* is greater when cells are frozen and stored at -18°C rather than -198°C. Repeated freezing at -18°C and thawing at 35°C produced more injury than the same cycle at -198°C (El-Kest and Marth, 1992).

Several studies have been conducted to compare the effectiveness of the FDA and the USDA methods with stressed *Listeria* cells. Bailey et al. (1990a) compared the ability of FDA and USDA broths to recover heat-injured *L. monocytogenes* in pure culture and in Brie cheese and chicken. Both broths were satisfactory for recovery of viable *L. monocytogenes* from foods. However, with low levels of heat-injured cells of *L. monocytogenes*, the USDA enrichment allowed superior recovery. Lovett et al. (1991) found results contrary to those of Bailey et al. (1990a). They compared both, FDA and USDA enrichment protocols, for their ability to isolate *L. monocytogenes* from raw and cooked seafood. Cooked crab meat, raw and cooked shrimp and surimi were each inoculated with *L. monocytogenes* Scott A and strain SE68 was obtained from raw shrimp. The FDA procedure used enrichment intervals of 24 h, 48 h, and 7 days. The results indicated that 24 h was the least effective interval. The FDA procedure was more sensitive for isolating heated cells at a lower level than the USDA method, but both enrichments permitted isolation of unheated cells equally well. Warburton et al. (1992)

reported no significant difference between modified versions of the FDA and USDA methods in the ability to detect stressed and low levels of *L. monocytogenes* in food and environmental samples. Modifications included additional plating agars for both procedures, Fraser broth in the FDA method, and extended incubation for enrichment in the USDA method. All plating media (OXA, LPM, MOX and PALCAM) were comparable in quantitative recovery of stressed and non-stressed cells. Busch and Donnelly (1992) formulated *Listeria* repair broth (LRB) in which, following repair of the heat-injured *Listeria* cells within 5 h, selective agents are added and incubation is continued up to 24 h. When LRB was compared to FDA, USDA and UVM broth, they observed no repair of heat-injured cells in FDA, USDA or UVM broth, however, heat-injured cells resuscitated in LRB. Therefore, Busch and Donnelly (1992) recommended this medium for recovery of heat stressed *L. monocytogenes*. When comparing LCA agar to LPM agar to quantitatively recover *L. monocytogenes* from foods (Lachica, 1990) a higher efficiency in the recovery of sublethally heat-injured cells was reported for LCA agar, with the further advantage of a more distinct bluish hue of the colonies when observed with oblique incandescent light. LCA facilitated formation of larger colonies while suppressing growth of other food-borne microorganisms. Budu-Amoako et al. (1992a) evaluated the efficiency of trypticase soy broth yeast extract (TSBYE) and LEB to recover heat-injured and freeze-injured cells of *L. monocytogenes*. They reported better performance of TSBYE in recovery of both heat-injured and freeze-injured cells. Tran and Hitchins (1993) reported an enhanced recovery of heat-injured cells after enrichment in LEB for 48 h, as compared to 24 h, when *L. monocytogenes* is in the absence of microflora. However, in the presence of food microflora this effect is diminished.

Yu and Fung (1993) inoculated cooked, chopped ham, with a mixture of three strains of *L. monocytogenes* at levels of $\leq$ 350 CFU/25 g, subjected to either heat-injury (65°C, 30 min) or freeze-injury (-18°C, 14 days) and studied their survival at 5°C for 5 weeks. They reported that low numbers of *L. monocytogenes* surviving sublethal heat or freeze-injury could grow after recovery in chopped ham. They also indicated the inability

to recover *L. monocytogenes* at low levels ($\leq$ 100 CFU/25 g) by direct plating, whereas MPN counts using the Fung-Yu five-tube method, was successful.

Sörqvist (1993) compared the efficiency of blood agar and tryptose phosphate agar with ferric citrate and esculin in the recovery of heat-injured cells of *L. monocytogenes* reporting better performance of blood agar. The optimum temperature range for recovery of heat-injured *L. monocytogenes* has been reported to be 20-25°C. Among the recovery medium tested: blood agar, TSA, TSA + catalase, TSA + pyruvate, and TSB, blood agar was the best recovery medium. However, good recoveries were obtained in the TSB using a MPN technique (Mackey et al., 1994).

McCarthy et al. (1990) developed a method to enhance recovery of thermally stressed *L .monocytogenes* from internally contaminated shrimp. By combining cold incubation with primary and secondary enrichment at 30°C, recovery of thermally injured *L. monocytogenes* was enhanced.

Knabel and Thielen (1995) developed a strictly anaerobic method for the enhanced recovery of heat-injured *L. monocytogenes* from USDA and FDA primary enrichment media. They used a combination of purging the headspace with nitrogen gas and adding cysteine to the media. Compared to conventional methods, the following treatments significantly improved recovery: addition of filter-sterilized cysteine + N_2 purging > pre-reduced Hungate media + N_2 purging = Oxyrase® + lactate + N_2 purging > filter-sterilized cysteine - N_2 purging > Oxyrase® - lactate + N_2 purging. Recovery from USDA UVM broth was increased from 0% to 60% with the addition of filtered cysteine to a final concentration of 0.5 g/l and subsequent N_2 purging. The same treatment increased the recovery from 11 to 100% in FDA LEB. The addition of either 1% pyruvate or a concentration of cysteine greater than 10-fold, completely inhibited recovery.

5. Typing methodology

Typing of *L. monocytogenes* strains involved in foodborne listeriosis outbreaks or associated with food products, beyond the species and serotype levels, is required to establish relationships between the outbreak and sources of the pathogen (Estela et al., 1992). Traditional methods used for typing *Listeria* spp. include: biotyping and serotyping (Seeliger and Jones, 1986); phage typing (Ortel, 1989a; Audurier and Martin,

1989); and antibiotic susceptibility testing and bacteriocin production (Seeliger and Jones, 1986; Ortel, 1989b). Serotyping is difficult and of limited value because of the small number of serotypes with pathogenic strains (Estela et al., 1992). Phage typing is successful only for strains that are typeable, sometimes very few (between 50% to 90%), and it is affected by factors such as the origin of the culture. In 1987, the FDA obtained the International *L. monocytogenes* Phage Typing Set in an attempt to phage-type isolates for use in its regulation of consumer protection efforts. Estela et al. (1992) determined the phage spectra found in *L. monocytogenes* cultures isolated from 227 seafood products. Of the 227 cultures subjected to phage typing, 173 were typeable (76%). Overall the majority of the typeable strains were *L. monocytogenes* type ½ (64%) and the remainders were type 4b (36%).

Newer methods for subtyping *Listeria* spp. include plasmid profile analysis (Mayer, 1988; Kolstad et al., 1992), plasmid chromosomal DNA restriction enzyme analysis (Chenevert et al., 1989; Datta et al., 1990; Loncarevic et al., 1996; Boerlin et al., 1997), multilocus enzyme electrophoresis (Pinner et al., 1992; Slade, 1992; Kolstad et al., 1992; Boerlin et al., 1997), and pulse-field gel electrophoresis (Buchrieser et al., 1993; and Loncarevic et al., 1997).

E. *Listeria* species in foods

The organism is widely distributed in the environment being found most commonly in cool, damp environments, on both food contact and non-food contact surfaces, especially conveyors, floors and drains (Slade, 1992). Soil is an important reservoir and the bacterium is frequently carried in the intestinal tract of animals, including healthy humans. Jackson et al. (1993b) surveyed one hundred and ninety-five residential refrigerators for the presence of *L. monocytogenes* and were not able to recover the organism. However, the presence of *L. monocytogenes* has been reported in household dishcloths and refrigerators (Doyle, 1991). Pinner et al. (1992) in an evaluation of the role of foods in sporadic listeriosis, reported that *L. monocytogenes* was detected from at least one food specimen in the refrigerators of 79 (65%) of 123 listeriosis patients and that 11% of more than 2000 food specimens collected in the study contained *L. monocytogenes*. Twenty-six (33%) of 79 refrigerators with foods that grew *L. monocytogenes* contained at least one food isolate of the same strain as that in the corresponding patient. Cox et al. (1989) studied the occurrence of *Listeria* spp. in 35 Dutch households. Seven (20%) of the household kitchens were found to be contaminated with *Listeria*. Six of seven dishcloths were positive for *Listeria*, as were swabs from two dustbins and one refrigerator. Sergelidis et al. (1997) studied the prevalence of *Listeria* in home and food store refrigerators in Greece. They found 2 (1.5%) out of 136 households had refrigerators that harbored *L. monocytogenes*, and out of 335 samples taken from 212 food stores, 3.1% were found to be positive for *Listeria* spp., and 1.7% were positive for *L. monocytogenes*.

Lachia (1990) has reported no effect of a large number of microflora (SPC > 10^8 CFU/g) in the ability of enumerating *Listeria* spp. in artificially contaminated foods (ca. 10^3 CFU/g) and indicated the problem as being food samples with *Listeria* contamination levels below the sensitivity of the direct plating technique (<100 CFU/g), in which cases a recovery and selective-enrichment phase is required prior to plating.

1. Vegetables

Several large outbreaks of listeriosis have been attributed to contaminated vegetables. The first outbreak implicating vegetables occurred in Boston in 1979, lettuce, celery, and tomatoes were involved; 23 cases resulted in 5 fatalities (Ho et al., 1986). Coleslaw, made from cabbage contaminated with manure from sheep with listeriosis, led to the 1981 outbreak in Nova Scotia which resulted in 41 cases, 18 of those resulting in death (Schlech et al., 1983). Beuchat (1995) compiled an extensive list of pathogens associated with vegetables, this list includes the country and prevalence of each pathogen. *L. monocytogenes* was found in the following: bean sprouts (Malaysia 85%); cabbage (Canada 2.2%, U.S. 1.1%); cucumber (Malaysia 80%, Pakistan 6.7%, and U.S. 2.2%); leafy vegetables (Malaysia 22.7%); potatoes (U.S. 21.7% and 21.2%); prepackaged salads (Northern Ireland 14.3%, UK 13.3%); radishes (U.S. 36.8% and 14.4%); salad vegetables (Germany 2.3%, Northern Ireland 25%); tomatoes (Pakistan 13.3%); and miscellaneous vegetables (Italy 6.9%, Spain 7.8%, Taiwan 12.2%, and UK 6.2%). *L. monocytogenes* has also been isolated from fruits and vegetables in the refrigerators of listeriosis patients (Pinner et al., 1992).

Berrang et al. (1989) studied the effect of controlled atmosphere storage (CAS) on the growth of *L. monocytogenes* on fresh vegetables. They determined that CAS did not influence the rate of growth, positive or negative, of *L. monocytogenes*, but did lengthen the time that the vegetables were considered acceptable for consumption based on subjective inspection. Beuchat and Brackett (1990) also showed that the growth rate of *L. monocytogenes* was not affected by modified atmosphere packaging (MAP) (3% O_2, 97% N_2), as well as, chlorine treatment or shredding, all of which are common procedures used in the food industry.

Odumeru et al. (1997) studied the microbiological quality, including *L. monocytogenes*, of ready-to-use vegetables for health-care facilities. Vegetables studied included: salad mix; coleslaw mix; broccoli florets; carrot sticks; cauliflower florets; sliced celery; sliced green peppers; salad mix; and coleslaw mix, both before and after processing. Microbial analysis was performed before processing and 24 h after processing and on days 4, 7, and 11 after storage at 4 and 10°C (to simulate temperature abuse). Six

of the 8 vegetable types tested were positive for *L. monocytogenes*: broccoli florets; green peppers; chopped lettuce; salad mix; coleslaw mix; and unprocessed celery. Of the samples store at 4°C, 5 (2.8%) were positive for *L. monocytogenes*. The positive samples included: chopped lettuce at day 0 and 11 with recovery levels of 3.6 and 11.0 MPN/g, respectively; and salad mix on days 4, 7, and 11 with counts of 64, 9.1, and 240 MPN/g, respectively. At 10°C, *L. monocytogenes* was isolated from two separate batches of salad mix on days 4, 7, and 11 with recovery levels of 460, 1100, and 460 MPN/g, respectively for batch 1, and 1100, 23, and 43 MPN/g, respectively, for batch 2; chopped lettuce on days 4, 7, and 11 with recovery of 460, 1100, and 460 MPN/g, respectively; coleslaw mix on day 4 at 210 MPN/g; broccoli florets on days 4 and 7 both at > 1600 MPN/g; and green peppers on day 7 at 3.6 MPN/g. In addition, salad mix, coleslaw mix, cauliflower florets, and sliced green peppers were distributed to 14 participating hospitals and were analyzed after 7 days. The analysis showed that 5 (3.7%) of the 135 vegetable samples contained *L. monocytogenes*.

Several studies have shown vegetables to be free of *L. monocytogenes*. Farber et al. (1989) analyzed lettuce, celery, tomatoes, and radishes for *Listeria* spp., including *L. monocytogenes*, and found all of them to be free of the microorganism. Similar results were produced by Tiwari and Aldenrath (1990), studying lettuce, broccoli, carrots, tomatoes, cabbage, cauliflower, radishes, brussel sprouts, and other vegetables, as well as, prepared salads.

2. Dairy Products

Dairy products have been implicated as the source of several listeriosis outbreaks. Flemming et al. (1985) reported an outbreak in Boston, Massachusetts, due to the consumption of pasteurized milk; although the organism was not cultured from the suspect brand of milk. Forty-nine cases were confirmed in immunocompromised adults and infants, of these 14 individuals died. Linnan et al. (1988) reported an outbreak in California due to Mexican-style soft cheese. This outbreak produced 142 cases with 48 fatalities. In 1994, 52 individuals developed gastrointestinal listeriosis from temperature abused chocolate milk (Shank et al., 1996). *L. monocytogenes* has also been isolated from dairy products in the refrigerators of patients with listeriosis (Pinner et al., 1992). These

large outbreaks have lead several countries to place a zero-tolerance level for *L. monocytogenes* in certain dairy products (soft cheeses) (Shank et al., 1996; Farber and Harwig, 1996; and Qvist, 1996).

Tiwari and Aldenrath (1990) studied the prevalence of *Listeria* spp. in raw milks from both farm bulk and silo tanks. They found that 10 (4%) of the 252 samples from raw bulk tanks and 5 (33%) of the 15 silo tank samples were positive for *Listeria* spp. with 4 (1.6%) and 4 (26.6%) being positive for *L. monocytogenes*, respectively. Eight samples of raw cream produced 2 samples positive for *Listeria* spp. but *L. monocytogenes* was not found. Other dairy products sampled were pasteurized milks, hard and soft ice creams, and soft and hard cheeses. All of these products were negative for *Listeria*.

A survey of dairy products by Farber et al. (1989), found very low contamination with *L. monocytogenes*. Only 2 out of 530 samples of ice creams and pasteurized milks were positive for *L. monocytogenes*; these included one ice cream novelty and one ice cream sample.

Rosso et al. (1996) compared the growth of *L. monocytogenes* at 4 and 8°C in milk (whole, skimmed and chocolate), cream, and soft cheese. Using experimental growth curves, they showed that increasing the temperature from 4 to 8°C led to a decrease in the time required to reach a given density level. The decrease was calculated to be 60 to 65% for milk and cream and 50% for soft cheeses such as Camembert. They applied this information to a hypothetical situation involving a dairy dessert. If the dairy dessert had an initial contamination of 1 *L. monocytogenes* cell per g, then it could lead to a population of 100 cells per g after 5 days at 8°C, as opposed to 2 weeks at 4°C.

3. Meat and poultry

Meat and poultry have been the source of several outbreaks and sporadic cases of listeriosis. Between 1987 and 1989 there were 366 cases reported in the UK that resulted from consuming contaminated pate (McLauchlin et al., 1991). This has been the largest outbreak to date (Jay, 1996). In light of this large outbreak many countries have placed a zero tolerance of *L. monocytogenes* on pate (Farber and Harwig, 1996; Shank et al., 1996; and Qvist, 1996). In 1992, a large outbreak involving 279 people was contributed to contaminated jellied pork tongue (Jacquet et al., 1995). This outbreak resulted in 63

deaths and 22 miscarriages. The first meat-associated case in the U.S. was a sporadic case in 1988 involving a turkey frank (Barnes et al., 1989). The victim was a female cancer patient and samples of the turkey franks from her refrigerator had levels of *L. monocytogenes* exceeding 1100 MPN/g (Wenger et al., 1990). Pinner et al. (1992) isolated *L. monocytogenes* from beef, poultry, pork, and luncheon meat from the home refrigerators of listeriosis patients.

Farber et al. (1989) studied ground meats (beef, pork, and veal), fermented sausages, and chicken legs for the presence of *L. monocytogenes*. There were 22, 19, and 3 samples taken of ground beef, pork, and veal, respectively, with 17 (77.3%), 18 (94.7%), and 3 (100%), respectively, positive for *L. monocytogenes*. These high numbers may be due to the method of enumeration used; instead of selecting 5 suspect colonies per plate, as in the USDA method (McClain and Lee, 1987), 10-30 colonies were selected. *L. monocytogenes* was isolated from 20% of the dry-cured sausages sampled. Sixteen chicken leg samples were taken, of these, 9 (56.3%) were positive for *L. monocytogenes*. This high percentage is supported by the frequent occurrence of *L. monocytogenes* in bird feces.

Jay (1996) presents on overview on the prevalence of *Listeria* in poultry and meat products based on studies from around the world. The overall prevalence of *L. monocytogenes* in both fresh and frozen pork was found to be 20%. *L. monocytogenes* was found in fresh and frozen beef and lamb at a rate of 16%. Seventeen percent of fresh and frozen poultry contain *L. monocytogenes*. Processed meats (pork sausages, bacon, salami, pate, etc.) had a 16% prevalence of *L. monocytogenes*. *L. monocytogenes* and *L. innocua* are the most reported species of *Listeria* found in meats, followed by *L. welshimeri*, *L. seeligeri*, and *L. ivanovii*. Large counts, those greater than 10^3/g, have mostly been found in processed meats such as pate. It appears that in non-processed meats that the level of *L. monocytogenes* rarely exceeds 100/g. Most *L. monocytogenes* meat isolates are of serotype 1/2a, 1/2b, or 1/2c while most human listeriosis cases are caused by serotypes 1 and 4. In contradiction, Farber et al. (1989) found that most meat and poultry isolates belonged to serogroup 1.

Tiwari and Aldenrath (1990) studied the presence of *Listeria* spp. in raw meats and processed meats. Raw meats studied included: ground meats; meat cuts; poultry; moose; and sausages. Of the samples analyzed, at least 1 sample per product was positive for *Listeria* spp as well as *L. monocytogenes*. The rates of those positive for *L. monocytogenes* were 63.6%, 44.4%, 16.7%, 100%, and 100% for ground meats, meat cuts, poultry, moose, and sausages, respectively. Unpackaged processed products (cooked meats and bulk RTE) were all found negative for all *Listeria* spp. All packaged process products (wieners, luncheon meats, and sliced meats) except sausages were found to be positive for *Listeria* spp.; with rates of *L. monocytogenes* being 21%, 13.1%, and 13.8% for wieners, luncheon meats and sliced meats, respectively.

Beumer et al. (1996) researched the growth of *L. monocytogenes* on luncheon meat, ham and cooked chicken breast. Each of these products were inoculated with low levels (100µl of 10^4), packaged under modified atmosphere (30% CO_2/70% N_2, or vacuum), and stored at 7°C. During the shelf life of the products, the levels of *L. monocytogenes* increased up to 10^8 CFU/g. They also found that neither the location of the inoculation nor the composition of the modified atmosphere, affected the growth of *L. monocytogenes*. An increase of a competitor (*lactobacilli*), by a factor of 100, decreased the initial levels of *L. monocytogenes*, however, during the storage period, the levels of *L. monocytogenes* reached 10^7 to 10^8 CFU/g. This may be important when investigating MAP foods due to the fact that consumers may judge products safe even in the presence of high levels of pathogens.

