THE PRESENCE AND GROWTH CHARACTERISTICS OF *LISTERIA MONOCYTOGENES* IN BLUE CRAB (*CALLINECTES SAPIDUS*) MEAT AND THE EFFECTIVENESS OF MICROWAVE ENERGY IN A PASTEURIZATION PROCESS

by

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ABSTRACT

In this study, the incidence and occurrence levels of *Listeria monocytogenes* in fresh blue crab (*Callinectes sapidus*) meat and its behavior in the presence of *L. innocua* and competing microflora were determined. An attempt was made to develop a process using microwave energy to destroy, or significantly decrease, naturally occurring levels of *L. monocytogenes* in crab meat. Data for the growth of *L. monocytogenes* under refrigeration conditions were fitted to the logistics and Gompertz functions, to a developed modified non-linear Gompertz function including the temperature effect, and to a developed extended modified Gompertz function that combined temperatures in the refrigeration range used.

Listeria was found in 10% of the 126 samples of fresh blue crab meat analyzed, with an 8% incidence of *L. monocytogenes*. The levels of occurrence were in all but one sample less than 100/g, the exception had an MPN of 1,100 /g. Growth curves were obtained for *L. monocytogenes* in pasteurized crab meat and generation times of 68.7, 31.4, and 21.8 h at 1.1, 2.2, and 5 °C respectively were

observed. When *L. monocytogenes* was simultaneously inoculated with *L. innocua* in pasteurized crab meat, no significant effect ($p > 0.05$) was observed on their repective growth curves. This was observed for three mixture proportions of *L. monocytogenes* and *L. innocua*, during 15 days, at 4 and 36 °C. Fresh blue crab meat, classified according to the initial levels of microflora (APCs) into three different categories (< 15,000, 15,000-60,000 and, 60,000-100,000 cfu/g) was inoculated with *L. monocytogenes* at less than 50 cfu/g and incubated at 1.1, 2.2 and 5 °C for 21 days. A significant ($p \leq 0.05$) inhibitory effect on the growth rate of *L. monocytogenes* was observed with decreasing temperatures. The effect of microflora population upon the growth of *L. monocytogenes* was observed after 13 days of storage, however, the levels of microflora were such that the meat was rendered inedible.

Fresh crab meat (454 g) was inoculated with *L. monocytogenes* (less than 50 cfu/g), packed in a polyethylene bag (18 x 20 cm) or in a polypropylene tub (11 cm top diameter, 9 cm bottom diameter, 7 cm height,) and microwaved (2,450 MHz) for 3 min. *L. monocytogenes* was not detected in the product stored at 0 °C, 3 h or 5 d after microwave application. The best fit for the growth of *L. monocytogenes* in fresh crab meat during refrigerated storage was obtained by the developed modified non-linear Gompertz function. Correlation of the estimates to the experimental growth values ($\log_{10}$ cfu/g) were between 0.82 and 0.99. An extended modified Gompertz equation was developed for the estimation of growth of *L. monocytogenes* in the range of storage temperatures between 1.1 to 5 °C.

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INTRODUCTION

Listeria is not a new microorganism. The disease, listeriosis, was recognized in sheep in 1925 and the first confirmed human listeriosis was reported in 1929 (Wehr, 1987). Foodborne transmission was finally confirmed by an outbreak of community-acquired perinatal and adult listeriosis traced to ingestion of contaminated cabbage from a farm on Prince Edward Island (Schlech, 1992). Recent association of *L. monocytogenes* with several large foodborne outbreaks, suggests that contaminated food may be the primary source of the organism in human infections (Schlech et al., 1983; Flemming et al., 1985; James et al., 1985; Datta and Wentz, 1989; Farber and Peterkin, 1991; Dever et al., 1993). The majority of cases of human listeriosis occur in immunocompromised individuals who have an underlying condition such as cancer, cirrhosis, transplant patients, pregnant women and their fetuses or neonates or the elderly (Doyle, 1991; National Advisory Committee on Microbiological Criteria for Foods, 1991; Schuchat et al., 1992). Mortality in predisposed groups may exceed 25% (Health and Welfare Canada, 1988). *L. monocytogenes* can be responsible for health problems that include meningoencephalitis, flu-like low grade septicemia, pneumonia, endocarditis, urethritis and abortion (El-Shenawy and Marth, 1988; National Advisory Committee on Microbiological Criteria for Foods, 1991).