4. Seafood

L. monocytogenes has been associated with three sporadic cases of seafood-borne listeriosis (Facinelli et al., 1989; Frederiksen, 1991; Baker et al., 1993). The incidence of *Listeria* spp. in seafood has been reported to be approximately 28% (Buchanan et al., 1989). A survey of home refrigerators of listeriosis patients, by Pinner et al. (1992), showed that 12% of seafood samples tested were positive for *L. monocytogenes*. Differences in clones may explain why, despite frequent contamination with *Listeria*, RTE seafood products are rarely associated with clinical listeriosis (Boerlin et al., 1997).

Commodities found to contain *L. monocytogenes* include raw and cooked shrimp, crab meat, lobster tails, squid, finfish, and surimi analogs (Farber, 1991; NACMCF, 1991). Weagant et al. (1988) tested samples of frozen seafood products from various countries for the presence of *Listeria* spp., including *L. monocytogenes*. They isolated *Listeria* spp. from 61% of the samples with 26% being positive for *L. monocytogenes*. *L. monocytogenes* was found in samples of raw shrimp, cooked and peeled shrimp, cooked crab meat, raw lobster tails, langostinos, scallops, squid and surimi-based imitation seafood. Fresh and frozen fish vary in their prevalence of *L. monocytogenes,* between 4 to 12%, whereas raw and heat treated (listericidal) shrimp show a prevalence of 1.5-20% (Ben Embark, 1994). The existence of *Listeria* in frozen seafood with a previous heat treatment indicates possible post-process contamination. Buchanan et al. (1989) determined that seafood has the second highest rate (28%) of *Listeria* spp., with meat being first. They isolated *Listeria* spp. from uncooked frozen shrimp, cooked and non-pasteurized crab meat, flounder, monkfish, and catfish, yet only two finfish samples tested positive for *L. monocytogenes*. *L. innocua* was the *Listeria* spp. most often isolated from seafood. Rawles et al. (1995b) studied the relationship between *L. monocytogenes* and *L. innocua* in pasteurized crab meat, and found no significant difference in the rate of growth of either organism.

Wang and Shelef (1992) reported the ability of *L. monocytogenes* to grow and multiply in raw cod fish stored at 20 and 5°C. However, at a refrigeration temperature the lag phase of *Listeria* took longer than the deterioration of the shelf life induced by the natural microflora of the fish.

Ben Embark (1994), in a review of the incidence of *L. monocytogenes* in seafood, indicated that *Listeria* spp. can be isolated from polluted water and waters with a high content of organic material, but that its natural presence in clean open seawater remains to be established. He suggested that *Listeria* spp. are likely to occur naturally on freshwater finfish but are unlikely to occur in pelagic fish or fish living in clean seawater; thus, the contamination of fish with *Listeria* is most likely dependent on the presence of the bacteria in its surrounding waters. Arvanitidou et al. (1997) found that *L. monocytogenes* could be isolated from river waters (3.9%), but not from lake samples. A low incidence (5%) of *L.*

monocytogenes in shellfish growing waters was reported by Motes (1991). Shrimp samples harvested from the same water were positive for *L. monocytogenes* (11%) yet the organism was not detected in oysters. They also reported that the highest incidence of *Listeria* spp. from water and shrimp occurred at water temperatures $\leq 20°C$.

     L. monocytogenes can survive light preservation processes such as marinating and curing, as well as the cold-smoking process, while the overall isolation rate of *L. monocytogenes* in cold and hot smoked fish are 10% and 9%, respectively (Ben Embark, 1994). Significant growth of *L. monocytogenes* on smoked salmon has been reported by Farber (1991), Fletcher and Rogers (1991), and Rörvik and Yndestad (1991). Guyer and Jemmi (1991) reported that *L. monocytogenes* survives the brining and smoking stages that occur during cold-smoking of salmon, and that the organism can grow on the final product, under refrigeration conditions, increasing approximately 4 log cycles over a 30 day period of storage. The growth of *L. monocytogenes* on refrigerated vacuum-packed cold-smoked salmon has been reported by Hudson and Mott (1993) and Rörvik et al. (1991). Jemmi and Keusch (1992) showed that *L. monocytogenes* cannot survive the hot-smoking process, if the internal temperature reaches 65°C for 20 min. Therefore, low levels of initial contamination would easily be eliminated and findings of *L. monocytogenes* in finished product would indicate post-processing contamination. Storage of hot-smoked trout at 8-10°C resulted in a significant increase of *L. monocytogenes*, indicating the importance of storing hot-smoked fish at temperatures of 4°C or lower. Fish and shrimp samples inoculated with *L. monocytogenes* (10^3 of rinse buffer) and held in ice for 21 days, showed no increase in population (Harrison et al., 1991).

     Farber (1991) researched the potential for *L. monocytogenes* growth in seafood products such as cooked lobster, crab meat, shrimp, and smoked salmon. Fifteen samples out of 113 total samples were found to be positive for *L. monocytogenes* at the retail level; these samples included RTE shrimp, crab, and salmon. The naturally contaminated products showed only low levels of the organism. Growth studies with the naturally contaminated shrimp showed that levels of *L. monocytogenes*, after a week of storage at 4°C, remained below 100 MPN/g. Artificial inoculation studies were also performed with initial inoculation levels of 10^2-10^3 CFU/g. Within 7 days at 4°C, *L. monocytogenes*

increased 2-3 logs in each product. Storage of the products at room temperature for 6 h, a simulation of consumer abuse, the levels of *L. monocytogenes* on shrimp, crab, lobster, and salmon increased by 1.0, 1.0, 0.2, and 0 to 1.0 logs, respectively.

The NACMCF (1990) published and adopted the recommendations of the Committee to address the microbiological safety of cooked RTE shrimp and cooked RTE crab meat. Selection of these products was done on the basis of their consumption with minimal or no additional heat processing and the consequent inherent risk to the consumer. Commonly *L. monocytogenes* can be found in finfish and shellfish products (Hudson et al., 1992). The organism was found in 35.7% of smoked mussels and in 75% of the salmon samples tested. *Listeria* was present in 11.3% of the smoked seafood products surveyed from Newfoundland retail markets (Dillon et al., 1992). Ryu et al. (1992) isolated *L. monocytogenes* in 6.1% of the samples of fish and fish products, including RTE foods, in a study of retail foods in Japan. Levels of *L. monocytogenes* were estimated to be less than 50 CFU/g.

Rawles et al. (1995a) researched the occurrence and growth at refrigeration temperatures of *L. monocytogenes* in blue crab meat. Ten percent of the samples taken from processing facilities were positive for *Listeria* spp., with 10 samples positive for *L. monocytogenes* while three samples were positive for *L. innocua*. All positive samples were found at levels less than 100 CFU/g, with the exception of one sample which attained the level of 1100 CFU/g. The growth rate of *L. monocytogenes* increased as storage temperature was increased, when inoculated in crab meat. At temperatures of 1.1°C, 2.2°C, and 5°C, the generation rates were 68.7, 31.4, and 21.8 h, respectively.

Rosso et al. (1996) compared the growth of *L. monocytogenes* at 4 and 8°C in smoked salmon. Using experimental growth curves, they showed that increasing the temperature from 4 to 8°C led to a decrease in the time required to reach a given density level. The decrease was about 80% for the smoked salmon. An application of this information can be seen in the following example. If smoked salmon with a contamination level of 1 to 10 cells per g was incubated it at 4 and 8°C, then theoretically the initial contamination level would exceed 100 CFU/g after 21 days at 4°C, but the level would exceed 100 CFU/g in only 7 days if the salmon is stored at 8°C.

McCarthy (1997b) studied the effects of processing and post-processing storage on the incidence and survival of *L. monocytogenes* on smoked salmon, crab meat, and crawfish. The effects of temperature on the survival and growth of *L. monocytogenes* on RTE cooked crab meat and salmon showed that this variable was only significant on crab meat grown at 6°C. *L. monocytogenes* was recovered from 3.2% of the boiled whole crawfish, and from 16.7% of the peeled tails. Rapid boiling for 5 min or steeping for 20 min of raw inoculated crawfish, reduced *L. monocytogenes* to non-detectable levels. Post-process temperature abuse ($\geq$ 22°C) for prolonged periods resulted in an increase in the level of *L. monocytogenes* in inoculated crawfish meat.

F. *Listeria* **and food processing**

Fenlon et al. (1996) showed that low levels, in both distribution and numbers, of *L. monocytogenes* are found in plants and animals; yet once processing is initiated *Listeria* numbers and the percentage of positive samples increase significantly. This indicated that processing is a major source of cross-contamination and amplification of the organism. Two examples confirming this idea are: (1) the initial processing of grass into silage results in a burst of *L. monocytogenes* growth; and (2) the diet and feces of poultry entering a processing plant are rarely positive, however, five out of six birds sampled after processing tested positive for *L. monocytogenes*. They also note that most food products implicated in major listeriosis outbreaks have received a high degree of processing. Wentz et al. (1985), in a survey of establishments processing crab meat, showed that the microbial quality of the finished product correlates very well with the handling practices the crab meat is subjected to after cooking.

Several studies have shown the presence of *Listeria* spp. in various places in the processing plant. Locations yielding positive results include: floors (Tiwari and Aldenrath, 1990; Sammarco et al., 1997); storage coolers (Tiwari and Aldenrath, 1990; Sergelidis et al., 1997); refrigerator handles (Sergelidis et al., 1997); mechanical saws (Fenlon et al., 1996); hand basins (Sammarco et al., 1997); and shelves and tables (Menendez et al., 1997; Sammarco et al., 1997).

In attempt to determine how *L. monocytogenes* may survive in a plant, several researchers have investigated its attachment and growth on work surfaces (Spurlock and Zottola, 1991; Blackman and Frank, 1996; Hood and Zottola, 1997a; and Hood and Zottola, 1997b). Based on the work of Charlton et al. (1991), suggesting that floor drains may be a source of *Listeria,* Spurlock and Zottola (1991) determined the growth and attachment of *L. monocytogenes* to cast iron, commonly used in floor drains. They found that *L. monocytogenes* Scott A could survive in the cast iron drains regardless of growth media or pH. Blackman and Frank (1996) showed that *L. monocytogenes* could form as a biofilm, the result of both adherence and growth, on hydrophilic (stainless steel) and hydrophobic (Teflon®) surfaces. The attachment of *L. monocytogenes* to stainless steel, one of the most common surfaces found in food processing plants, was studied by Hood

and Zottola (1997a and 1997b). They found that the organism could adhere to stainless steel, with rates of adherence dependent on available organic material.

1. Control/reduction

NACMCF (1991) states that *L. monocytogenes* is difficult to control because many food-processing plants provide conditions conducive to the organism's proliferation. Many procedures which were deemed adequate in the past for controlling pathogens are not sufficient for *L. monocytogenes.* NACMCF (1991) presents recommendations for six areas of importance (plant design and layout, equipment design, process control, employee practices, cleaning and sanitation practices and procedures, and verification) in reduction and control of *L. monocytogenes* in the plant setting. It is important to follow such recommendations because once a plant is contaminated, *Listeria* can establish itself and can lead to cross-contamination. In the case of a persisting strain, cross-contamination between different products of a single producer may result (Boerlin et al. 1997). Loncarevic et al. (1996) found that once *L. monocytogenes* establishes itself in a processing plant, the risk of continual contamination is reasonable.

Proper cleaning is necessary to remove *L. monocytogenes*, as well as, other pathogens in the processing plant. Once cells have adhered to food-contact surfaces, traditional cleaning and sanitizing may prove ineffective (Hood and Zottola, 1997b). Blackman and Frank (1996) note that biofilm formation can in part be controlled by decreasing the levels of complex nutrients (food residue) on wet surfaces in the plant environment. Spurlock and Zottola (1991) suggest that the presence of *L. monocytogenes* in floor drains may lead to airborne contamination if the drains are not cleaned properly. Aerosols may be created when hoses are used to flush out drains because the air is forced out as the solution enters. Sammarco et al. (1997) stresses the importance of applying proper sanitizers to equipment and walls, as the practice of allowing sanitizers to flow off of walls and equipment may not provide adequate contact time; instead foam cleanser/sanitizers may work better.

Menendez et al. (1997) documented the removal of *Listeria* spp. in a cheese factory in Galicia, Spain. *L. innocua*, *L. monocytogenes*, and *L. welshimeri* were found in various places in the plant. Twenty percent of the raw milk entering the plant were

positive for *Listeria*, however, pasteurization effectively eliminated the *Listeria*. In the cheese manufacturing room, *L. innocua* was located on non-contact surfaces; this was removed by changing the disinfection solution from 400 ppm hypochlorite liquid to 400 ppm chlorine-based foam. The foam was also effective in removing *Listeria* from conveyor belts used in processing, as well as the floor and drains. Old iron screw presses were also found to be a source of contamination due to oxides that were difficult to clean; replacing the iron presses with stainless steel eliminated the problem. In the cheese ripening room wooden shelves used to store cheese were found to harbor all three *Listeria* species. *Listeria* was not detected after the wooden shelves were replaced with stainless steel. Milk cans and stacker trucker used in the quarg manufacturing room were found to house *Listeria* spp.; this was attributed to their storage outside of the plant. Internal stainless steel pipes replaced the milk cans and stacker trucks were housed inside to eliminate this problem. After all changes were implemented, none of the cheese or quarg samples tested positive for any *Listeria* species.

1.1 Thermal processing in the blue crab industry

Crabs are typically cooked, in retorts under steam with temperature ranges of 240-250°F or boiled to denature the meat which assists in its removal from the shell (Dickerson and Berry, 1976). Most crab meat is hand picked and packed, and ready to eat without any additional processing (Dickerson and Berry, 1976). Pasteurization was proposed as a method to increase the shelf life of the product. An arbitrary z-value of 8.9°C was selected, which based on historical data that gave a desired shelf life (Rippen and Hackney, 1992). Most of the spoilage microorganisms and pathogens are destroyed by low to moderate heat treatment.

L. monocytogenes is a concern to the crab processing facility, especially in fresh crab meat. Fresh crab meat receives an initial heat treatment, to coagulate the meat for ease of removal, but does not receive a secondary heat treatment after removal from the crab. Due to the ubiquitous nature of *L. monocytogenes*, it may be present on the raw crabs coming into the processing facility. Since *L. monocytogenes* is more heat resistant than many microorganisms, and can also survive in cold temperatures, questions arise as to whether the cooking process provides an adequate decimal reduction (D-value) of *L.*

monocytogenes. Farber (1992) states that crustaceans should be heat processed until a center temperature of at least 80°C (176°F) is achieved. Huss (1997) reports that guidelines should be 0.3-2 min at 70°C. The following table documents reported D-values and Z-values for *L. monocytogenes* in various products.

TABLE I - 1. D- and Z-Values for *L. monocytogenes* in Various Foods

Product / Culture type	D-value (min)							Z-value (°C)	Reference
	50°C	55°C	56°C	60°C	62°C	65°C	85°C		
Crab meat	40.43	12.0		2.61		39.8[a]	0.16[a]	8.4	Harrison and Huang, (1990)
Crawfish		10.23		1.98		0.19		5.5	Dorsa et al., (1993)
Cream						17.2[a]	0.02[a]		Bradshaw et al., (1987)
Ground meat			13.18		1.01				Farber, (1989)
Ham/heat resistant		13.4		0.97				4.38	Carlier et al., (1996)
Ham/heat shocked		19.2		3.48				6.74	Carlier et al., (1996)
Ham/untreated		17.8		1.82				5.05	Carlier et al., (1996)
Milk						11.2[a]	0.02[a]	7.2	Bradshaw et al., (1985)
Milk						28.2[a]	0.09[a]	8.0	Bunning et al., (1986)
Skim milk						0.19[a]	0.007[a]	5.8	Bradshaw et al., (1987)

[a] As calculated by Rippen and Hackney (1992)

1.2 Employee sanitation - hand washing

Microorganisms found on the hands can be divided into two groups, resident and transient. Resident organisms have been defined as bacteria representing particular species that are recovered on more than 75% of 25 sampling days over a 7 month period; while transient organisms are comprised of those bacteria that appear less than 25% of the time during this same sampling period (Nobel and Pitcher, 1978). In food production, resident bacteria usually do not pose any threat of infectious disease to consumers who come in contact with them, but instead contribute to food spoilage (Paulson, 1993a). Transient bacteria, specifically pathogens, pose a major concern to the food industry because these bacteria are loosely attached to the workers skin and can easily cross contaminate food products if proper hand washing is not established in the work place (Miller, 1994). Isolation of pathogenic bacteria from the hands can be demonstrated after using the restroom, touching soiled surfaces, or touching raw foods. Proper hand washing can

break the transmission of pathogens from the food handlers, the food they process, and the consumer (Paulson, 1993b).

Kerr et al. (1993) reported the prevalence of *Listeria* spp. on the hands of food workers. They found that 12% of the workers surveyed had *Listeria* spp. on their hands, with 7% being *L. monocytogenes*. A control group of clerical workers did not show any in the control group to be positive. Genigeorgis et al. (1990) found 30% of workers in a turkey processing plant had *Listeria* spp. on their hands or gloves. Kerr et al. (1993) also noted that of the workers found to carry *Listeria* on their hands, only one individual was deemed to have washed his hands adequately. Inadequate hand washing behavior observed included, failure to use soap/antibacterial hand wash, duration of washing lasted 10 s or less, and drying hands on visibly dirty paper towels.

Snelling et al. (1991) studied the survival of *L. monocytogenes* on fingertips. Their research found that when *L. monocytogenes* was suspended in saline, it could survive for up to 60 min on fingertips. When suspended in milk, there was a rapid decrease in the population during the first 30 min followed by a slower decline and a plateau was reached after 60-90 min. The fat content of the milk, activity of skin lipids, and commensal flora had no significant affect on the survival of *L. monocytogenes* on the fingertips. They suggest that dessication is the only mechanism responsible for the eradication of *L. monocytogenes* from the skin.

An effective hand wash is dependent on an effective hand wash regimen and personnel compliance, specifically self-motivation to perform the wash, and consistency in performance. Larson et al. (1997), after studying a multifaceted approach to changing hand washing behavior, concluded that intensive intervention, including education, feed back, and increased sink automation, had minor long-term effects on hand washing frequency. Instead, they indicated that the focus should be on making it more difficult not to wash hands, by means of environmental controls, automation, or management mandate. An automated hand washing system may increase hand washing compliance and consistency, because it is not dependent upon any personnel motivation (Paulson, 1993b). In an automated wash system, all the employee must do is place his or her hands in the machine and leave them in place for the duration of the wash. Paulson (1992)

demonstrated, based on his data, that the bacterial $\log_{10}$ reduction averages for a manual wash was statistically equivalent ($p > 0.05$) to automated wash methods.

The hand washing compound (i.e. soap, disinfectant) must effectively remove the contaminants from the hands. Washing with hand soap and water should significantly decrease the transient flora of the skin (Miller et al., 1994). Shekhawat et al. (1991) found that washing with soap and water decreased the bacterial hand counts by 93.80% in doctors, 94.09% in nurses, and 87.88% in ward nurses. Miller et al. (1994) found that bacterial populations were reduced by 39.5-43.9% after a soap and water hand wash, and that this was comparable to the results obtained from antibacterial hand soaps. On the contrary, Snelling et al. (1991) found that soap typically fails to remove *L. monocytogenes* suspended in milk, from the fingertips. They also found water-based chlorhexidine to be ineffective, however, a solution of chlorhexidine gluconate in methanol proved to be effective. McCarthy (1996) notes that hand sanitizers can reduce contamination in the processing plant, but they must be carefully chosen since their efficacy can be reduced in the presence of organic material.