Due to the ubiquitousness of *L. monocytogenes*, its ability to grow at refrigeration temperatures, and because the infective dose for pregnant women and the

immunocompromised are unknown, the United States Department of Agriculture (USDA), Centers for Disease Control (CDC) and the Food and Drug Administration (FDA) agreed on a zero tolerance in foods not intended for further heat treatment (McNamara, 1994). A study by Wilkins et al. (1972) reported that *L. monocytogenes* grows at temperatures between 3 and 45 °C with a longer lag phase with decreasing temperatures. The minimum temperature for growth was estimated to be 3.3 °C. A more recent study (Junttila et al., 1988), reported that the minimum growth temperature for *L. monocytogenes* is 1.1 °C and also that pathogenic *L. monocytogenes* strains grow at about 0.6 °C lower than non-pathogenic strains. Incidences of *L. monocytogenes* in raw milk, raw meats, poultry and seafoods have been widely reported (Tiwari and Aldenrath, 1990; Farber et al., 1989). In seafoods, *Listeria* spp. has been reported to have an incidence of 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; Hudson et al., 1992; National Advisory Committee on Microbiological Criteria for Foods, 1991;).

One promising processing technology that has potential in eliminating *L. monocytogenes* from cooked ready-to-eat seafood is a thermal process based on microwave energy. The major reason to consider pasteurization processes is the energy efficiency, the rapid development of temperature within a material without the normal thermal lag, and the bulk heating effect (Schiffmann, 1989). One of the

major concerns in microwave pasteurization is ensuring that each container receives the proper thermal lethality. Most authors have concluded that inactivation of microorganisms during microwave application occurrs solely from heat generation (Decareau, 1985; Heddleson and Doores, 1994; Mertens and Knorr, 1992).

The objectives of this research project were to determine the presence and growth characteristics of *L. monocytogenes* in fresh blue crab (*Callinectes sapidus*) meat; to obtain a better knowledge of the potential hazard of the microorganism in the product; and to determine the effectivenesss of microwave energy in killing or inactivating the microorganism so that a safe, cost-effective process for commercial production of the meat can be available to the crab processing industry.

SECTION I: LITERATURE REVIEW

A. GENERAL CHARACTERISTICS OF THE *LISTERIA* GENUS

1. Description

Listeria monocytogenes is a Gram positive, nonsporeforming, facultatively anaerobic, short diphtheroid rod which grows between -0.4 °C and 50 °C (Junttila et al., 1988; Walker and Stringer, 1987). It is catalase positive and oxidase negative and expresses a β-hemolysin, which produces clear zones on blood agar. The organism possesses peritrichous flagella which imparts it with tumbling motility in a narrow temperature range up to 30 °C. Between 20 °C and 25 °C flagellin is produced and assembled at the cell surface, but at 37 °C, flagellin production is reduced (Peel et al., 1988). The organism can multiply in high salt (Hudson 1992; Hefnawy and Marth, 1993) or bile concentrations and due to its ability to grow and reproduce at 1.1 °C, it is considered a psychrotrophic organism (National Advisory Committee on Microbiological Criteria for Foods, 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). *Listeria* colonies present a characteristic blue-green sheen when observed under obliquely transmitted light. *L. monocytogenes* is widely found in plant, soil, surface, and water samples (Weis and Seeliger, 1975), silage, sewage, slaughterhouse waste, milk of normal or mastitic cows, human and animal feces (McCarthy, 1990). As an

intracellular parasite, it can grow inside leukocytes of humans or animals (Farber, 1989; National Advisory Committee, 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, 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 in part done by using 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 *Staphylococcus aureus* and a strain of *Rhodococcus equi* are streaked in parallel on a sheep blood agar plate and then test cultures are streaked parallel to to one another between the *S. aureus* and *R. equi* streaks. Blood agar plates are incubated at 35 °C for 24-48 h and then examined for hemolysis. *L. monocytogenes* and *L. seeligeri'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 acriflavine 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 transferable 37-kbp plasmid carrying gene responsible for resistance to chloramphenicol, erythromycin, streptomycin and tetracycline.