When testing hand washing materials the Peterson glove juice test (Peterson, 1973), which has been adopted by the FDA, is widely used (Holloway et al., 1990). The glove juice method consists of placing a sterile surgical glove over the hand, instilling a surfactant to strip the bacteria from the hands, and subsequent plating of the "glove juice" (Paulson, 1993a). Other methods employed in hand sampling include swabbing and finger or hand press. The swab procedure begins by dampening a swab with saline and swabbing a predetermined area of the hand. After swabbing is complete, the swab is returned to the sterile container of saline, which is then vortexed and plated. In the press method, subjects gently press their finger tips or hands onto an agar plate, which is later incubated and checked for growth. Paulson (1993a) found the glove juice method to be consistent within ½ a $\log_{10}$ of the seeded population, whereas both the swab and finger press consistently underestimated the bacterial population by as much as 2 to 3 logs. The glove juice method typically covers more surface area and reaches subungual areas which may be missed by swabs or presses (Leyden et al., 1989).

G. Biological indicators

The use of biological indicators to provide information concerning the adequacy of a thermal process with respect to a target organism of concern is widely applied. Thus, pathogenicity of the target organism is avoided since biological indicators are non-pathogenic, and the information obtained gives the processor a margin of safety since biological indicators are typically more resistant than the pathogen. Fairchild and Foegeding (1993) evaluated the adequacy of *L. innocua* mutants to be used as a biological indicator for *L. monocytogenes*. They reported *L. innocua* M1, a natural mutant resistant to streptomycin and rifampin, to be useful as a biological indicator for the evaluation of pasteurization processes in the range of 61 to 71°C. Kamat and Nair (1996) showed that *L. innocua* could be used as a biological indicator for *L. monocytogenes* in heat, gamma radiation, lactic acid and sodium nitrite treatments. In order to be considered a good microbiological indicator, the level of the microorganism should remain stable for as long as the product is stored adequately and rapidly increase if the product is temperature abused. The use of microbiological indicators for process integrity was studied by Buchanan et al. (1992).

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Section II: The Effects of Varying Thermal Processing Schedules on *Listeria monocytogenes* and Indicative Microorganisms in Blue Crab (*Callinectes sapidus*) Meat.

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ABSTRACT

In this study, blue crabs (*Callinectes sapidus*) were cooked at 250°F, 15 psi, for 5, 7, and 8 minutes and evaluated for microbial reduction and time temperature profiles. Samples from each cooking time were analyzed for aerobic, anaerobic, coliform, fecal coliform, and *Escherichia coli* counts using the current methods of the Food and Drug Administration Bacteriological Analytical Manual. Surviving species were isolated and typed for identification. Time and temperature profiles were used to form a mathematical model for the decimal reduction of *Listeria monocytogenes*. Crabs from the 8 minute cook, the current industry practice, were profiled through cooling, overnight refrigerated storage, and storage on the picking table during the following day. Coliforms, fecal coliforms, and *E. coli* were eliminated during the 7 and 8 minute cooks, while found at low levels after a 5 minute cook. Reductions in the total microbial population were found with each cooking time. A minimum of 5 cooking minutes provided a sufficient reduction of *L. monocytogenes* and other microorganisms to meet regulatory action level criteria.

INTRODUCTION

In December of 1997, the United States Food and Drug Administration (FDA) mandated that a quality assurance program based on Hazard Analysis and Critical Control Points (HACCP) be implemented in all seafood processing. This implementation has produced the need for scientific data to determine critical control points and the subsequent critical limits. The FDA has determined that the cooking step in processing fresh crab meat (meat that is not pasteurized) is a critical control point (FDA, 1996). The minimum or maximum values for the critical limit must be established to ensure that the product is adequately processed. The critical limits for the cooking step of crab meat processing should be based on the time, or length of the cooking cycle and the

76

temperature of the steam used for cooking. In order to set such limits, pathogens which may be in the raw product, and thus need to be eliminated, must be considered.

Listeria monocytogenes has been associated with three sporadic cases of seafood-borne listeriosis (Facinelli et al., 1989; Frederiksen, 1991; Baker et al., 1993). The incidence of *Listeria* species in seafood has been reported to be approximately 28% (Buchanan et al., 1989a). Commodities found to contain *L. monocytogenes* include raw and cooked shrimp, crab meat, lobster tails, squid, finfish, and surimi analogs (Farber, 1991; NACMCF, 1991). Buchanan et al. (1989b) determined that seafood has the second highest rate (28%) of *Listeria* species, with meat being first.

L. monocytogenes is a concern to the crab processor due to its ability to grow at refrigeration temperatures (Junttila et al., 1988; Walker and Stringer, 1987; Wilkins et al., 1972). If *L. monocytogenes* comes in on the raw crabs and is not eliminated in the cooking process, then it may survive and multiply during the subsequent refrigeration and processing of the meat. It has been proposed that *L. monocytogenes* can multiply at 41-50°F, 51-70°F, and above 70°F within 2 days, 12 hours, and 3 hours, respectively (FDA, 1996). Since crab meat is considered ready-to-eat, it is likely that it will not receive a heat treatment by the consumer, and thus according to the FDA, the detection of *L. monocytogenes*, by the FDA method, is a violation of the Federal Food, Drug and Cosmetics Act, section 402(a) (1) and (4).

The objective of this study was to determine if a 5, 7, or 8 minute cook provided an adequate reduction of *L. monocytogenes* and indicator organisms to meet regulatory action level criteria. This study included plotting time temperature profiles for the cooking process, calculating process lethality (F value) for each cooking time, and microbial analysis. This information could be used to determine critical limits for the cooking step in crab processing.

MATERIALS AND METHODS

Preparation of samples for cooking

All cooking was done at one commercial processing facility, in the Hampton Roads area of Virginia, to ensure that there was no variability due to differences in retorts. Crabs were delivered to the plant during June to October, washed and placed in rims for

cooking. Randomly selected crabs were divided into two groups for internal and external temperature monitoring. Each crab in the internal group had a thermocouple inserted into the back fin muscle, between its back leg and swimmer. The thermocouple wire, once inserted, was wrapped around the crab and secured with a rubber band. Each crab in the external group had a thermocouple wrapped around its body and attached by a rubber band. Internal and external crabs were evenly distributed within and among each cooking rim. Three cooking rims, which held approximately 250 lb. each, were used for each cook. Eight to 15 thermocouples were used for each cook. These thermocouples were attached to a Squirrel® (Grant, Barrington, Cambridge) data logger, which recorded time and temperature every 10 seconds during the cook. Seven 5 minute cooks, five 7 minute cooks, and three 8 minute cooks were recorded.

The retort was equipped with an automatic timer and pressure gauge. The timer was set for either 5, 7, or 8 minutes for each cook with a minimum pressure of 15 psi ensuring a temperature of 250°F.

Collection of samples

After the cooking process was complete, each rim was removed from the retort and 5 crabs were selected randomly and removed aseptically with sterile tongs from each rim; notes were made as to the location of each sample came from (top, middle, or bottom rim). The crabs were placed in sterile aluminum containers and covered with sterile aluminum foil. The containers were immediately placed on ice in a cooler for transport to the Virginia Tech Agricultural Seafood Research and Extension Center, Hampton, VA, for microbial analysis.

Microbiological analysis

The whole crabs were weighed and placed in a sterile large metal Waring blender. Sterile peptone water (0.5%) was added in an amount twice the weight of the crabs. Two crabs were removed (for ease of blending) and the remaining crabs were blended for 1 ½ minutes at high speed. The two remaining crabs were added and the mixture was blended for an additional 1 ½ minutes. Thirty ml of the crab slurry was transferred into 70 ml 0.1% sterile peptone water; resulting in the equivalent of 50 g meat in 450 ml peptone

water. From this mixture, serial dilution were made for plating and the 3 tube Most Probable Number (MPN) tests. All serial dilutions were made in 0.1% peptone water.

Three pour plate controls were utilized to determine if there were contaminants from the air, agar, or peptone.

A sample from each rim of each cook was plated in duplicate on plate count agar, anaerobic agar, and brain heart infusion agar. Both the plate count agar plates and the brain heart infusion agar plates were incubated at 37°C for 48h. Anaerobic plates were incubated for 48h at 37°C under anaerobic conditions using anaerobe jars and GasPak Plus anaerobic system envelops (BBL).

Coliform, fecal coliform, and *Escherichia coli* tests were performed using the three tube MPN method as described in the FDA's Bacteriological Analytical Manual (BAM) (Hitchins et al., 1992).

Microorganisms that survived the cooking process were isolated and identified based on microbial cellular fatty acids using the MIDI software (Hewlett Packard) program.

Analysis of data

Data generated from the thermocouples was recorded by a data logger and analyzed by an F-value program using a Z-value of 13 and target temperatures 176°F, 185°F, and 250°F. Microbial counts were analyzed by an analysis of variance.

RESULTS AND DISCUSSION

Microbial analysis

Aerobic plate counts on plate count agar showed no significant difference (p>0.5) between rim position (top, middle, or bottom) or cooking time (5, 7, or 8 min). The average aerobic plate counts ranged between 2 and 4 logs. Brain heart infusion agar, which was also incubated aerobically, resulted in CFU counts that showed no significant difference (p>0.5) between rim position or cooking time. All brain heart infusion plate counts ranged between 2 and 3 logs. The anaerobic plate counts showed no significant difference (p>0.5) in CFU counts between rim position or cooking time, and counts ranged between 2 and 3 logs.

Coliforms were only found after the 5 minute cook, but not at significant (p>0.5) levels. Fecal coliforms and *E. coli* were below the limit of detection of the 3 tube MPN method.

Two bacteria from the *Bacillus* genus were found to survive the cooking process at 5, 7, and 8 minutes. These bacteria were identified as *B. licheniformans* and *B. spaericus*.

Cooking profiles

See appendix 1 for graphs. Graphs were grouped by cooking times. Many of the graphs show wide variations when the temperature begins to rise and when the temperature decreases after the designated cooking time. These variations are thought to be due to the rapid pressure change as the steam enters the retort. At least one of the environmental probes for each cook reached 250°F for the entire cooking time. None of the internal crab probes reached 250°F. Looking at 5 minute cook A (Appendix 1), a typical cooking profile, it is calculated that the fastest heating crab obtains a cumulative F-value of 3176075.03, 645034.67, and 6.45 for target temperatures 176°F, 185°F, and 250°F, respectively. The slowest heating crab obtains a cumulative F-value of 385718.20, 78336.19, and 0.78 for target temperatures 176°F, 185°F, and 250°F, respectively. A Z-value of 13 was used for calculations. The different levels in heat received by each crab is accounted for due to location within the cooking rim.

F-values for L. monocytogenes

F-values calculated were averaged (Table II-1). The averages were compiled from seven 5 minute cooks, five 7 minute cooks, and three 8 minute cooks. F-values were calculated using a Z- value of 13 and target temperatures of 176°F, 185°F, and 250°F. All of the F-values obtained were equivalent to a minimum 7 decimal reduction of *L. monocytogenes*, based on calculations of a 7D processing being 1.1 seconds at 185°F (Rippen, 1998). Since *L. monocytogenes*, when present, occurs only at low levels in seafood, including crab meat, (Ryu et al., 1992 and Rawles et al., 1995) it is apparent that each of these cooking times is sufficient to provide a product which eliminates *L. monocytogenes*.

With the implementation of HACCP into the seafood industry and the concern over *L. monocytogenes* in cooked ready-to-eat products, in this case crab meat, there is a need to establish guidelines to ensure that the product is properly processed. A cook as short as 5 minutes at 250°F, 15 psi, provides a crab which meets FDA regulatory action level criteria. The finding that several *Bacillus* species survived the cooking process, even at eight minutes, needs to be further studied. These may be spoilage organisms which may decrease the shelf-life of the crab meat.

Table II-1 Average Calculated Cumulative F Values
Z value =13

Cooking minutes	Rim	Target Temperatures		
		176	185	250
5	Top	780000.00	160000.00	1.60
5	Middle	400000.00	81000.00	0.81
5	Bottom	210000.00	53000.00	0.53
7	Top	1300000.00	270000.00	2.80
7	Middle	1000000.00	85000.00	1.20
7	Bottom	170000.00	35000.00	0.35
8	Top	1200000.00	240000.00	2.40
8	Middle	450000.00	90000.00	0.90
8	Bottom	240000.00	48000.00	0.48

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Section III: Effect of Hand Washing on Removal of *Listeria*, Manual Washing Versus Automated Washing

J.L. Smith, G. Flick, M. Jahncke, and R. Croonenberghs

ABSTRACT

An adequate hand wash is essential to any processing plant, especially for ready-to-eat (RTE) products that will not receive a final lethal thermal process. *Listeria monocytogenes* is a pathogen of concern in RTE products and thus employee hand washing should effectively remove the organism from their hands. Testing of employees in a crab plant showed that several employees in the facility had *Listeria* species present on their hands while working. Two hand washing procedures, one manual and one automated, were tested for efficiency of removal of *Listeria innocua* as a model for *L. monocytogenes*. A manual hand wash of 15 seconds with a non-detergent soap and an automated wash of 15 seconds with 2% chlorhexidine gluconate were evaluated. It was found that the manual hand wash was more effective than an automated wash.

INTRODUCTION

Microorganisms found on the hands can be divided into two groups, resident and transient. Resident organisms have been defined as bacteria representing particular species that are recovered on more the 75% of 25 sampling days over a 7 month period; while transient organisms are comprised of those bacteria that appear less than 25% of the time during this same sampling period (Nobel and Pitcher, 1978). In food production, resident bacteria usually do not pose any threat of infectious disease to consumers who come in contact with them, but instead contribute to food spoilage (Paulson, 1993a). Transient bacteria, specifically pathogens, pose a major concern to the food industry because these bacteria are loosely attached to the workers skin and can easily cross contaminate food products if proper hand washing is not established in the work place (Miller, 1994). Isolation of pathogenic bacteria from the hands can be demonstrated after using the restroom, touching soiled surfaces, or touching raw foods. Proper hand washing can

break the transmission of pathogens from the food handlers, the food they process, and the consumer (Paulson, 1993b).

Kerr et al. (1993) reported the prevalence of *Listeria* spp. on the hands of food workers. They found that 12% of the workers surveyed had *Listeria* spp. on their hands, with 7% being *L. monocytogenes*. A control group of clerical workers did not show any individuals in the control group to be positive. Genigeorgis et al. (1990) found 30% of workers in a turkey processing plant had *Listeria* spp. on their hands or gloves. Kerr et al. (1993) also noted that of the workers found to carry *Listeria* on their hands, only one individual was deemed to have washed his hands adequately. Inadequate hand washing behavior observations included, failure to use soap/antibacterial hand wash, duration of washing lasted 10 s or less, and drying hands on visibly dirty paper towels.

An effective hand wash is dependent on an effective hand wash regimen and personnel compliance, specifically self-motivation to perform the wash and consistency in performance. Larson et al. (1997), after studying a multifaceted approach to changing hand washing behavior, concluded that intensive intervention, including education, feed back, and increased sink automation, had minor long-term effects on hand washing frequency. Instead, they indicated that the focus should be on making it more difficult not to wash hands, by means of environmental controls, automation, or management mandate. An automated hand washing system may increase hand washing compliance and consistency, because it is not dependent upon any personnel motivation (Paulson, 1993b). In an automated wash system, all the employee must do is place his or her hands in the machine and leave them in place for the duration of the wash. Paulson (1992) demonstrated, based on his data, that the bacterial $\log_{10}$ reduction averages for a manual wash were statistically equivalent ($p > 0.05$) to automated wash methods.

When testing hand washing materials the Peterson glove juice test (Peterson, 1973), which has been adopted by the FDA, is widely used (Holloway et al., 1990). The glove juice method consists of placing a sterile surgical glove over the hand, instilling a surfactant to strip the bacteria from the hands, and subsequent plating of the "glove juice" (Paulson, 1993). Other methods employed in hand sampling include swabbing and finger or hand press. The swab procedure begins by dampening a swab with saline and swabbing

a predetermined area of the hand. After swabbing is complete, the swab is returned to the sterile container of saline, which is then vortexed and plated. In the press method, subjects gently press their finger tips or hands onto an agar plate, which is later incubated and checked for growth. Paulson (1993) found the glove juice method to be consistent within ½ a $\log_{10}$ of the seeded population, whereas both the swab and finger press consistently underestimated the bacterial population by as much as 2 to 3 logs. The glove juice method typically covers more surface area and reaches subungual areas which may be missed by swabs or presses (Leyden et al., 1989).

This study began with a preliminary investigation into the presence of *Listeria* species on the hands of workers in a crab processing facility, followed by an evaluation of two hand washing procedures. Both a manual hand wash and an automated wash were evaluated for the removal of a seeded population of *L. innocua* placed on the hands of subjects.

MATERIALS AND METHODS

Preliminary testing in a crab plant

All workers (n=34) were tested twice on two separate days, testing began approximately 30 minutes after processing started. Ninety-nine ml of sterile peptone water was added to a sterile Whirl-pak speci-sponge bag (Nasco). The worker was then instructed to remove the sponge from the bag and rub the sponge over the entire surface, palm, back, and between fingers, of both hands and then deposit the sponge back into the bag. All samples were placed on ice and transported to the laboratory for analysis. Sponges were massaged inside the bag for 1 minute to loosen any material picked up by the sponge. A 25 ml aliqout was removed by sterile pipette and tested for the presence of *Listeria* species using the Reveal® for *Listeria* Test System (Neogen, Lansing, MI). This system is a rapid test which allows for detection of *Listeria* species, via flagellar antigen, within 43 hours.

Manual and Automated laboratory wash study

Bacterial preparation

       Listeria innocua was grown in Tryptic Soy Broth plus Yeast Extract (TSBYE) at 35°C overnight. After 24h the culture was approximately 10^8 CFU/ml. This was diluted to 10^2 CFU/ml in peptone immediately before applying to subjects hands.

Method of contamination

       An aliquot of the *L. innocua* suspension was pipetted onto the subjects' cupped hands. One half of a milliliter was placed in each hand. The suspension was applied over the subjects' hands by instructing the subjects to rub the suspension over the fronts and backs of their hands. The suspension was then allowed to dry on their hands before washing.

Stripping solution

       This solution was poured into the glove to remove bacteria from the hands and neutralize the cleaning agent, specifically chlorhexidine gluconate (CHG). The solution was composed of the following: 0.1% peptone, 0.1% triton X-100, 1.0% Tween 80, and 0.3% lecithin. The media was dispensed into individuals bottles (50ml/hand) and autoclaved 15 min.

Manual hand washing procedures

       Subjects were instructed to wash their hands in warm water with Ivory liquid soap. They were timed, and given 15 sec for the washing procedure.

Automated hand washing procedure

       The machine employed was the Cleantech 2000S (Meritech, Englewood, CO), which was set up according to specifications with water pressure of 50, warm water connection and 2% CHG cleaner (Meritech, Englewood, CO). Subjects were instructed to place their hands in the machine and leave them there for the duration of the wash process. The hand washing system was set for a 15 second wash cycle.

Glove juice sampling procedure

       The glove juice method was utilized as the method of hand sampling for both the manual and automated wash. A sterile latex glove was placed on one hand (randomized left and right) and 50 ml of the stripping solution was injected into the glove. The glove

was massaged, or the subject gently squeezed their hand for 1 minute. After 1 minute, the glove was removed and the cuff was secured tightly with a rubber band until further processing.

The subject was then instructed to perform either a manual or automated wash. After the wash they were allowed to dry their hands with plain paper towels. A second sterile glove was placed on the hand opposite that which was tested before the wash. The same procedure for stripping the bacteria was employed as listed above.

After all samples were taken, the solution from each glove was individually analyzed. One of the glove fingertips was sterilized with ethanol and sterile scissors were used to snip the fingertip and allow the sample to be drained into a Whirl-pak bag containing *Listeria* Enrichment Broth (LEB) for a primary incubation of 48h at 35°C.

 Following the LEB incubation, a loopful of the suspension was removed and streaked onto Oxford agar. After 24-48 h at 35°C the plates were check for black color, thus indicating the presence of *Listeria*.