2. Taxonomy

The genus *Listeria* is found in the *Bergey's Manual of Systematic Bacteriology* together with *Lactobacillus* in a section entitled "Regular, Nonsporing, Gram positive rods". Eight species are listed for the genus: *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. murrayi*, *L. grayi* and *L. denitrificans* (Seeliger and Jones, 1986). In 1987, *L. denitrificans* was reclassified as *Jonesia denitrificans* (Rocourt et al., 1987). Because of existing similarities; both are non-pathogenic (Farber and Speirs, 1987) and very rarely isolated from foods, *L. murrayi* and *L. grayi* are being considered for reclassification into a single species named *L. grayi* (Rocourt et al., 1992). *L. ivanovii*, *L. seeligeri*, and *L. monocytogenes* are the only species of *Listeria* pathogenic for humans and animals. Although *L. ivanovii* has been linked with sporadic illnesses 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 *L. monocytogenes*

Most *Listeria* species, with the exception of *L. monocytogenes*, can be regarded as harmless to man (Lovett, 1991). Besides, not all strains of *L. monocytogenes* are pathogenic; rough variants possess only 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). At least 13 serotypes of *L. monocytogenes* have been described, three of which (4b, 1/2b, and 1/2a) have been associated with 90% of all cases of human listeriosis. The virulence mechanisms of *L. monocytogenes* are not fully understood; however, the single recognized factor proven to be crucial for the virulence of *L. monocytogenes* is listeriolysin O (Cossart, 1988); a chromosomal bound hemolytic extracellular 60 kDa protein (National Advisory Committee on Microbiological Criteria for Foods, 1991; Kolstad et al., 1992). This hemolysin is biochemically very similar to streptolysin O and pneumolysin, and cross-reacts antigenically with these hemolysins and with the hemolysins of *L. ivanovii* and *L. seeligeri* (Geoffroy et al., 1989). Mutants of transposon-induced Hly^- (hemolysin defficient) *L. monocytogenes* are avirulent (Gaillard et al., 1986; Katharious et al., 1987), cannot grow in host

tissue or in human enterocytes (Gaillard et al., 1987), and cannot induce T-cell mediated immunity (Berche et al., 1987); however, their entry into macrophages is not affected (Gaillard et al., 1987; Kuhn et al., 1988). Therefore, it has been concluded that once *L. monocytogenes* enters the macrophage, listeriolysin O is required to lyse the phagosomes, facilitating the release of the bacterium into the cytoplasm so that they can multiply (Datta et al., 1990). A recently identified protein, internalin, appears to play an important role in the entrance of *L. monocytogenes* to 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). Schlech et al. (1993) developed a model for *L. monocytogenes* infection using the Sprague-Dawley rat. By 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 a Gram positive bacteria; a thick homogeneous structure surrounding the cytoplasmic membrane and without the outer membrane characteristic of Gram negative bacteria. Isolated dry cell walls are composed of approximately 35% peptidoglycan (cross-linked meso-diaminopimelic 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 lypoteichoic acids, in which a glycolipid 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 lipopolysaccharides 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 (National Advisory Committee on Microbiological Criteria for Foods, 1991).

Del Corral et al. (1990) quantitatively comparing thirty food and clinical isolates of *Listeria*, found all *L. monocytogenes* to be hemolytic, invasive, weakly cytotoxic and lethal to immunocompromised mice. No significant difference between the clinical and food isolates were 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 high temperature-short time pasteurization treatment (Mackey and Bratchell, 1989; Lovett et al., 1990; Farber, 1991a). 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 new factors have been found to influence the thermoresistance of the cell (Farber, 1991a); 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, aquired thermotolerance of *L. monocytogenes* after short pretreatment at sublethal temperatures in broth (Knabel et al., 1990; Linton et al., 1990) and meat systems have been described (Farber and Brown, 1990). A greater increase in thermotolerance was observed (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. 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. According to Knabel et al. (1990), a larger number of heat-injured cells of *L. monocytogenes* can be recovered by strictly anaerobic procedures than by conventional aerobic recovery procedures. The physiological condition of the microorganism, the medium 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). Wang and Hitchins (1994) demonstrated that survivors of lethal heating were injured heterogeneously. The minority of the survivors were the slightly injured cells and they were recovered after 6 h of incubation in Tryptic Soy Broth with 6% Yeast Extract (TSBYE) at 30 °C. In contrast, the majority of survivors were severely injured cells and these required at least 20 h of incubation in TSBYE at 30 °C for recovery.

5. Resistance against 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 materia. Best et al. (1990) found microorganisms dried onto surfaces to be more resistant to disinfectants than those in suspension. From 14 disinfectants they studied, only three (povidone-iodine, chlorhexidine gluconate, and glutaraldehyde) were effective against *Listeria* spp. in the carrier test in 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 tests, regardless of the content in organic matter. *L. monocytogenes* appeared to be slightly more resistant to disinfection than *L. innocua*. Four commonly used sanitizers, two quaternary ammonium compounds (QAC), an acid anionic sanitizer and a chlorine-based sanitizer, were evaluated for their efficacy against strains of *L. monocytogenes* (2) and *L. innocua* (1) (Sallam and Donnelly, 1992). All sanitizers showed efficacy of >99.999% against all test organisms, regardless of type of sanitizer, concentration or exposure time. The ability of two enrichment broths, University of Vermont (UVM) and *Listeria* repair broth (LRB), to recover injured *Listeria* was also tested. In all instances LRB recovered larger numbers of cells, inducing 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 disochlorine tablets. No particular resistance of listerias to disinfectants was found, but a decrease in efficacy on 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 48 h old culture, grown in tryptose broth rather than on tryptose agar slant, washed and suspended using a 20 rather than 0.312 Mm phosphate buffer solution, and that reduction in the cell number occurred rapidly on 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).

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 microorganism. 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*. Sasahara 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 and the studies of Frank and Koffi (1990) and McCarthy (1992) confirmed that attachment of cells to surfaces can provide protection against chemical sanitizers. Mafu et al. (1990) using scanning electron microscopy technique, investigated the attachment capabilities of *L. monocytogenes* strain Scott A to stainless steel, glass, polypropylene, and rubber surfaces after short contact times at ambient (20 °C) and cold storage (4 °C) temperatures. Attachment to all surfaces, at both temperatures, at either 20 min or 1 h and presence of extracellular material, especially in glass and polypropylene was observed.

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 that 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).

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, 1991a). *L. monocytogenes* can be responsible for health problems that include meningoencephalitis, flu-like low grade septicemia, pneumonia, endocarditis, urethritis and abortion (El-Shenawy and Marth, 1988; National Advisory Committee on Microbiological Criteria for Foods, 1991).

Virulence of the genus *Listeria* which is associated with their ability to produce hemolysis in blood agar has been demonstrated for the species *L. monocytogenes* and *L. ivanovii*. *L. seeligeri* is the only specie which is hemolytic but

apathogenic (Schonberg, 1989). Isolates of *L. monocytogenes* from outbreaks of listeriosis indicate that serotype 4b is the main participant in epidemic disease in North America, in comparison to serotypes 1/2a or 1/2b (Schlech, 1992).

Due to the ability of *L. monocytogenes* to grow at refrigeration temperatures and because the infective dose for pregnant women and the immunocompromised are unknown, the USDA, CDC and the FDA agreed on a zero tolerance in foods not intended for further heat treatment (McNamara, 1994). However, facts such as that in a large population as that of the U.S., there are only few cases of listeriosis per year and most of these cases involve immunocompromised people; the ubiquitousness of the organism; and that most sporadic cases involve only certain foods, mainly soft cheeses, undercooked chicken and poorly reheated hot dogs; have put the "zero tolerance" policy under vigorous challenge. The FDA is now considering possible changes to the current policy. These changes would include: categorization of foods by known, established risks; maintenance of a strict standard for those products subjected to a listericidal process or which would support the growth of the microorganism to higher numbers; and the allowance of low numbers of *L. monocytogenes* in foods demonstrated to be listericidal, but in which the microbe is found in low numbers due to the unavoidable presence of the organism in the raw food (Madden, 1994).

C. 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 and Brackett, 1989).

1. Enrichment procedures

The use of cold enrichment (Gray et al., 1948; Gray and Killinger, 1966; 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 and Brackett, 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* species to hydrolyze esculin to esculetin (6,7-dihydroxycoumarin) which reacts with ferric ions in the medium to produce a black color (McFaddin, 1980).

Bailey and Cox (1992) developed a universal preenrichment (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/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 described 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 (1990, 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 acriflavine-Hcl (15 g/ml), nalidixic acid (40 g/ml), and cycloheximide (50 g/ml) as selective agents. EB is incubated for 24 and 48 h at 30 °C and then streaked onto two selective agars, Lithium Chloride-Phenylethanol-Moxalactam (LPM) and Oxford agar (OXA) at each time. These agars are incubated at 30 °C and 35 °C, respectively, for 24-48 h. Ferric iron salt and esculin may be added to LPM to avoid use of Henry's illumination. Suspect colonies from either agar must be streaked onto nonselective 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 continued as described above.