RESULTS AND DISCUSSION

Preliminary testing in the crab plant

Out of 34 plant workers tested, 3 workers were found to have *Listeria* species on their hand, yet none of these persons tested positive on both testing days. This finding indicates the need for adequate hand washing in the plant setting, whether by strict enforcement of hand washing procedures or by implementing an automated wash system which standardizes the procedure for all employees. Aerobic plate counts from hands sampled before and after washing did show an average reduction of 1 log.

Manual hand wash

The manual hand wash showed the removal of *L. innocua* on most subjects. After inoculation and before the wash 15 and 16 subjects tested positive for *L. innocua*. In runs 1 and 2, respectively. The remainder of the participants, 5 and 4, respectively, may have had natural flora that out competed *L. innocua.* After washing with a mild non-detergent soap 19 subjects in the first run tested negative, while all 20 subjects in the second run tested negative. It was found that the one positive test can from an enrichment incubation

bag that had a hole in it and therefor must be disregarded in the results because it may have become contaminated during the incubation period.

Automated hand wash

The automated study also employed 20 subjects for each of its two runs. Before the wash all 20 subjects tested positive for *L. innocua* in both runs. After the 15 second wash cycle, all 20 subjects tested positive for *L. innocua*, in both runs. This signifies that the automated wash was not effective in the removal of *L. innocua* from the hands.

This study shows that there is a need for adequate hand washing, in the processing facility, which removes *Listeria*. By using *L. innocua* as a model for *L. monocytogenes*, it was shown that a manual hand wash with soap and warm water for 15 seconds was superior to a pre-set automated wash of 15 seconds employing 2%CHG and warm water.

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Section IV: Evaluation of Sanitation Based on Environmental Testing for the Presence of Adenosine Triphosphate, Microbial Populations, and *Listeria* Species

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ABSTRACT

Processing plant sanitation is essential for producing a product of high quality. Proper sanitation procedures should remove microorganisms as well as any food residual. A crab processing facility has been evaluated for sanitation based on remaining adenosine triphosphate (ATP) levels as well as microbial counts and the presence of *Listeria* spp. In most areas of the plant with high ATP readings the microbial counts were less than 10 CFU/cm^2; thus the ATP levels were a result of residual crab meat. *Listeria* was tested for since the primary product of the plant was a ready-to-eat product, in which there is a zero tolerance of *Listeria monocytogenes*. *Listeria* species were found under picking tables and on cooler doors, as well as on several employees aprons.

INTRODUCTION

Fenlon et al. (1996) showed that low levels, in both distribution and numbers, of *L. monocytogenes* are found in plants and animals; yet once processing is initiated *Listeria* numbers and the percentage of positive samples increase significantly. Thus indicating that processing is a major source of cross-contamination and amplification of the organism. Two examples confirming this idea are (1) the initial processing of grass into silage which results in burst of *L. monocytogenes* growth; and (2) the diet and feces of poultry entering a processing plant are rarely positive, however, five out of six birds sampled after processing test positive for *L. monocytogenes*. They also note that most food products implicated in major listeriosis outbreaks have received a high degree of processing. Wentz et al. (1985), in a survey of establishments processing crab meat, showed that the microbial quality of the finished product correlates very well with the handling practices the crab meat is subjected to after cooking.

Several studies have shown the presence of *Listeria* spp. in various places in the processing plant. Locations yielding positive results include: floors (Tiwari and Aldenrath, 1990; Sammarco et al., 1997), storage coolers (Tiwari and Aldenrath, 1990; Sergelidis et

al., 1997), refrigerator handles (Sergelidis et al., 1997), mechanical saws (Fenlon et al., 1996), hand basins (Sammarco et al., 1997), shelves and tables (Menendez et al., 1997; Sammarco et al., 1997).

In attempts to determine how *L. monocytogenes* may survive in a plant, several researchers have investigated its attachment and growth on work surfaces (Spurlock and Zottola, 1991; Blackman and Frank, 1996; Hood and Zottola, 1997b; and Hood and Zottola, 1997a). Based on the work of Charlton et al. (1991), suggesting that floor drains may be a source of *Listeria,* Spurlock and Zottola (1991) determined the growth and attachment of *L. monocytogenes* to cast iron, commonly used in floor drains. They found that *L. monocytogenes* Scott A could survive in the cast iron drains regardless of growth media or pH. Blackman and Frank (1996) showed that *L. monocytogenes* could form as a biofilm, the result of both adherence and growth, on hydrophilic (stainless steel) and hydrophobic (Teflon®) surfaces. The attachment of *L. monocytogenes* to stainless steel, one of the most common surfaces found in food processing plants, was studied by Hood and Zottola (1997a and 1997b). They found that the organism could adhere to stainless steel, with rates of adherence dependent on available organic material.

NACMCF (1991) states that *L. monocytogenes* is difficult to control because many food-processing plants provide conditions conducive to the organisms proliferation. Many procedures which were deemed adequate in the past for controlling pathogens are not sufficient for *L. monocytogenes.* NACMCF (1991) presents recommendations for six areas of importance (plant design and layout, equipment design, process control, employee practices, cleaning and sanitation practices and procedures, and verification) in reduction and control of *L. monocytogenes* in the plant setting. It is important to follow such recommendations because once a plant is contaminated, *Listeria* can establish itself and can lead to cross-contamination. In the case of a persisting strain, cross-contamination between different products of a single producer may result (Boerlin et al., 1997). Loncarevic et al. (1996) found that once *L. monocytogenes* establishes itself in a processing plant, the risk of continual contamination is reasonable.

Proper cleaning is necessary to remove *L. monocytogenes*, as well as, other pathogens in the processing plant. Once cells have adhered to food-contact surfaces,

traditional cleaning and sanitizing may prove ineffective (Hood and Zottola, 1997b). Blackman and Frank (1996) note that biofilm formation can in part be controlled by decreasing the levels of complex nutrients (food residue) on wet surfaces in the plant environment. Spurlock and Zottola (1991) suggest that the presence of *L. monocytogenes* in floor drains may lead to airborne contamination if the drains are not cleaned properly. Aerosols may be created when hoses are used to flush out drains because the air is forced out as the solution enters. Sammarco et al. (1997) stresses the importance of applying proper sanitizers to equipment and walls, as the practice of allowing sanitizers to flow off of walls and equipment may not provide adequate contact time, instead, foam cleanser/sanitizers may work better.

MATERIALS AND METHODS

Environmental sampling with Hy-Lite[®]

Hy-Lite (Neogen, Lansing, MI), is an adenosine triphosphate (ATP) detection system that detects the presence of ATP on surfaces. Adenosine triphosphate is a substance found in all vegetable and animal matter, including food and food debris, in addition to bacteria, fungi, and other microorganisms. Levels of ATP can be used to indicate the amount of this matter on surfaces that come in contact with food, thus giving a measure of their cleanliness. The system used for this study uses a chemical reagent that reacts with ATP to form light. The light produced is measured in relative light units (RLU). A swab moistened with sterile saline was rubbed across the area to be sampled. Each area sampled was a 10 cm^2 area, designated by a sterile aluminum template. Areas sampled included all picking tables, packing tables, scales, steam cabinet, sinks, crab bins, floors, and doors (table 4.1).

Two crab processing facilities (plant A and plant B) were evaluated by the ATP detection system. Each plant was evaluated after routine cleaning was completed. Plant A was evaluated on two separate days. After the first evaluation the results were reported to the cleaning personnel so that areas that did not pass could be paid special attention to during cleaning. One week later the second evaluation was made and those areas that did not pass were swabbed to assess bacterial populations. Plant B was evaluated on three separate occasions. The results from test one were reported to cleaning personnel before

test two was run. The following week a third evaluation was made, but cleaning personnel were not informed before hand that the test would be conducted.

Environmental sampling for microorganisms

Areas throughout the plant were sampled for bacteria populations; these areas are listed in table 4.2; these areas included tables, floors, sinks and doors. A 10 cm^2 area was swabbed with a sterile swab moistened in 10 ml sterile peptone. The area sampled was designated by a sterile aluminum template. The swabs were returned to the 10 ml peptone blanks and subsequently diluted and plated onto plate count agar. Aerobic plate counts were taken after a 2 day incubation at 37°C.

Environmental testing for Listeria *species in the processing plant*

Areas throughout the plant were tested for the presence of *Listeria* species. The Reveal® for *Listeria* polyclonal antibody test (Neogen, Lansing, MI) was used for analysis. Samples to be analyzed by the polyclonal antibody test for *Listeria* were taken using the speci-sponge bag (Nasco) with 99ml sterile peptone. The peptone was added just before sampling to saturate the sponge. The sponge was removed by hand using sterile latex gloves; excess peptone was squeezed from the sponge and an area of approximately 1 ft^2 tested. The sponge was rubbed over the designated area and returned to the bag and placed on ice for transport to the lab for analysis. A 50 ml aliqout was used for the test.

Apron sampling for Listeria *species and total bacterial populations*

Workers' aprons were sampled for *Listeria* species using the polyclonal antibody test for *Listeria* species. Bacterial populations were grown on plate count agar.

Samples from aprons to be analyzed by the polyclonal antibody test for *Listeria* were taken using the speci-sponge bag (Nasco) with 99ml sterile peptone. The peptone was added just before sampling to saturate the sponge. The sponge was removed by hand using sterile latex gloves; excess peptone was squeezed from the sponge and an area of approximately 1 ft^2 tested. The sponge was rubbed over the designated area and returned to the bag and placed on ice for transport to the lab for analysis. A 50 ml aliqout was used for the test. Both the front, that which may come into contact with the crab meat, and the back, that which comes in contact with the employees' clothing, were sampled.

A swab sample was also taken of both the front and the back of each employee's apron. A sterile swab dipped in a 10 ml peptone blank was used to swab a 10 cm^2 area outlined by a sterile aluminum template. The swab was returned to the peptone blank and placed on ice until analysis. Serial dilutions were made in peptone and plated on plate count agar. Plates were counted after a 2 day incubation at 37°C.

RESULTS and DISCUSSION

Hy-lite testing

The range of this test is 0-99000 RLU. Pass, caution , and fail limits were set as follows: 50-500 RLU, 501-1499 RLU, and 1500-99000 RLU, respectively.

Plant A showed improvement between the first and second test. Test 1 resulted in 33% passing, 10% caution, and 57% failures while test 2 resulted in 41% passing, 21% caution, and 38% failures. This shows that intervention, in this case informing the personnel of areas that did not pass, can improve sanitation levels. The microbial analysis of those areas not passing showed that most areas had low microbial counts, less than 10^2 CFU/cm^2, and thus the high RLU reading was a result of residual crab meat. High microbial counts, up to 10^4 CFU/cm^2, were found under several of the picking tables, sink handles, the canning machine and the floor.

Plant B showed improvement between the first and second test but regression between the second and third test. Test 1 yielded 17% passing, 11% caution and 72% failure, while test 2 resulted in 50% passing, 13% caution and 37% failure. Test 3 resulted in 22% passing, 15% caution and 63% failure. The increase in samples passing in the second test is attributed to employee knowledge of the areas that failed and knowledge that all areas would be tested again the next week. The unannounced third test shows that employee motivation is essential to good cleaning practices. Unannounced inspections are more reflective of day to day operations. Microbial analysis of those areas that failed showed that there was a high level of microorganisms, 10^4 CFU/cm^2, under the picking tables. Other areas tested did not yield high microbial counts and thus residual crab debris was assumed to give the high RLU readings.

Although few places in either plant had high microbial counts (over 10^3 CFU/cm^2) after cleaning, the residual crab debris remains a problem. If this is not effectively removed from the work area each day, it may build up and become a haven for microorganisms to multiply.

Microbiological analysis

All microbial samples were taken after cleaning and sanitizing except where noted. High microbial counts associated with high RLU counts are noted above. High counts typically were found under tables, with counts as high as 10^4 CFU/cm^2. Table tops were found to have low counts with less than 10 CFU/cm^2. Employees' chairs ranged from 10^0-10^1 CFU/cm^2. The weigh up and packing area was found to have low microbial counts of less than 10 CFU/cm^2. Cooler doors, sink handles and rest room doors all fell within the 10^3-10^4 CFU/cm^2 range. Samples taken of the hand dips, cup dips, and crab shovel dips were taken in the middle of the processing day, to assess their effectiveness after continued use. Two hand dips out of 7 tested showed no microbial presence. These two hand dips were rarely used due to their location in the plant. All other hand dips had microbial populations of 10^2 CFU/ml, with the cup and shovel dip also falling within this range. Most dips contained crab debris and paper towels which contributed to the higher counts. All dips used in the plant contained a minimum of 400 ppm quaternary ammonia compound.

Environmental testing for Listeria

All areas that were evaluated for microbial populations and general sanitation, were tested for the presence of *Listeria* species. Three picking table tops were found to be positive, even though these tables were found to have low microbial counts, and two of the tables had passed the ATP test. The underneath of every picking table was found to be positive for *Listeria* species. Visible crab meat was found on the underneath edge of every picking table. This buildup harbored the bacteria and as a result of this finding all tables were dismantled steam cleaned and repainted. In addition, daily cleaning now includes scrubbing the underneath edge of each table, with the entire table top being removed for thorough cleaning once a week. Both doors to the cooler were also found to be positive. Despite cleaning, these doors were always found to be positive for *Listeria*.

All crab meat samples taken during this study were also found to be positive for *Listeria* species, this may be attributed to the presence of *Listeria innocua*.

Apron study

Out of 38 employees' aprons tested, two were found to be positive for *Listeria* species on the front side. The two positive aprons had microbial counts of less than 10 CFU/cm^2. Microbial counts were found to be higher on the front side of the apron, with an average of 430 CFU/cm^2; whereas the back side had an average of 150 CFU/cm^2.

Table 4.1 Areas Tested for the Presence of ATP in Plant A

Picking tables, both top and underneath (12)
Cart
Sink (4)
Floor
Steam cabinet
Wench
Crab bin
Crab shovel (2)
Plastic curtains (2)
Chairs (4)
Packing table
Scales (2)
Ice shovel
Ice bin
Multivac
Lids
Canning machine
Floor drain
Weigh up window

Table 4.2 Areas tested for the presence of ATP in Plant B

Picking tables, both top and underneath (12)
Chairs (5)
Packing table
Weigh up window
Scales (3)
Cooler doors (3)
Plastic curtain (2)
Crab bin
Sinks (3)
Gloves (2)
Underneath refrigeration unit
Vending machines (2)
Telephone
Dolly

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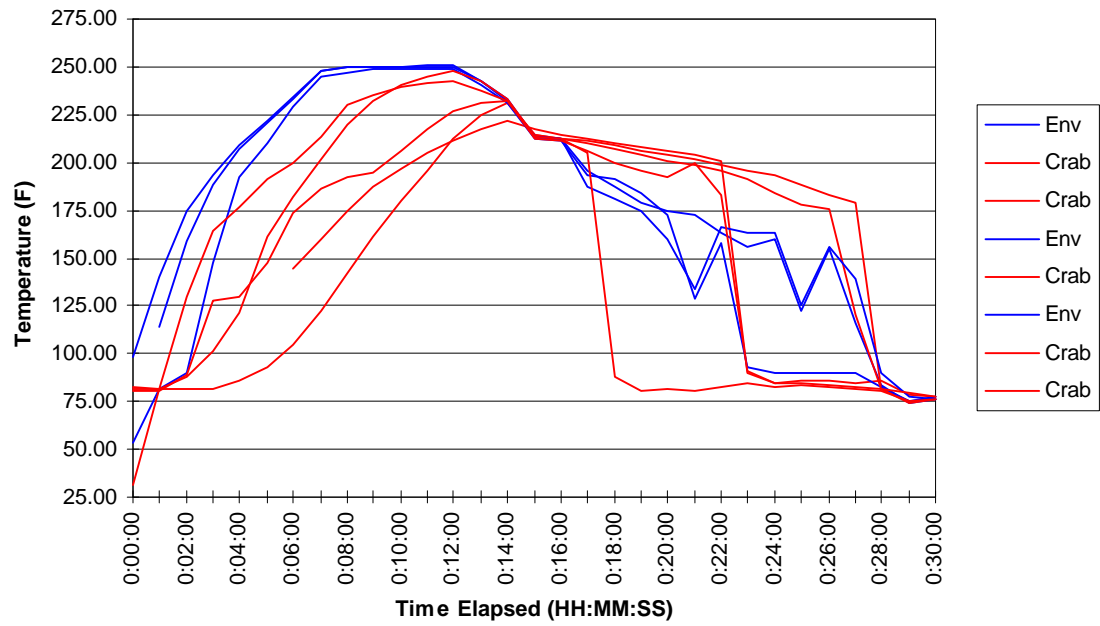
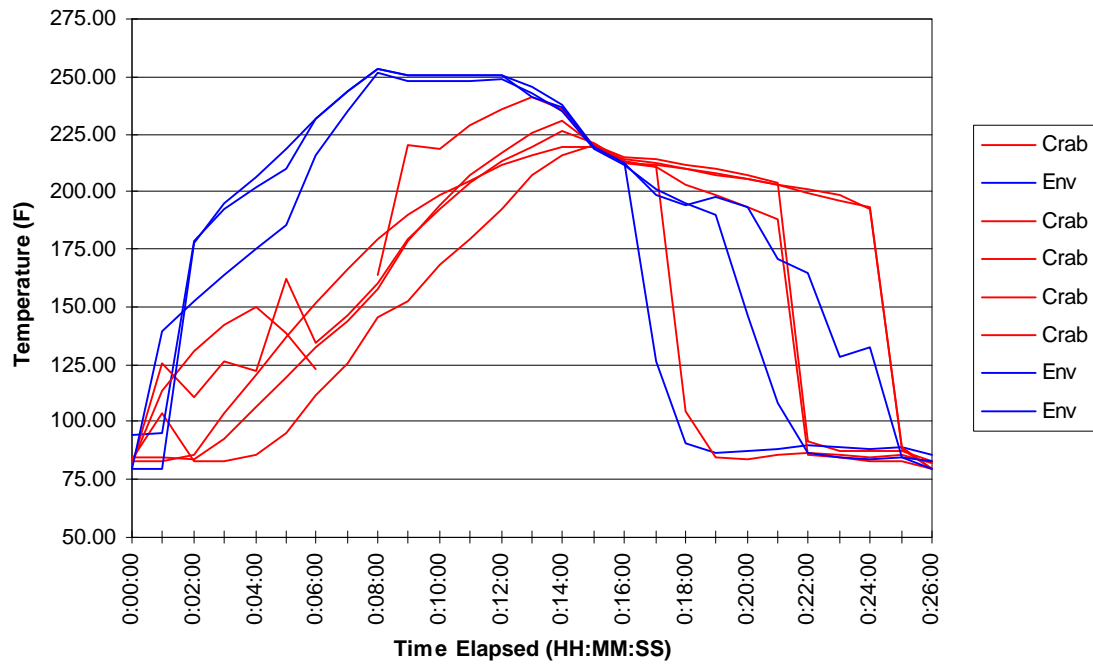**Appendix 1: Cooking Graphs**