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 proteose peptone, tryptone, Lab Lemco powder (Oxoid, Unipath, Ogdensburg, NY), yeast extract, sodium chloride, buffers, esculin, nalidixic acid (20 g/ml) and acriflavine (12 g/ml). The primary enrichment is transferred to a secondary broth, Fraser medium (FM) (Fraser and Sperber, 1988), which uses LEB as base with addition of increased acriflavine-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. (1989a) compared Modified Vogel Johnson (MVJ) agar and LPM agar in their efficiency to detect *Listeria* spp. in foods, including seafoods, when using both direct plating and direct plating with a three-tube most-probable-number enrichment. They reported 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., 1989a; Budu-Amoako et al., 1992a), making it impossible to detect them by direct plating. Modified Vogel Johnson (MVJ) agar presenta an advantage in the enumeration of *Listeria* spp. from foods due to its capability to differentiate *Listeria* spp. from other microorganisms, based in its characteristic tellurite-positive, mannitol-negative reactions. *Listeria* isolates result in black colonies on a red background (Buchanan et al., 1989a).

Westöö and Peterz (1992) developed a collaborative study between eleven Nordic laboratories to compare qualitative methods for the detection of *L. monocytogenes* in foods. They compared two enrichment methods (USDA for meats and FDA for dairy) and two agar media, LPM and Oxford agar, finding no difference in performance between the two enrichments but a better recovery of *L. monocytogenes* when using Oxford agar. Comparisons of the FDA and USDA methods have been conducted, but few comparisons have been identical. Lowry and Tiong (1989) and Norrung et al. (1991) found the USDA method more sensitive for detecting *L. monocytogenes* from artificially and naturally contaminated minced meat when present in numbers of < 3 cells/g. Fernandez-Garayzabal and Genigeorgis (1990) and Yu and Fung (1991) evaluated the performance of different combinations of selective enrichments and agars for enumerating *L. monocytogenes*. Yu and Fung (1991) found all media recovered *L. monocytogenes* strains well, and that the use of EB (FDA) with MOX (USDA) combined, provided the best recovery of all *L. monocytogenes* strains. Fernandez-Garayzabal and Genigeorgis (1990) reported that all enrichment broths were equally effective for enrichment of *Listeria* species, with the FDA broth providing faster growth than the others. The FDA enrichment was the most favorable of four enrichments evaluated by Noah et al. (1991) for recovery of *Listeria* species from raw and processed seafoods when used in with a commercially available enzyme-linked immunosorbent assay (ELISA) kit. Most investigators have found the USDA and FDA methods to be equivalent in their ability to recover unstressed

Listeria cells. The USDA method provides more selectivity for foods and environmental samples with high aerobic plate counts (Dever et al., 1993). Slade (1992a) reviewed comparisons of different protocols for *Listeria* isolation and concluded that, based on the work of Lovett et al. (1991), the USDA method is more applicable than the FDA method for samples where more selectivity is required. However, the FDA method may be appropriate for samples containing stressed cells or with less background contamination. According to a study by Hayes et al. (1992), comparing the USDA, FDA and the Netherlands Government Food Inspection Service (NGFIS) procedures, no single method for isolating *L. monocytogenes* is 100% sensitive. Based on the results obtained by these investigators, the CDC adopted the simultaneous use of the USDA and the NGFIS method to isolate *L. monocytogenes* from foods.

2.2 Rapid methods

Among methods developed for the rapid detection and identification of *Listeria* are the use of antibody-based assay (enzyme-linked immunosorbent assay: ELISA, RIA) (Mattingly et al., 1988; Kerr et al., 1990a; Vanderlinde and Grau, 1991), nucleic acid probe-based assays (DNA hybridization) (Pusch, 1989; Datta and Wentz, 1989; Chenevert et al., 1989; Emond et al., 1993) and a modified microtiter plate procedure (MMP) for characterization of *Listeria* spp. (Siragusa and Nielsen, 1991). Recently, new kits such as Accuprobe by Gen-Probe, Inc. (San Diego, CA), have

been developed to specifically detect *L. monocytogenes* in foods (Dever et al., 1993; Farber, 1993; Okwumabua et al., 1992).