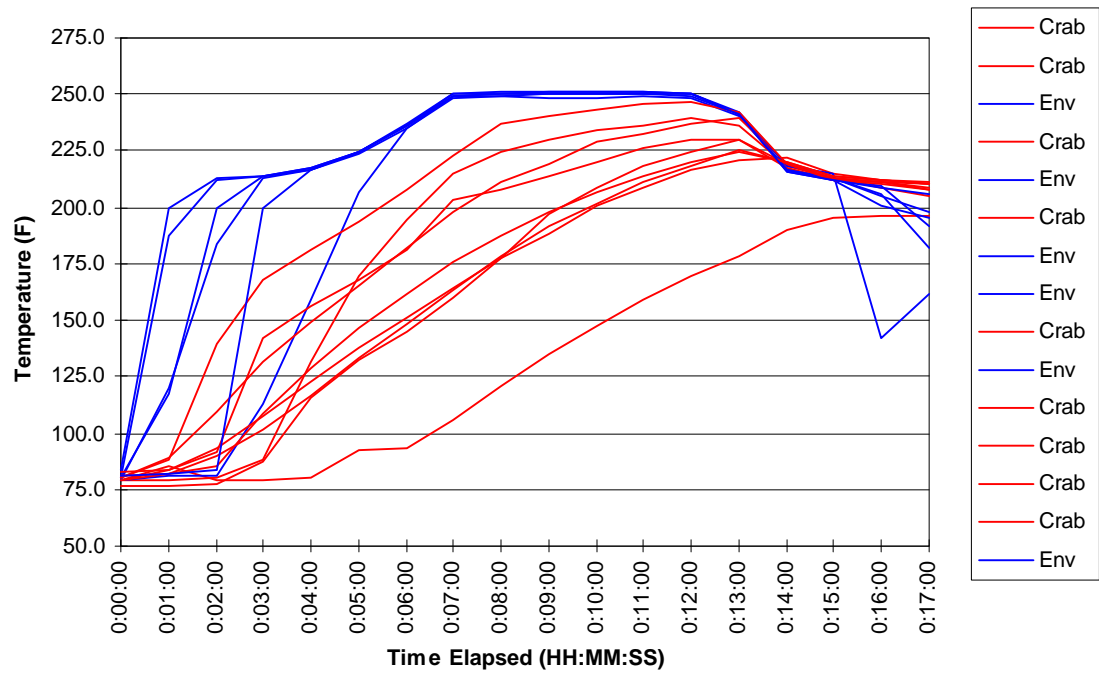**5 Minute Cook (A)**
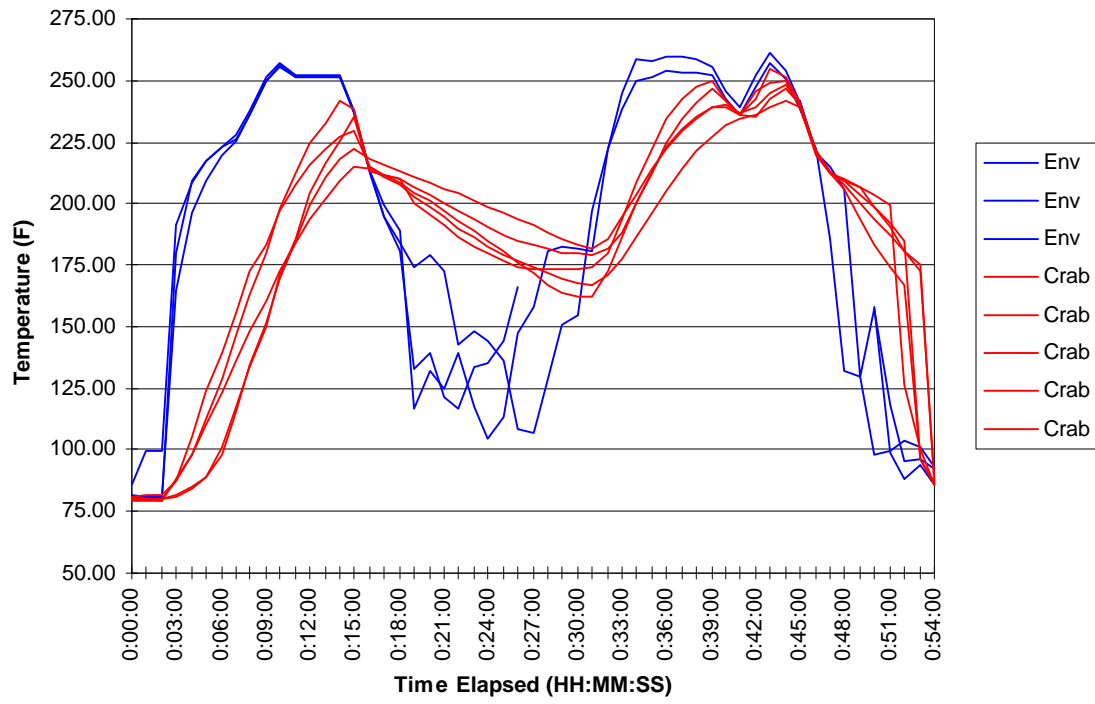
5 Minute Cook (B)

5 Minute Cook (C)

Legend:
- Env
- Crab
- Env
- Crab
- Crab
- Env
- Crab
- Crab

Y-axis: Temperature (F)
X-axis: Time Elapsed (HH:MM:SS)
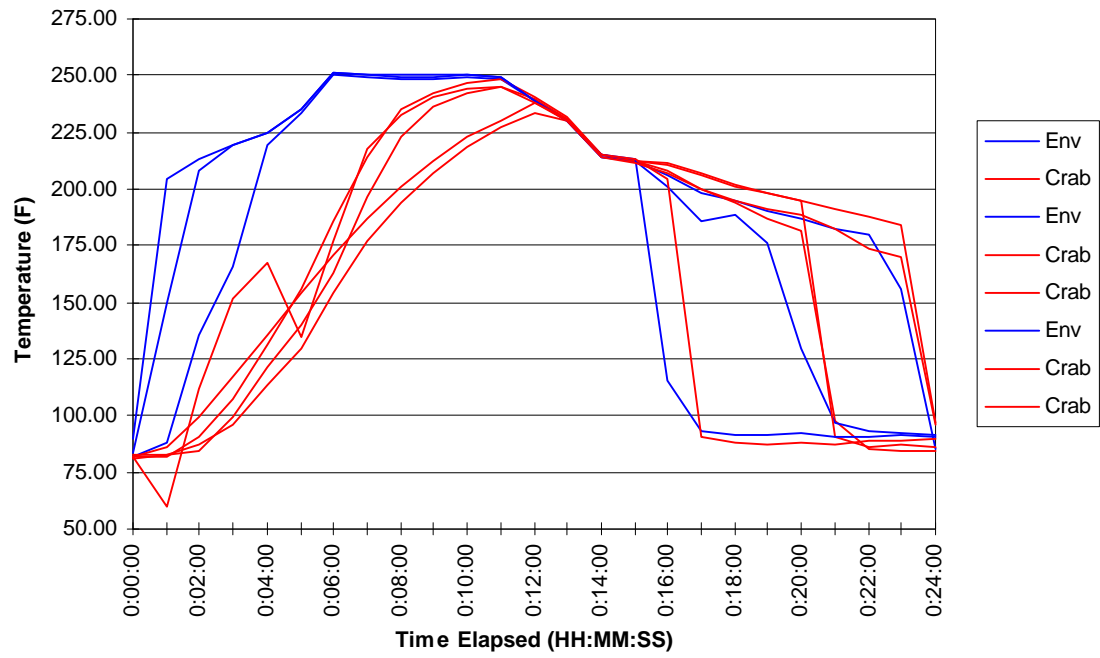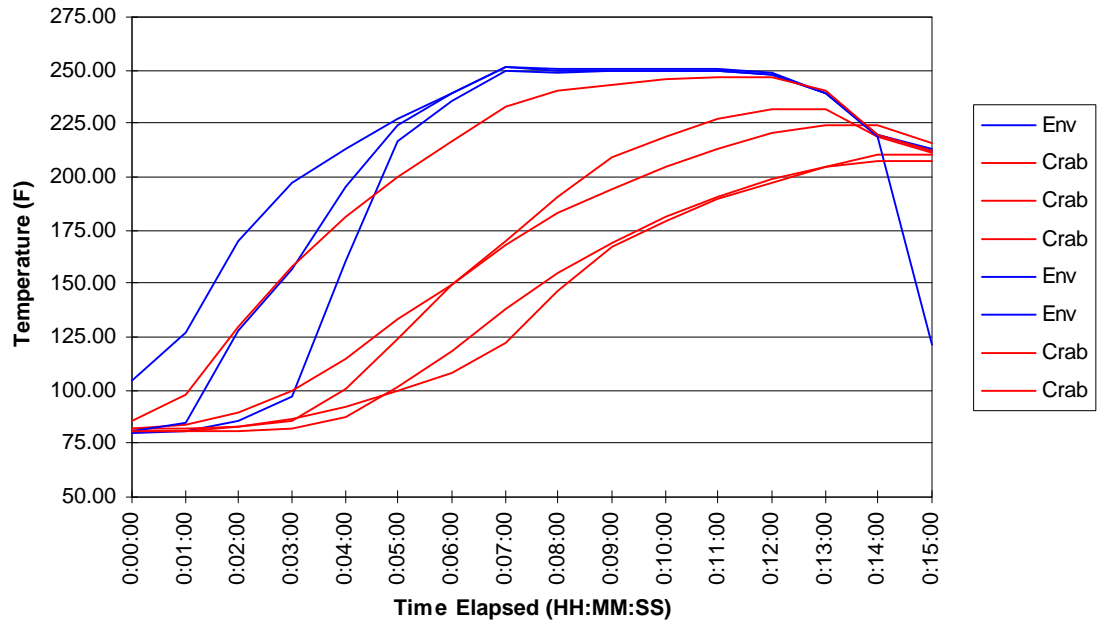
5 Minute Cook (D)

5 Minute Cook (E)

5 Minute Cook (F)

Legend:
- Crab
- Crab
- Env
- Crab
- Env
- Crab
- Env
- Env
- Crab
- Env
- Crab
- Crab
- Crab
- Crab
- Env

Y-axis: Temperature (F)

X-axis: Time Elapsed (HH:MM:SS)

5 Minute Cook (G)

Legend (top to bottom): Env, Crab, Env, Crab, Env, Crab, Env, Env, Env, Crab, Crab, Env, Crab, Env

X-axis: Time Elapsed (HH:MM:SS)
Y-axis: Temperature (F)
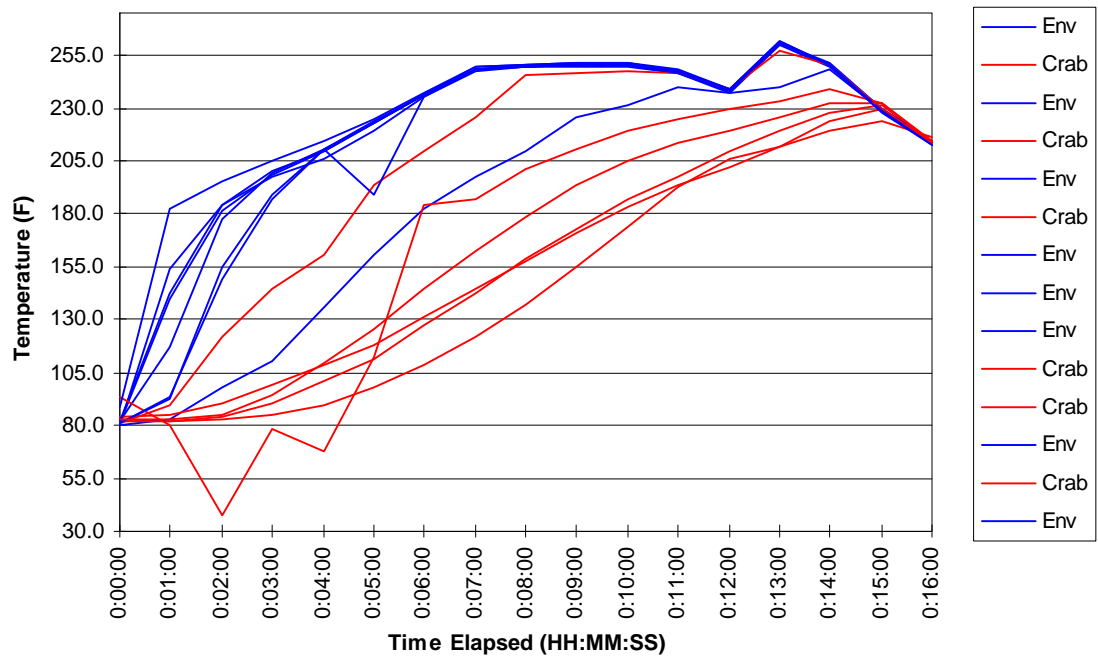
7 Minute Cook (A)

7 Minute Cook (B)

Legend:
- Env
- Env
- Crab
- Crab
- Env
- Crab
- Env
- Crab
- Crab

X-axis: Time Elapsed (HH:MM:SS) from 0:00:00 to 0:16:00
Y-axis: Temperature (F) from 50.0 to 275.0

7 Minute Cook (C)

Legend:
- Env
- Crab
- Crab
- Crab
- Crab
- Crab
- Env
- Env
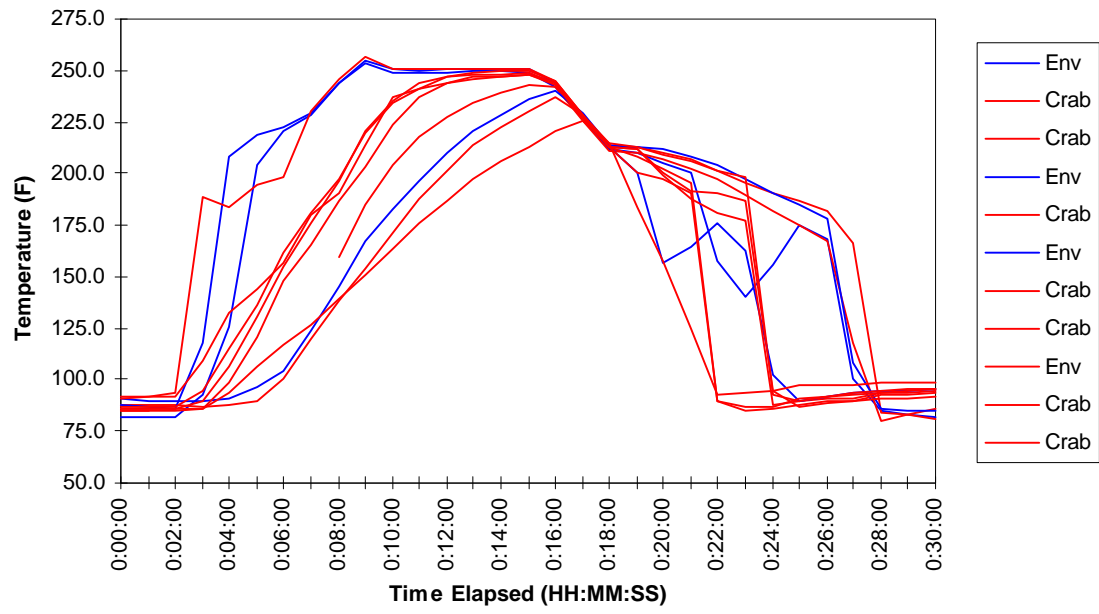- Crab
- Crab
- Crab