Listeria-Tek, an ELISA manufactured by Organon Teknika Corp. (Durham, NC), can detect *Listeria* species 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 are enriched in broth (FDA or USDA), heated, and added to polystyrene microtiter plate well coated with antibodies. Immune complexes form between the *Listeria*-specific antigen in the sample and the *Listeria*-specific monoclonal antibody bound to the well. A different enzyme labeled monoclonal antibody binds to 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 specie (Dever et al., 1993). The detection limit of the ELISA test for *L. monocytogenes* in pure cultures is approximately 10^6 CFU/ml (Norrung et al., 1991). Development of kits that do not require enrichment steps and which confirm the identity of *Listeria*

species within 24 h includes the Listertest (VICAM, Watertown, MA). Listertest consist of two separate tests that can be run simultaneously. Listertest Lift identifies all *L. monocytogenes* and several non-monocytogenes *Listeria* species, while pathogenic strains are identified by Listertest Mac. 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. (1993b). 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 and quantitative results were obtained within 24 h. Mc Lauchlin and Pini (1989) reported on the use of two monoclonal antibodies in a direct immunofluorescent test to detect *L. monocytogenes* 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. monocytogenes* 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). The most recent version detects *Listeria* species using *Listeria* specific DNA probes, and a colorimetric, nonradioactive, 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 saline which would provide a detection limit of approximately 10^6 CFU/ml (Dever et al., 1993).

2.2.1 Comparison of rapid methods

Both ELISA and DNA hybridization assays have been evaluated in

studies comparing rapid methods to cultural methods. Heisick 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 86% of the 44 positive vegetable samples, the FDA culture procedure, 75%, the *Listeria*-Tek ELISA 68%, and the Gene-Trak DNA probe, 45%. Although ELISA tests have not received approval of the Association of Official Analytical Chemists (AOAC) for foods, studies indicate that ELISA kits give similar results to those obtained with official cultural methods for the analysis of foods and are useful screening devices (Dever et al., 1993).

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 occasions more sensitive than the conventional procedures for detecting *Listeria* (Dever et al., 1993).

3. Confirmation of *Listeria* spp.

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. monocytogenes* 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 occur. Colonies were more easily detected by this method compared to using red cells incorporated into the medium.

The CAMP test is used to detect or confirm hemolysis, being useful in differentiating species. The test is based in the hemolysis enhancement by the metabolites of *Staphylococcus aureus* and *Rhodococcus equi* onto 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 *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*.

Poysky 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 at al. (1990b) found good correlation when comparing the API 50CH (BioMerieux, La Balmeles-Grottes, France) and MAST ID systems (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 646 *Listeria* strains at the species and subspecies level was obtained by the 10-test strip system. This system uses the arylamidase test (DIM) to differentiate *L. innocua* and *L. monocytogenes* strains. Arylamidase is present in *L. innocua* and many others *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.

Wiedmann et al. (1992) described a ligase chain reaction assay based on a single base-pair difference 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 polymerase chain reaction (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 primers 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

L. 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 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 of cells results in a removal of water with the 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 and 35 °C (El-Kest and Marth, 1992).

Several studies have been conducted to compare the effectiveness of the FDA and 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, the FDA and USDA enrichment protocols, for their ability to isolate *L. monocytogenes* from raw and cooked seafoods. Cooked crab meat, raw and cooked shrimp and surimi were each inoculated with *L. monocytogenes* Scott A and strain SE68 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 PALCALM) were comparable in quantitative recovery of stressed and nonstressed cells. Busch and Donnelly (1992) formulated

Listeria repair broth (LRB) in which, following repair of 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) recommend 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 incident light. LCA facilitated formation of larger colonies while suppressing growth of other food-borne microorganisms. Budu-Amoako et al. (1992a) evaluated the efficiency of TSBYE and LEB to recover heat-injured and freeze-injured cells of *L. monocytogenes*. They reported better performance of trypticase soy broth-yeast extract in recovering both, heat-injured or freeze-injured cells. An enhanced recovery of heat injured cells after enrichment in LEB for 48 h, as compared with 24 h, when *L. monocytogenes* is in the absence of microflora was reported by Tran and Hitchins (1993). 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/25g, subjected to either heat-injury (56 °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 level ($\leq$ 100 CFU/25g) 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 aesculin 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 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.

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); 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 seafoods products. Of the 227 cultures subjected to phage typing, 173 were typeable (76%). Overall the majority of the typeable strains were *L. monocytogenes* type 1/2 (64%) and the remainder 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), and multilocus enzyme electrophoresis (Pinner et al., 1992; Slade, 1992b; Kolstad et al., 1992). Buchrieser et al. (1993) used pulsed-field gel electrophoresis of large chromosomal DNA restriction fragments to analyze 75 *L. monocytogenes* strains isolated during recent listeriosis outbreaks.