Y-axis: Temperature (F)
X-axis: Time Elapsed (HH:MM:SS)
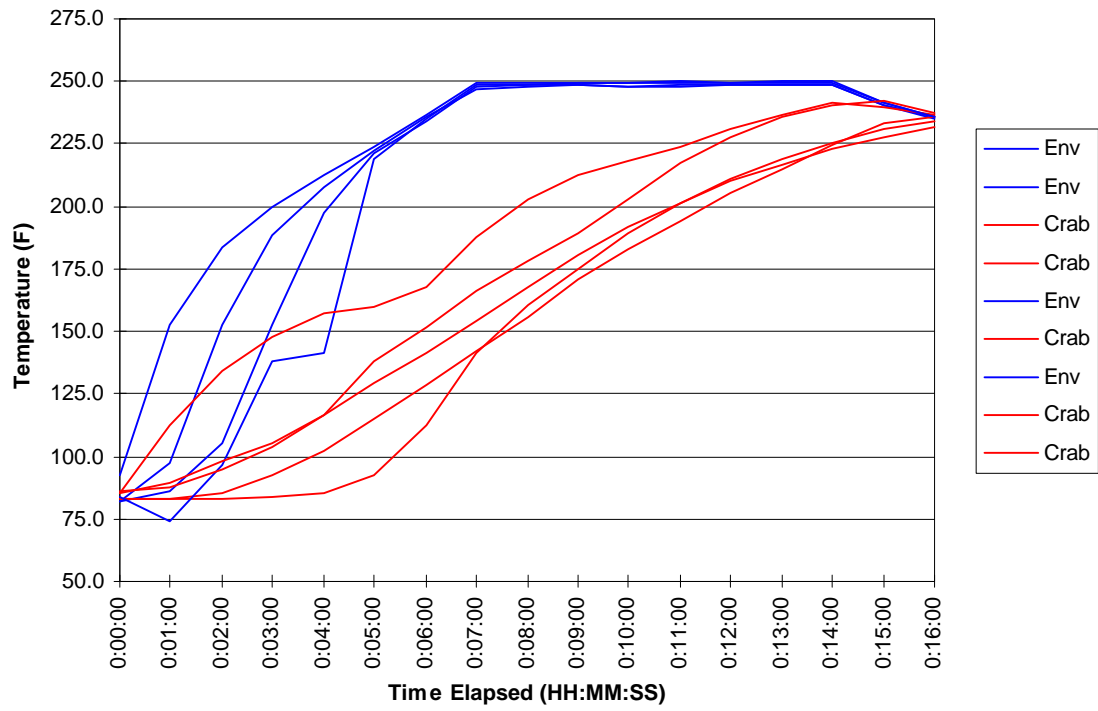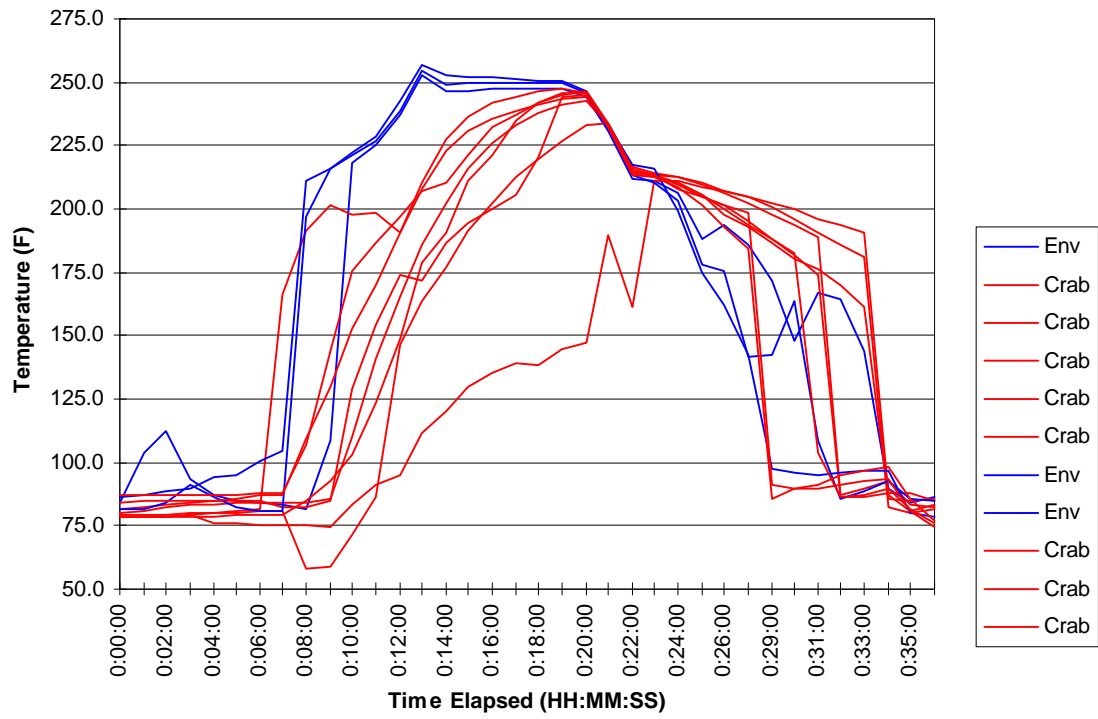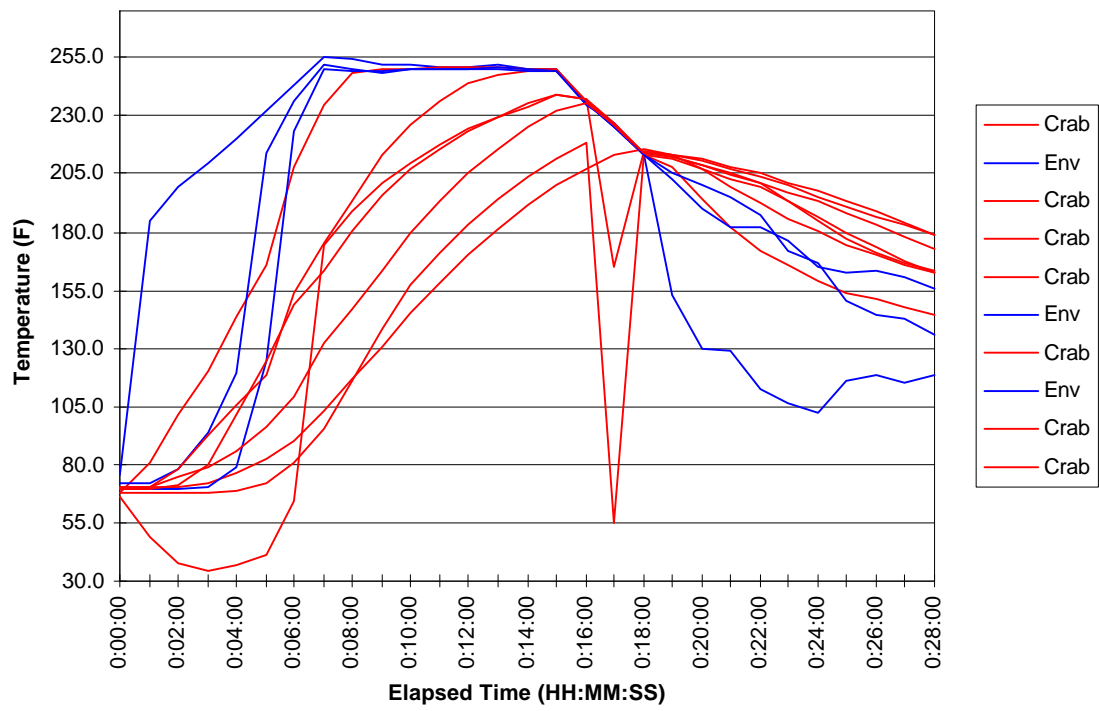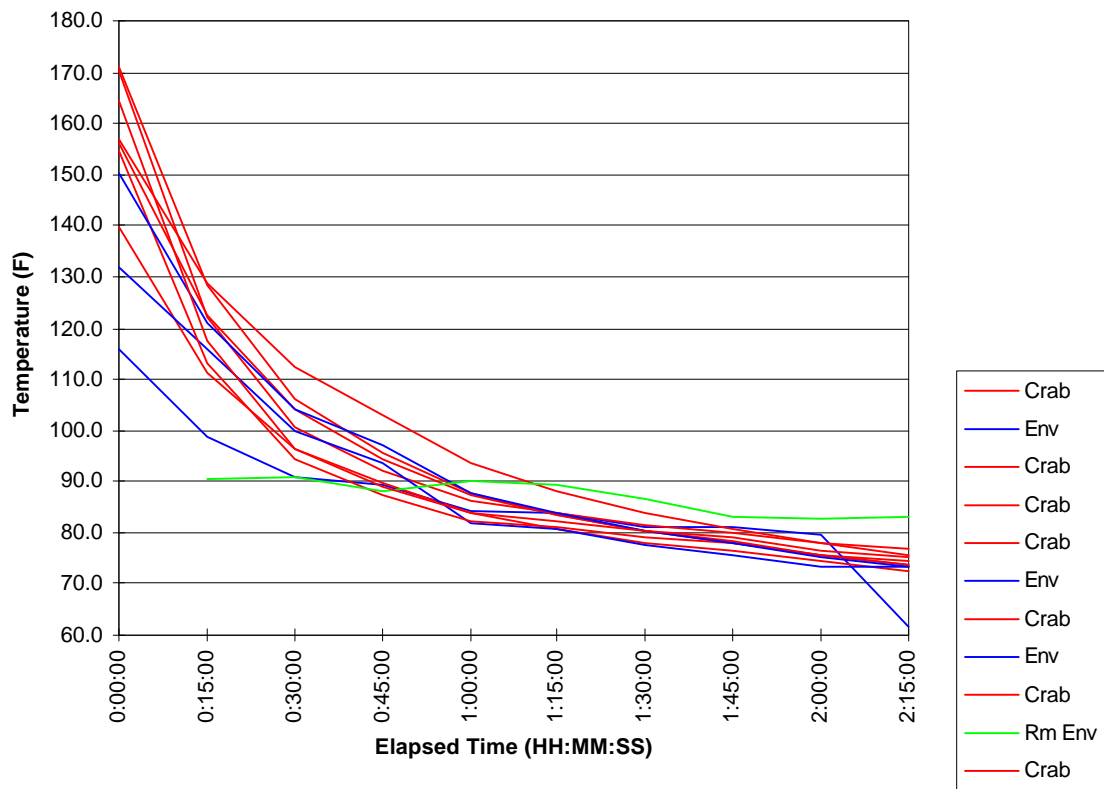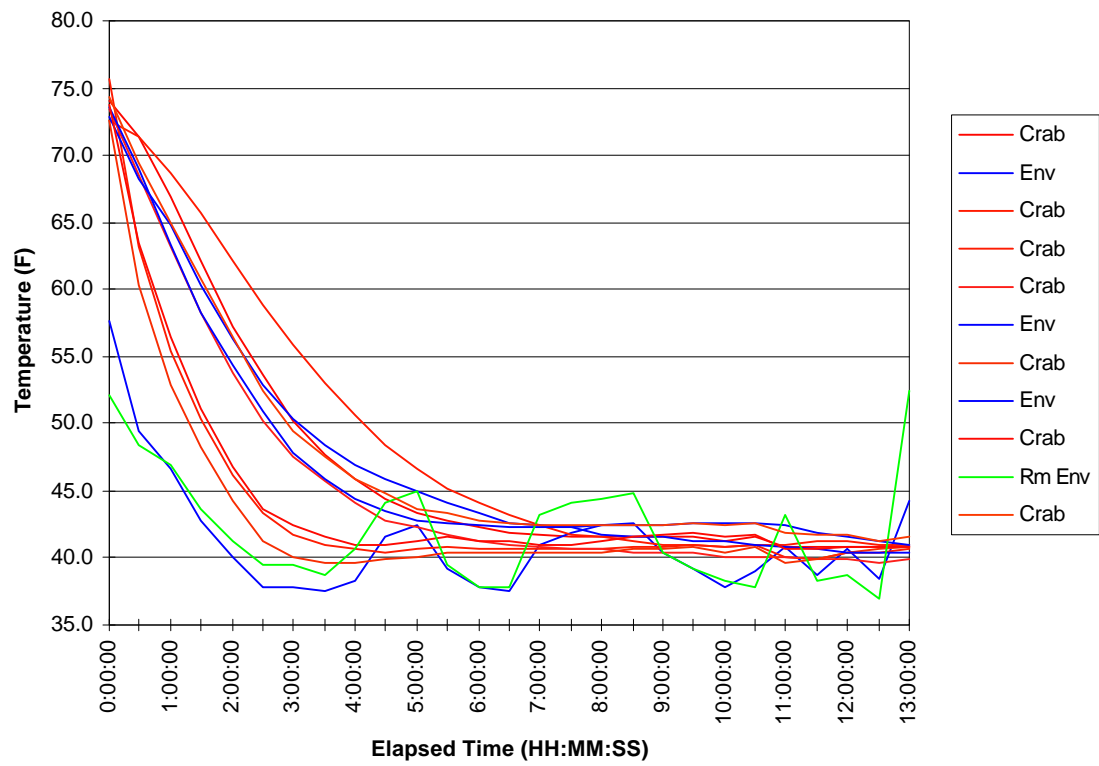
7 Minute Cook (D)

7 Minute Cook (E)
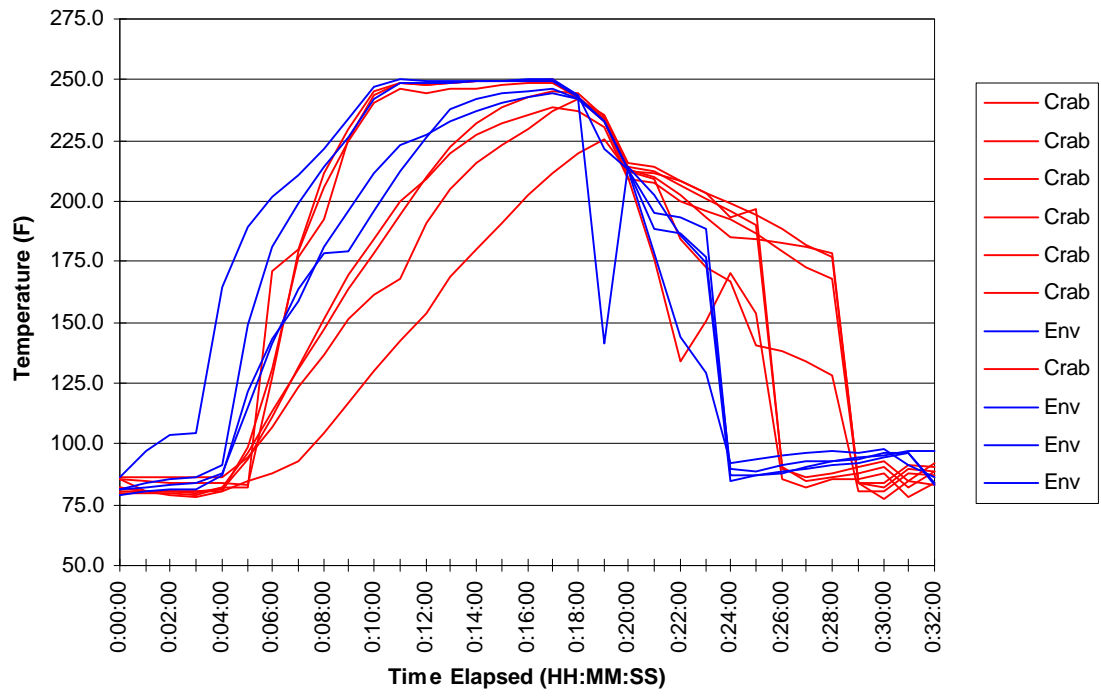
8 Minute Cook (A)

Air Cool (A)

Refrigerated Cooling (A)

Crabs on the Picking Table (A)
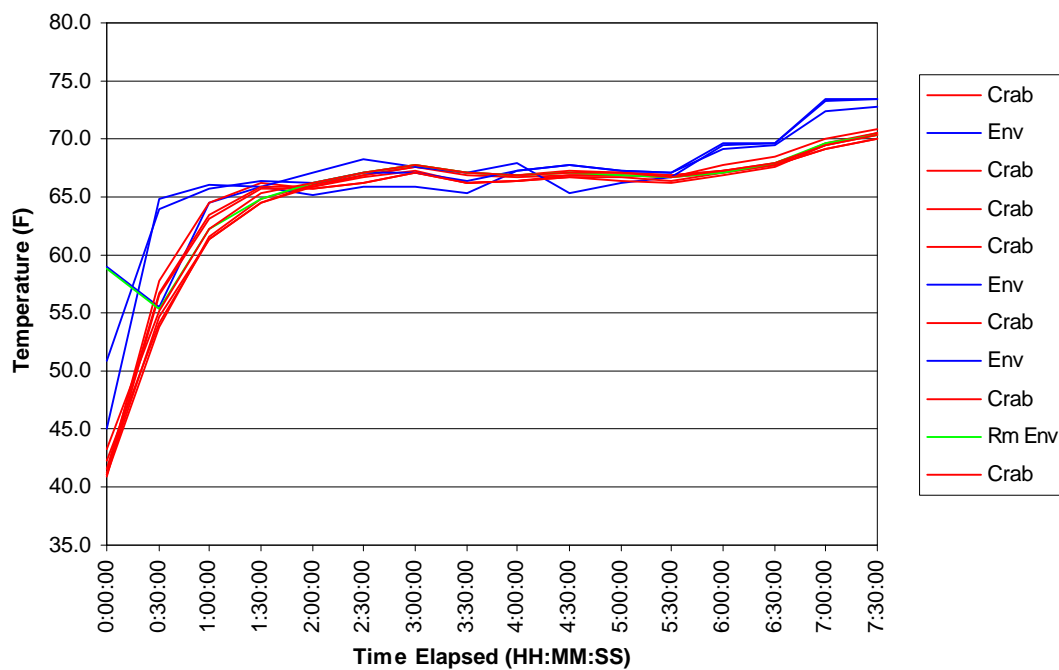
8 Minute Cook (B)
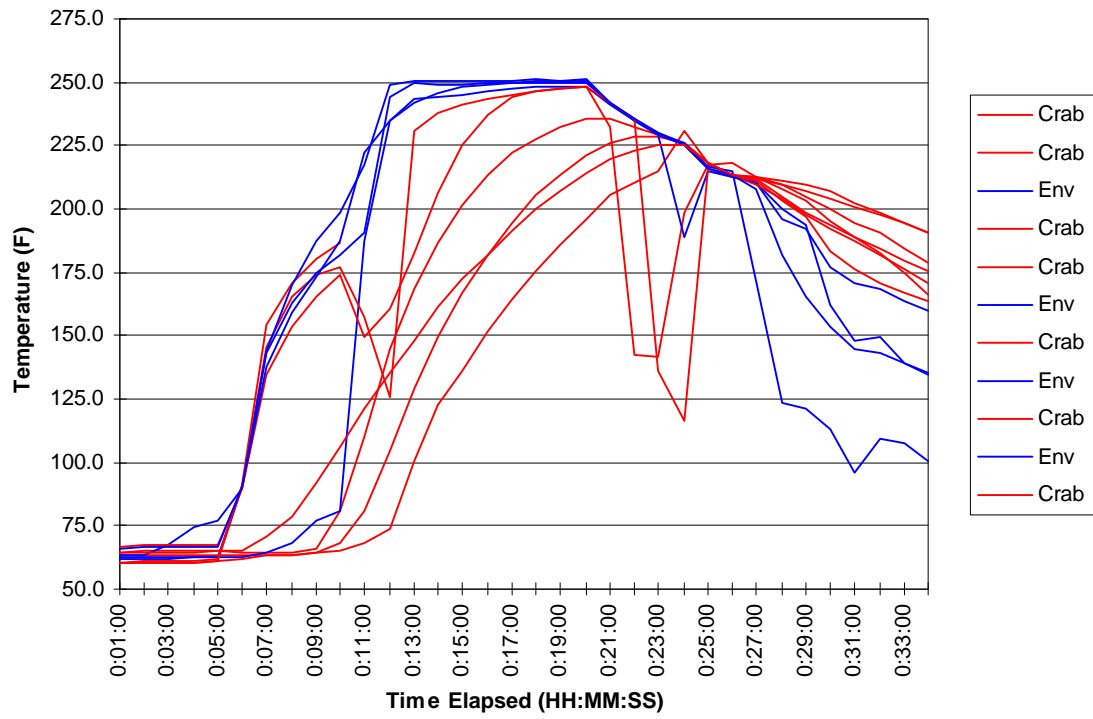
Air Cool (B)

Refrigerated Cooling (B)

Chart showing Temperature (F) on the y-axis (ranging from 35.0 to 90.0) versus Time Elapsed (HH:MM:SS) on the x-axis (ranging from 0:00:00 to 14:00:00). Legend entries from top to bottom: Crab, Crab, Env, Crab, Crab, Env, Crab, Rm Env, Crab, Env, Crab.
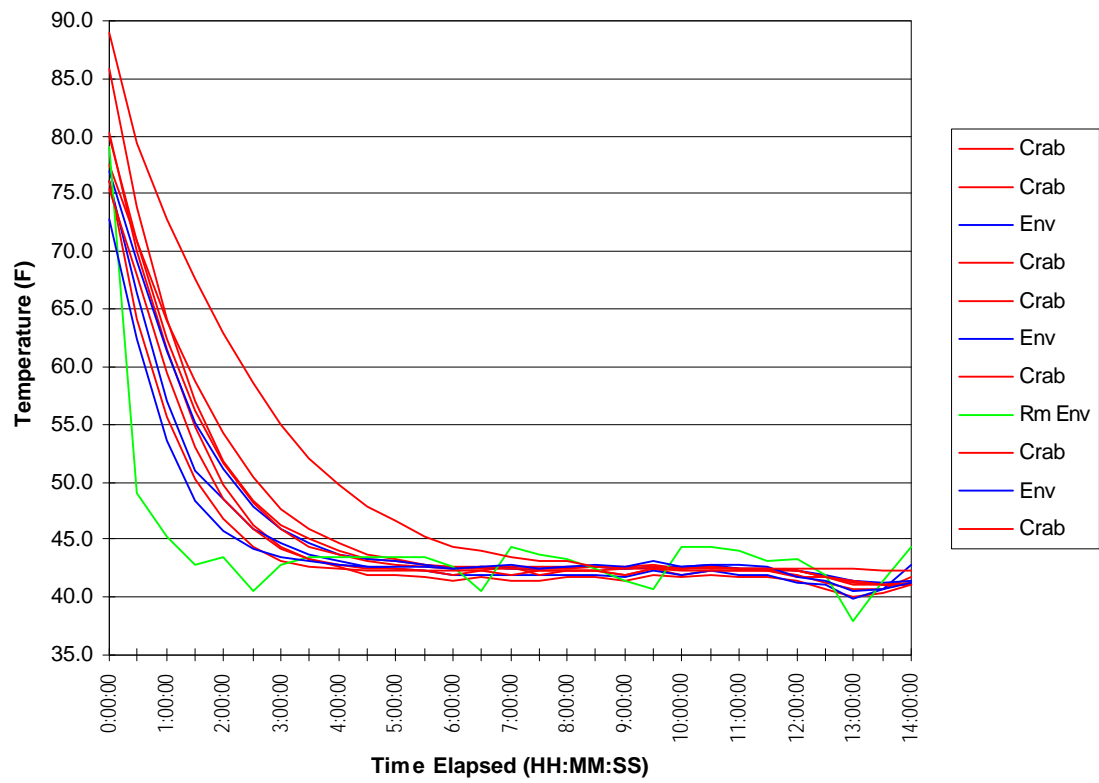
Crabs on the Picking Table (B)
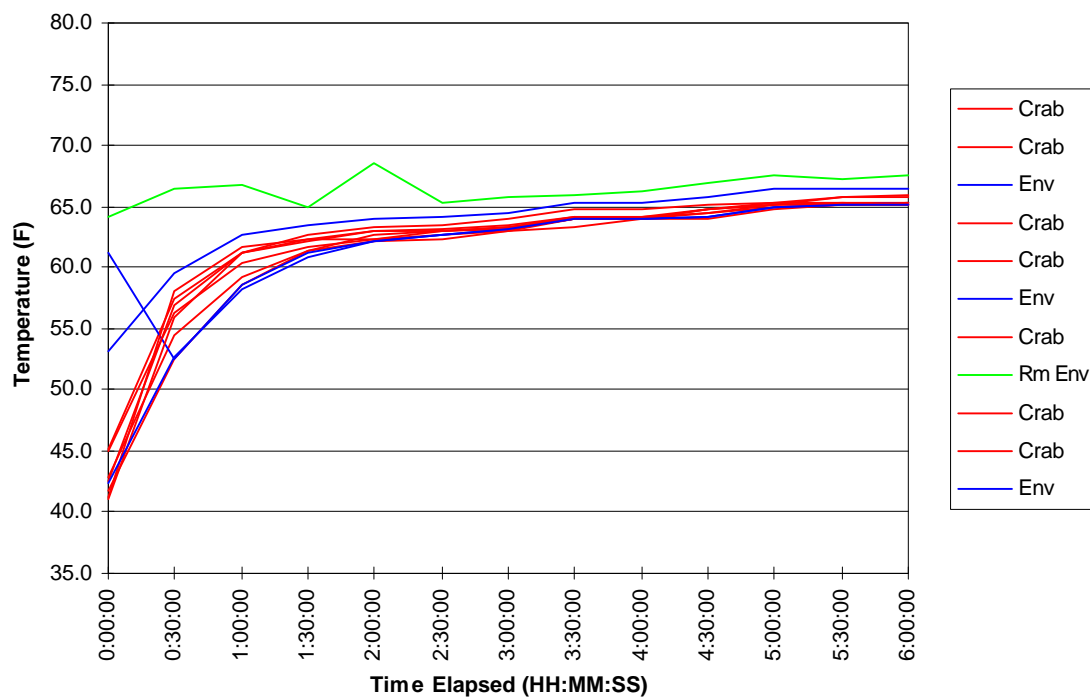
8 Minute Cook (C)
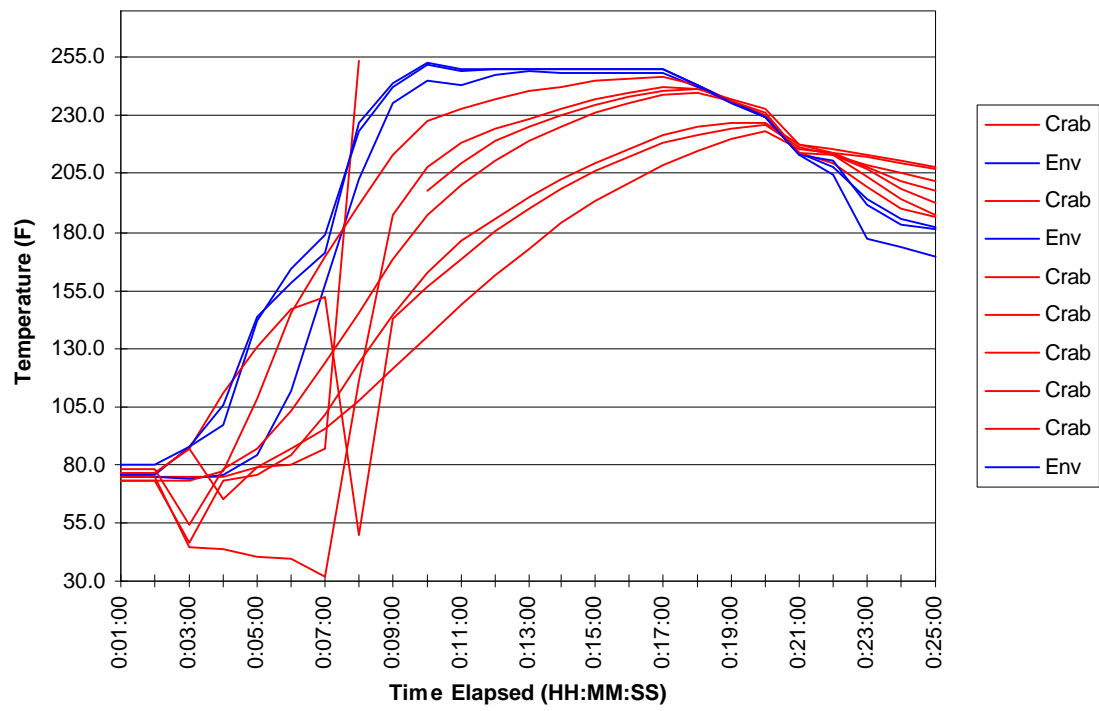
Air Cool (C)
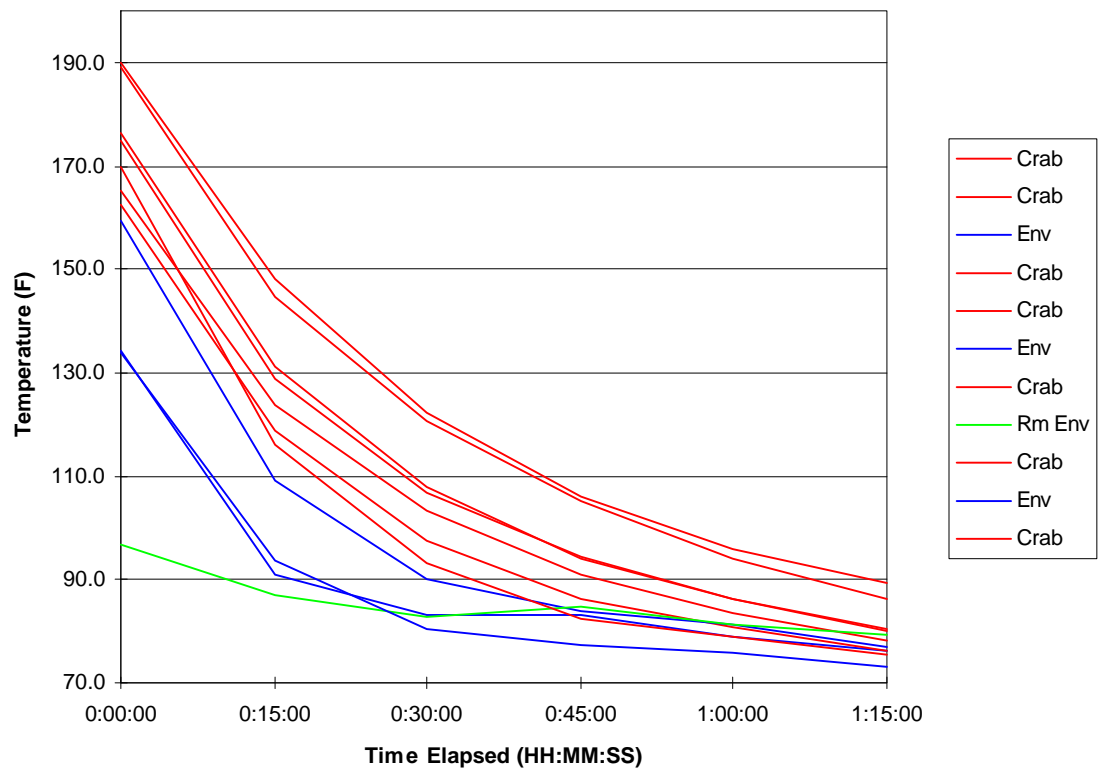
Refrigerated Cooling (C)

Legend: Crab, Env, Crab, Env, Crab, Crab, Crab, Crab, Crab, Env, Rm Env

X-axis: Time Elapsed (HH:MM:SS)

Y-axis: Temperature (F)
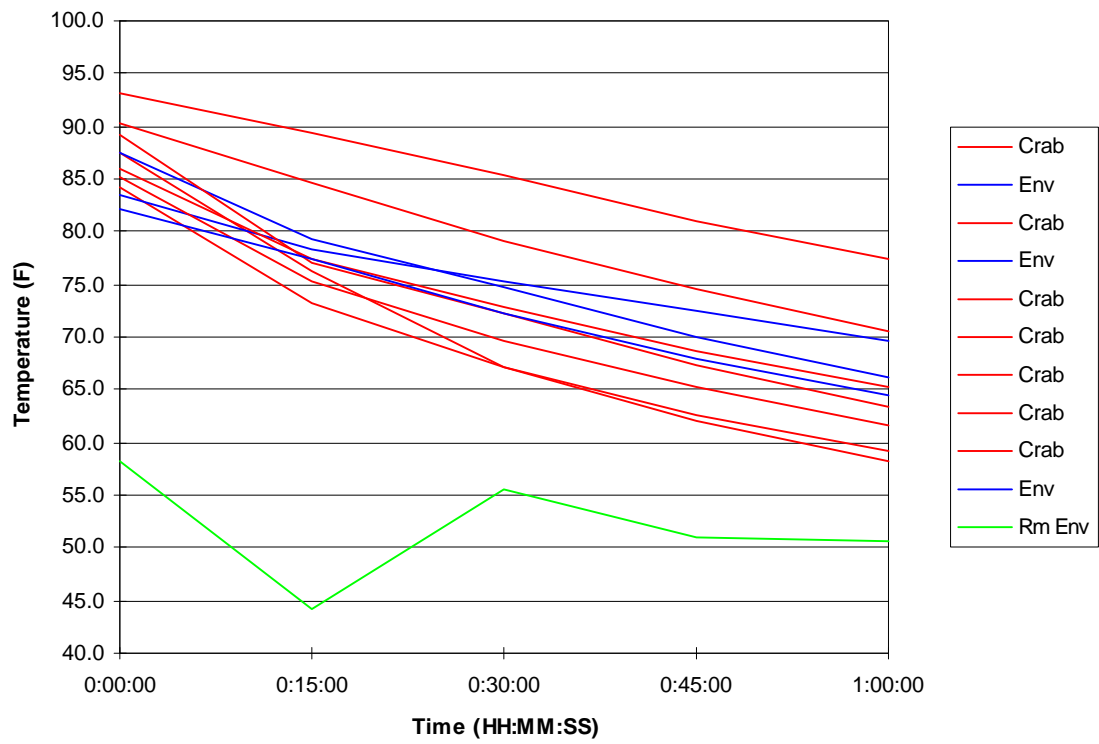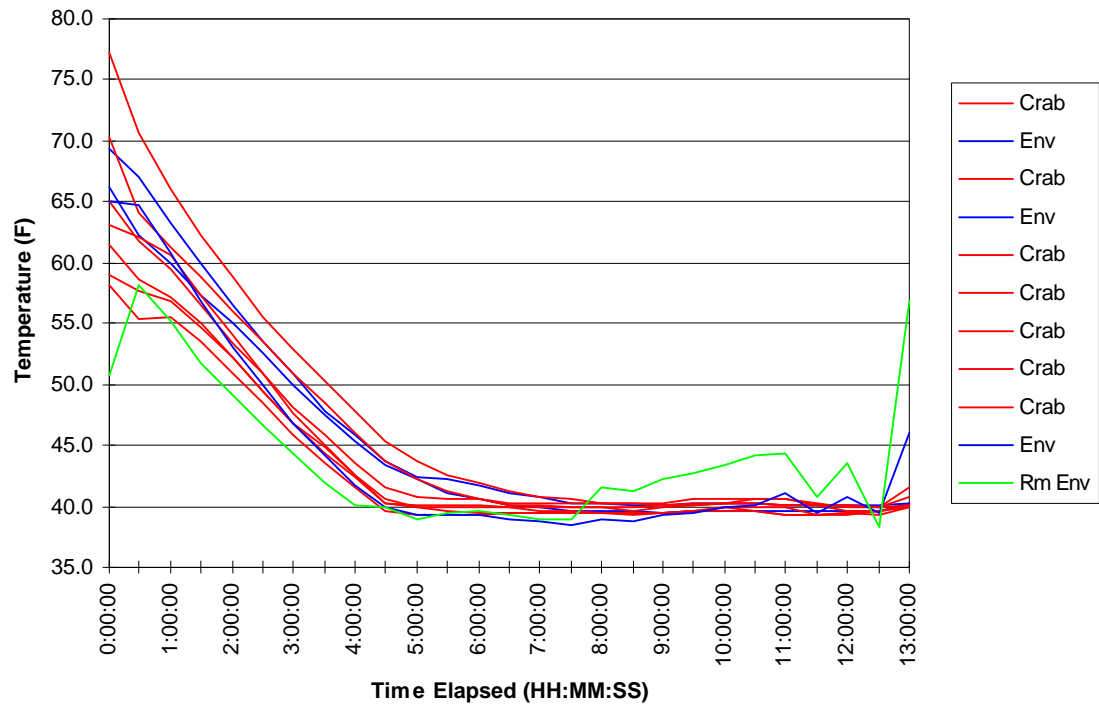
Crabs on the Picking Table (C)
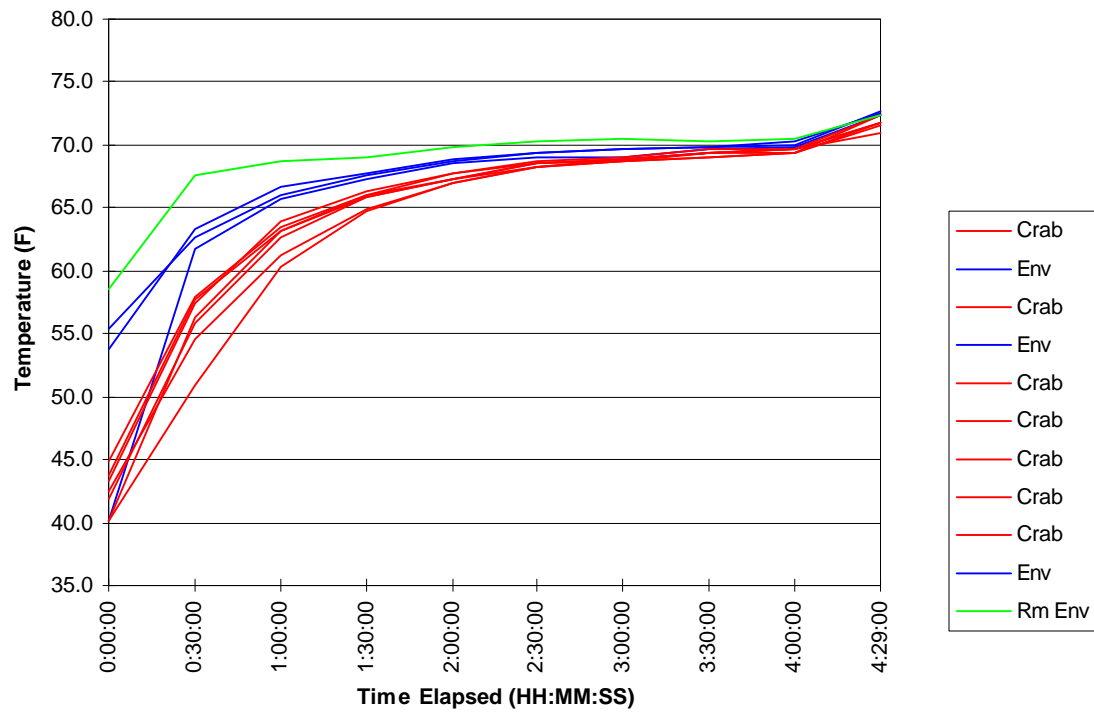
Appendix 2: Evaluation of Picked Blue Crab (*Callinectes sapidus*) Meat as Effected by Ambient Temperature Over Time

The objective of this study was to determine if the microbial population in fresh picked blue crab meat increases when exposed to ambient temperatures over a period of four hours.

MATERIALS AND METHODS

Picked crab meat was collected from pickers at weigh up. Each time the study was run, meat was collected from three different pickers; designated sample 1, sample 2, and sample 3. The meat was taken from the top of the pickers' bowl to assume a starting time of 0 hours. The meat was placed 1" deep in a sterile aluminum container by a packing room employee.

The three aluminum containers were placed on a picking table in the middle of the picking room. Samples were taken from each container every hour for four hours. Every hour, the temperature of the meat and the air temperature were recorded. Samples were removed by sterile scoops. A new scoop was used for each sample each hour. The meat taken was placed in a sterile Whirl-Pak (Nasco) bag and placed on refrigerant bricks for transport to the laboratory for analysis. All samples were plated within 6 hours.

Fifty grams of crab meat were aseptically removed from each bag for analysis. The 50 g sample was placed in a sterile glass blender jar with 450 ml of 0.5% sterile peptone and blended in a Waring blender at high speed for 60 seconds. This mixture was serially diluted in peptone and plated on plate count agar, anaerobic agar and brain heart infusion agar. A most probable number (MPN) series was also run for coliforms, fecal coliforms and *Escherichia coli* as described by the FDA Bacteriological Analytical Manual.

A second study was run as a control. Three pickers were selected to participate in this study. Meat was picked and directly placed by the picker into a sterile aluminum container. The containers were collected after the meat was 1" deep. Each pan was treated as above, and the same analysis performed.

Statistical analysis

A statistical analysis was conducted on both studies. Study 1 was composed of 6 trials resulting in 18 samples. Plate counts (aerobic, anaerobic, and brain heart infusion)

and MPNs (coliforms and fecal coliforms) obtained were analyzed based on time, air temperature, and meat temperature, using SAS, as follows: 2 way ANOVA with time and temperature as factors; two way ANOVA of the natural log of each value with time and temperature as factors; Kruskal-Wallis procedure using time as the factor; Robust technique using huber m-estimates; Kruskal-Wallis procedure with outliers removed; Wilcoxon rank sum for air temperature; Robust technique for air temperatures; two sample t-test for meat temperatures; Wilcoxon rank sum for meat temperatures; and the Robust technique for meat temperatures.

The control study, study 2, consisted of three runs, for a total of seven observations. One participant dropped out after the first run. The plate counts and MPNs obtained were analyzed based on time using the following tests: Barlett's test, Levene's test, ANOVA, Robust technique, and Welch's ANOVA.

RESULTS AND DISSCUSSION

Study 1

Study 1 was composed of 18 observations. The plate counts and MPNs were analyzed by type. *Escherchia coli* was dropped from the analysis because its presence was not detected in any of the samples.

At hour 1, the average air temperature was found to be 70°F with a range of 67-72°F. The average meat temperature was found to be 66°F with a range of 64-70°F. For hours 2, 3 and 4 the average air temperatures were found to be 71°F, 72°F, and 72°F, respectively with ranges of 67-70°F, 68-74°F, and 69-74°F, respectively. Average meat temperatures were found to be 67°F, 68°F and 69°F for hours 2, 3 and 4, respectively. Temperature ranges were found to be 65-70°F, 66-70°F, and 67-71°F for hours 2, 3 and 4, respectively.

Aerobic Plate Count

Average aerobic plate counts were found to be 470000 CFU/g (range 6000 - 4000000), 310000 CFU/g (range 6000 - 3300000), 400000 CFU/g (range 2600 - 3000000), and 510000 (range 2000 - 3400000) for hours 1, 2, 3, and 4, respectively. The

aerobic plate counts were first analyzed by a box plot graph, it was noted that outliers may be a problem. To over come this problem the two way ANOVA was carried out on both the original data and the natural log of the data. Using air temperature and time as factors, both of these tests resulted in no affect due to air temperature or time. The Kruskal-Wallis procedure run with time as the only factor resulted in no significant difference due to time (p=0.3416). A Robust technique, to account for outliers was applied to the data set. A p-value of 0.07843 indicated that there may be a difference due to time. P-values of 0.0506, 0.0820, and 0.01363 for hours 1, 2, and 3, respectively were contrasted with that of 4 hours. This suggests that time 4 was different from the others. The Kruskal-Wallis test run after outliers were removed resulted in a p-value of 0.076, thus suggesting that there are differences due to the time factor. Air temperature tested by the Wilcoxon rank sum test implied that there was no affect due to air temperature (p=0.9834). With the robust technique applied to account for outliers, there was still no affect due to air temperature (p=0.2895). A two sample t-test for the meat temperature indicated no significant affect (p=0.8465). The Wilcoxon rank sum test for the meat temperature showed no affect (p=.7101). Application of the robust technique to the meat temperature also implied no affect on the aerobic plate count due to the temperature of the meat (p=0.8199).

Anaerobic Plate Count

Average anaerobic plate counts for hours 1, 2, 3, and 4 were found to be 150000 CFU/g (range 1000 - 2500000), 32000 CFU/g (range 1000 - 250000), 9900 CFU/g (range 1000 - 52000), and 31000 CFU/g (range 1000 - 330000), respectively. Using air temperature and time as factors, two way ANOVA resulted in no affect due to air temperature or time. The Kruskal-Wallis procedure run with time as the only factor resulted in no significant difference due to time (p=0.2416). A Robust technique, to account for outliers was applied to the data set, and indicated no affect (p=0.3364). The Kruskal-Wallis test run after outliers were removed resulted in a p-value of 0.292, thus suggesting no affect due to the time factor. Air temperature tested by the Wilcoxon rank sum test implied that there was no affect due to air temperature (p=0.3442). With the robust technique applied to account for outliers, there was still no affect due to air

temperature (p=0.2295). A two sample t-test for the meat temperature indicated no significant affect (p=0.5527). The Wilcoxon rank sum test for the meat temperature showed no affect (p=.5467). Application of the robust technique to the meat temperature also implied no affect on the anaerobic plate count due to the temperature of the meat (p=0.5000).

Brain Heart Infusion Agar Count

Average brain heart infusion agar counts were found to be 530000 CFU/g (range 15000 - 3900000), 95000 CFU/g (range 9000 - 480000), 260000 CFU/g (range 8000 - 2000000), and 410000 CFU/g (range 18000 - 2500000) for hours 1, 2, 3, and 4, respectively. Using air temperature and time as factors, two way ANOVA resulted in no affect due to air temperature or time. The Kruskal-Wallis procedure run with time as the only factor resulted in no significant difference due to time (p=0.3194). A Robust technique, to account for outliers was applied to the data set, and indicated no affect (p=0.2414). The Kruskal-Wallis test run after outliers were removed resulted in a p-value of 0.573, thus suggesting no affect due to the time factor. Air temperature tested by the Wilcoxon rank sum test implied that there was no affect due to air temperature (p=0.5789). With the robust technique applied to account for outliers, there was still no affect due to air temperature (p=0.4108). A two sample t-test for the meat temperature indicated no significant affect (p=0.8278). The Wilcoxon rank sum test for the meat temperature showed no affect (p=.8270). Application of the robust technique to the meat temperature also implied no affect on the brain heart infusion agar plate count due to the temperature of the meat (p=0.8136).

Coliforms

For hours 1, 2, 3 and 4, average colifom counts were found to be 330 MPN/g (range 4.3 - 1100), 380 MPN/g (range 2.1 - 1100), 250 MPN/g (range 7.5 - 1100), and 390 MPN/g (range 2.1 - 1100), respectively. Using air temperature and time as factors, two way ANOVA resulted in no affect due to air temperature or time. The Kruskal-Wallis procedure run with time as the only factor resulted in no significant difference due to time (p=0.7766). The Robust technique, to account for outliers was omitted because there were no outliers. The Kruskal-Wallis test run after outliers were removed resulted

in a p-value of 0.753, thus suggesting no affect due to the time factor. Air temperature tested by the Wilcoxon rank sum test implied that there was no affect due to air temperature (p=0.7583). The robust technique, applied to account for outliers,was omitted. A two sample t-test for the meat temperature indicated no significant affect (p=0.7592). The Wilcoxon rank sum test for the meat temperature showed no affect (p=0.6618) on the coliform MPN.

Fecal Coliforms

For hours 1, 2, 3 and 4, average fecal colifom counts were found to be 16 MPN/g (range 0.4 - 210), 18 MPN/g (range 0.3 - 75), 4 MPN/g (range 0.3 - 43), and 3 MPN/g (range 0.3 - 9.3), respectively. Using air temperature and time as factors, two way ANOVA resulted in no affect due to air temperature or time. The Kruskal-Wallis procedure run with time as the only factor resulted in no significant difference due to time (p=0.5131). A Robust technique, to account for outliers was applied to the data set, and indicated no affect (p=0.6417). The Kruskal-Wallis test run after outliers were removed resulted in a p-value of 0.1298, thus suggesting no affect due to the time factor. Air temperature tested by the Wilcoxon rank sum test implied that there was no affect due to air temperature (p=0.1316). With the robust technique applied to account for outliers, there was still no affect due to air temperature (p=0.3191). A two sample t-test for the meat temperature indicated no significant affect (p=0.1279). The Wilcoxon rank sum test for the meat temperature showed no affect (p=.1234). Application of the robust technique to the meat temperature also implied no affect on the aerobic plate count due to the temperature of the meat (p=0.3191).

Controlled study (study 2)

The controlled study was analyzed with time as the factor. This was chosen based on the results obtained from the first study, indicating that time may have affect on the aerobic plate count at hour 4. Fecal coliform data was omitted from this study due to observations of MPNs below the limit of detection. Average air temperatures were found to be 65°F (range 64 - 67), 65°F (range 63 - 66), 65°F (range 64 - 67), and 66°F (range 65 - 67) for hours 1, 2, 3 and 4, respectively. Average meat temperatures were found to

be 60°F (range 55 - 62), 63°F (range 59 - 64), 64°F (62 - 66), and 66°F (range 64 - 68) for hours 1, 2, 3 and 4, respectively.

Aerobic Plate Count

Average aerobic plate counts were found to be 560000 CFU/g (range 2000 - 3600000), 400000 CFU/g (range 11 - 2600000), 1300000 CFU/g (range 2000 - 3600000), and 1300000 CFU/g (range 1000 - 5000000) for hours 1, 2, 3 and 4, respectively. Bartlett's test for normal distribution (p=0.292) and Levene's test for any continuous distribution (p=0.726) suggested that the variances were not significantly different. Analysis of variance based on time show that there is no significant effect due to time (p=0.729). Applying the Robust technique to account for outliers a p-value of 0.7768 is obtained, thus indicating no significant effect due to time. Welch's ANOVA also agreed with these findings.

Anaerobic Plate Count

For hours 1, 2, 3 and 4, average anaerobic plate counts were found to be 9400 CFU/g (range 1000 - 55000), 2600 CFU/g (range 1000 - 7500), 39000 CFU/g (range 2000 - 190000), and 240000 CFU/g (range 1000 - 1300000), respectively. Bartlett's test for normal distribution (p=0.000) and Levene's test for any continuous distribution (p=0.248) suggested that the variances were not significantly different. Analysis of variance based on time show that there is no significant effect due to time (p=0.257). Applying the Robust technique to account for outliers a p-value of 0.2134 is obtained, thus indicating no significant effect due to time. Welch's ANOVA also agreed with these findings (p=0.4185).

Brain Heart Infusion Plate Count

For hours 1, 2, 3 and 4, brain heart infusion agar counts were found to be 190000 CFU/g (range 5000 - 1200000), 450000 CFU/g (range 2000 - 2700000), 39000 CFU/g (range 2000 - 2700000), and 1400000 CFU/g (range 200 - 5800000), respectively. Bartlett's test for normal distribution (p=0.003) and Levene's test of any continuous distribution (p=0.458) suggested that the variances were not significantly different. Analysis of variance based on time show that there is no significant effect due to time (p=0.467). Applying the Robust technique to account for outliers a p-value of 0.7536 is

obtained, thus indicating no significant effect due to time. Welch's ANOVA also agreed with these findings (p=4663).

Coliform MPN Count

Average MPN counts for coliforms were found to be 330 MPN/g (range 9.3 - 1100), 290 MPN/g (4.3 - 1100), 270 MPN/g (range 7.5 - 1100), and 120 MPN/g (range 15 - 240), respectively. Bartlett's test for normal distribution (p=0.019) and Levene's test of any continuous distribution (p=0.569) suggested that the variances were not significantly different. Analysis of variance based on time show that there is no significant effect due to time (p=0.728). Applying the Robust technique to account for outliers a p-value of 0.7848 was obtained, thus indicating no significant effect due to time. Welch's ANOVA also agreed with these findings.

Based on the findings of these two studies, it has been shown that air temperature and meat temperature do not have an affect on microbial counts over the four hour test period. Time may significantly affect the aerobic microbial count at hour 4.

Appendix 3: Statistical Analysis of a Database Containing Information on Microbial Counts in Blue Crab (*Callinectes sapidus*)

The objective of this study was to statistically analyze microbiological and product temperature in Blue Crab (*Callinectes sapidus*) processing data collected over a seven year period by the Virginia Department of Health Division of Shellfish Sanitation.

MATERIAL AND METHODS

The Virginia Department of Health Division of Shellfish Sanitation took monthly samples from crab processing plants in Virginia. Samples were taken by inspectors and analyzed according to the American Public Health Association guidelines. Data is entered into the database as follows: sampled by (inspector's initials), date, air temp, product temp, (Virginia crab plant) certification number, product type, sample location, lab number, lab record date, lab record hour, lab record minute, examined by (laboratory personnel), control agar (to determine if agar was contaminated), dilution water (to determine if dilution water was contaminated), fecal coliforms, *Escherichia coli*, total plate count. For the purpose of this analysis only air temp, product temp, product type, sample location, fecal coliforms, and total plate count were used.

Product type was divided into nine categories: whole crab, special, regular, lump, backfin, pasteurized, claw hand, claw machine, and other. All products sampled fell into one of these categories.

Sample location was divided into six categories: cook (room), air cool (room), (cooked crab) cooler, pick (picking room), pack (packing room), and pasteurized (meat) cooler. All entries in the sample location column were entered into one of the categories listed above. Sample location cook was omitted from the analysis due to its small sample size.

All statistical analysis was performed using the SAS system.

RESULTS AND DISCUSSION

Total plate count (TPC) by location was analyzed by rank analysis with log transformation of the values. When analyzed using Tukey's Studentized Range (HSD) Test, there were no significant affects based on location of the product. The Wilcoxon

Rank Sum showed the pasteurized cooler to have the smallest mean score. Using General Linear Models on ranks provided a better estimation of separation via Tukey's test. The following comparisons were made: the packing room had a significantly higher total plate count than the picking room, cooler, air cool room and the pasteurized cooler; the picking room had a significantly higher plate count than the cooler, air cool room, and the pasteurized cooler; the cooler had a significantly higher plate count than the air cool room and the pasteurized cooler; and the air cool room had a sginificantly higher plate count than the pasteurized cooler (Table A1).

Fecal coliforms (FC) by location was analyzed by rank analysis with log transformation of the values. When analyzed using Tukey's Studentized Range (HSD) Test, there were no significant affects based on location of the product. Using General Linear Models on ranks provided a better estimation of separation via Tukey's test. The following comparisons were made: the packing room had a significantly higher fecal coliform count than the picking room, the cooler, and the pasteurized cooler; the picking room had a significantly higher count than the cooler and the pasteurized cooler; and the air cool room had a significantly higher count than the pasteurized cooler (Table A2).

Comparison of TPC by product were analyzed as follows, due to extreme values which distort the means, ranks must be compared; the data is log transformed with large values deleted followed by rank analysis. The ultra transformed model with extreme values omitted gave the results listed in Table A3 for Tukey's Studentized Test. Ranking by least square means from 1 to 9 was as follows: Claw hand, Other, Regular, Claw machine, Backfin, Special, Lump, Whole crab, Pasteurized. Ranking by mean, from 1 to 9, using all data was as follows: Whole crab, Pasteurized, Claw hand, Regular, Backfin, Claw machine, Special, Other. This was the result of sample sizes which were 1017, 144, 475, 629, 707, 64, 551, 38, and 260, respectively. Due to the data skewing by sample size, non-parametric ranking was employed. The non-parametric rankings were Claw hand, Claw machine, Regular, Backfin, Special, Other, Lump, Whole crab, Pasteurized. These results were as expected, with 1 having the highest plate count and 9 having the lowest plate count.

Comparison of FC by product type was evaluated by Tukey's Studentized Test with the large outlying values omitted. The only significant comparisons seen were between Regular and Whole crab, Regular and Backfin , and Regular and Lump. All of these comparisons were positive and significant at the 0.05 level.

The final analysis was Kendall Correlations of TPC and FC with temperature, both air and product, by product type. Correlations which were greater than 0.1 are noted below. Each product type was addressed.

Backfin

TPC and product temp showed a correlation of 0.13643 with a p-value of 0.0001. FC showed correlation with both air temperature and product temperature with correlations of 0.11280 and 0.11164, respectively and p-values of 0.0025 and 0.0003 respectively. This indicates that product temperature has an affect on both TPC and FC counts and air temperature has an affect on FC counts.

Claw hand

There was no correlation between TPC and air temperature or product temperature. No correlation was seen between FC and air temperature of product temperature.

Claw machine

TPC and product temperature show a correlation of 0.26024 with a p-value of 0.0053. Due to the small sample size, n=58, this may not be a true correlation. No correlation was seen between TPC and air temperature, FC and air temperature, or FC and product temperature.

Lump

There was no correlation between TPC and air temperature or product temperature. No correlation was seen between FC and air temperature or product temperature.

Claw hand

TPC and product temperature show a correlation of 0.18579 with a p-value of 0.0001. No correlation was seen between TPC and air temperature, FC and air temperature, or FC and product temperature.

Other

TPC and product temperature had a correlation of 0.27015 with a p-value of 0.0261, and FC and product temperature also showed a correlation of 0.41705 with a p-value of 0.0021. This finding may be due to the small sample size, n=35. No correlation was seen between TPC and air temperature or FC and air temperature.

Pasteurized

Both TPC and FC showed correlations with air temperature. Correlations were 0.26968 with a p-value of 0.0005, and 0.25783 with a p-value of 0.0025, respectively. No correlation was seen between TPC or FC with product temperature.

Regular

A correlation was seen between TPC and air temperature with a correlation value of 0.12705 and a p-value of 0.0004. TPC also showed a correlation with Product temperature with a value of 0.16245 and a p-value of 0.0001. FC indicated a correlation with air temperature and product temperature. The correlation values were 0.15475 with a p-value of 0.0001 for air temperature, and 0.12318 with a p-value of 0.0001 for product temperature.

Special

No correlations were seen between FC and Air temperature or product temperature. However, correlations were noted for TPC in regards to both air temp, with a correlation of 0.12583 and a p-value of 0.0015, and product temperature, with a correlation of 0.13889 and a p-value of 0.0001.

Whole crab

There was no correlation between TPC and air temperature or product temperature. No correlation was seen between FC and air temperature or product temperature.

Table A1: Comparisons of Location for Total Plate Count Based on Tukey's Studentized Range Test Using Ranks

Location Comparison	Difference Between the Means	Significant at the 0.05 Level
Pack - Pick	Positive	Yes
Pack - Cooler	Positive	Yes
Pack - Air Cool	Positive	Yes
Pack - Pasteurized Cooler	Positive	Yes
Pick - Pack	Negative	Yes
Pick - Cooler	Positive	Yes
Pick - Air Cool	Positive	Yes
Pick - Pasteurized Cooler	Positive	Yes
Cooler - Pack	Negative	Yes
Cooler - Pick	Negative	Yes
Cooler - Air Cool	Positive	No
Cooler - Pasteurized Cooler	Positive	Yes
Air Cool - Pack	Negative	Yes
Air Cool - Pick	Negative	Yes
Air Cool - Cooler	Negative	No
Air Cool - Pasteurized Cooler	Positive	No
Pasteurized Cooler - Pack	Negative	Yes
Pasteurized Cooler - Pick	Negative	Yes
Pasteurized Cooler - Cooler	Negative	Yes
Pasteurized Cooler - Air Cool	Negative	No

Table A2: Comparisons of Location for Fecal Coliform Count Based on Tukey's Studentized Range Test Using Ranks

Location Comparison	Difference Between the Means	Significant at the 0.05 Level
Pack - Pick	Positive	Yes
Pack - Cooler	Positive	Yes
Pack - Air Cool	Positive	No
Pack - Pasteurized Cooler	Positive	Yes
Pick - Pack	Negative	Yes
Pick - Cooler	Positive	Yes
Pick - Air Cool	Positive	No
Pick - Pasteurized Cooler	Positive	Yes
Cooler - Pack	Negative	Yes
Cooler - Pick	Negative	Yes
Cooler - Air Cool	Positive	No
Cooler - Pasteurized Cooler	Positive	Yes
Air Cool - Pack	Negative	No
Air Cool - Pick	Negative	No
Air Cool - Cooler	Negative	No
Air Cool - Pasteurized Cooler	Positive	Yes
Pasteurized Cooler - Pack	Negative	Yes
Pasteurized Cooler - Pick	Negative	Yes
Pasteurized Cooler - Cooler	Negative	Yes
Pasteurized Cooler - Air Cool	Negative	Yes

Table A3: Comparisons of Product Type for Total Plate Count Based on Tukey's Studentized Range Test Using an Ultra-transformed Model

Product Type Comparison	Difference Between the Means	Significant at the 0.05 Level
Claw hand - Other	Positive	No
Claw hand - Regular	Positive	No
Claw hand - Claw machine	Positive	No
Claw hand - Backfin	Positive	Yes
Claw hand - Special	Positive	Yes
Claw hand - Lump	Positive	Yes
Claw hand - Whole crab	Positive	Yes
Claw hand - Pasteurized	Positive	Yes
Other - Claw hand	Negative	No
Other - Regular	Positive	No
Other - Claw machine	Positive	No
Other - Backfin	Positive	No
Other - Special	Positive	No
Other - Lump	Positive	No
Other - Whole crab	Positive	No
Other - Pasteurized	Positive	No
Regular - Claw hand	Negative	No
Regular - Other	Negative	No
Regular - Claw machine	Positive	No
Regular - Backfin	Positive	Yes
Regular - Special	Positive	Yes
Regular - Lump	Positive	Yes
Regular - Whole crab	Positive	Yes
Regular - Pasteurized	Positive	Yes
Claw machine - Claw hand	Negative	No
Claw machine - Other	Negative	No
Claw machine - Regular	Negative	No
Claw machine - Backfin	Positive	No
Claw machine - Special	Positive	No
Claw machine - Lump	Positive	No
Claw machine - Whole crab	Positive	No
Claw machine - Pasteurized	Positive	No
Backfin - Claw hand	Negative	Yes
Backfin - Other	Negative	No
Backfin - Regular	Negative	Yes
Backfin - Claw machine	Negative	No
Backfin - Special	Positive	No
Backfin - Lump	Positive	No
Backfin - Whole crab	Positive	No
Backfin - Pasteurized	Positive	No
Special - Claw hand	Negative	Yes
Special - Other	Negative	No
Special - Regular	Negative	Yes
Special - Claw machine	Negative	No
Special - Backfin	Negative	No
Special - Lump	Positive	No

Special - Whole crab	Positive	No
Special - Pasteurized	Positive	No
Lump - Clawhand	Negative	Yes
Lump - Other	Negative	No
Lump - Regular	Negative	Yes
Lump - Claw machine	Negative	No
Lump - Backfin	Negative	No
Lump - Special	Negative	No
Lump - Whole crab	Positive	No
Lump - Pasteurized	Positive	No
Whole crab - Claw hand	Negative	Yes
Whole crab - Other	Negative	No
Whole crab - Regular	Negative	Yes
Whole crab - Claw machine	Negative	No
Whole crab - Backfin	Negative	No
Whole crab - Special	Negative	No
Whole crab - Lump	Negative	No
Whole crab - Pasteurized	Positive	No
Pasteurized - Claw hand	Negative	Yes
Pasteurized - Other	Negative	No
Pasteurized - Regular	Negative	Yes
Pasteurized - Claw machine	Negative	No
Pasteurized - Backfin	Negative	No
Pasteurized - Special	Negative	No
Pasteurized - Lump	Negative	No
Pasteurized - Whole crab	Negative	No

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PRESENTATIONS *Abstract: The effects of varying thermal processing schedules on Listeria monocytogenes and indicative microorganisms in blue crab (Callinectes sapidus) meat*

Interstate Seafood Seminar, October 1998
Invited Speaker
Topic: I. Statistical analysis of a state database
        II. Data collection and database set up and management

HONORS Gamma Sigma Delta, Agricultural Honor Society
ACTIVITIES Phi Sigma, Biological Honor Society
Institute of Food Technologists, Student Member
IAMFES, Student Member

American Society for Microbiology, Student Member
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