D. *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, specially conveyors, floors and drains (Slade, 1992a). Soil is an important reservoir and the bacterium is frequently carried in the intestinal tract of animals, including healthy humans. Jackson et al. (1993a) 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 dish cloths 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 (64%) 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 17 food factories (representing 6 different groups of food products) and 35 Dutch households. In food factories, listerias were found in drains, floors, standing water, residues, and food contact surfaces in descending order of frequency. Seven (20%) of 35 households kitchens were found to be contaminated with listerias. Six of seven dish cloths were positive for *Listeria*, as were swabs from two dustbins and one refrigerator. 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). Since 1981 up to date, foodborne outbreaks of listeriosis documented in the U.S. involving coleslaw (Schlech et al., 1983), pasteurized milk (Fleming et al., 1985), and Mexican-style cheese (James et al., 1985) have caused concern among producers, regulators and public health professionals. Incidences of *L. monocytogenes* in raw milk, raw meats, poultry and seafoods have been widely reported (Tiwari and Aldenrath, 1990). Farber et al. (1989) reported the presence of *L. monocytogenes* in samples of chicken legs (56.3%), ground meats (86.4%) and fermented sausages (20%) as a survey result of several retail foods. The same study reported samples of lettuce, celery, tomatoes and radishes to be free of the microorganism. *L. monocytogenes* and other *Listeria* spp. have been isolated mainly from freshwater samples and from seawater from coastal areas subject to pollution or contamination from industrial, human or animal sources (Motes, 1991; Ben Embarek, 1994).

In a study done by Wilkins et al. (1972), *L. monocytogenes* grew at temperatures between 3 and 45 °C. The duration of the lag phase increased with decreasing temperature and 3.3 °C was estimated as the minimum temperature for growth. Junttila et al. (1988) reported the minimum growth temperature for *L. monocytogenes* to be 1.1 °C and also observed that *L. monocytogenes* strains grew at about 0.6 °C lower than the non-pathogenic strains.

Johnson et al. (1990), in a review of the incidence of *L. monocytogenes* in meat and meat products, remarked the fact that the microorganism's presence in these food products is influenced by factors such as geographical differences, differences in animal rearing, handling and slaughtering practices and differences in food handling practices.

1. Bacteriological Quality of Seafoods

Bacterial counts of foods are widely used as general indicators of hygiene status and bacteriological quality. The most important fish spoilage bacteria show the ability to produce H_2S and reduce trimethylamine oxide (TMAO) (Gram, 1992); thus, these characteristics are used in the development of agar media and specific chemical and physical assays. It has been reported that the expected shelf life of chilled cod fish can be predicted by the number of specific spoilage bacteria (Ravn-Jørgensen et al., 1988). The estimation of the number of viable bacteria relies on the capability of each cell to form a visible colony. In examining seafoods held at chill temperatures, incubation temperature at and above 30 °C are inappropriate. Gram (1992) indicates the use of pour plating and a 3-4 day incubation at 25 °C for routine analysis and determination of plate counts in seafoods.

A survey of establishment processing crab meat was conducted by Wentz et al. (1985). The study showed that the microbial quality of the finished product correlates very well with the handling practices the crab meat is subjected after cooking. Fagri

et al. (1984) studied the incidence of potential human pathogens in edible crab meat obtained from cold marine ecosystems. They found that crabs collected from regions close to human habitation contained higher bacterial counts than those collected away from those regions. The bacteria found in the contaminated crabs included *Yersinia enterocolitica*, *Klebsiella pneumoniae* and coagulase negative *Staphylococcus* species. Ben Embarek (1994) in a review of the incidence of *L. monocytogenes* in seafood, indicated that it appears that *Listeria* spp. can be isolated from polluted waters and waters with high content of organic material, but that its natural presence in clean open seawater remains to be established.

2. Seafoods associated with *Listeria* spp.

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 seafoods 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; National Advisory Committee on Microbiological Criteria for Foods, 1991). The National Advisory Committe on Microbiological Criteria for Foods (1990) published and adopted the recommendations of the Committee to address the microbiological safety of cooked ready-to-eat shrimp and cooked ready-to-eat crab meat. Selection of these products was done on the basis of their consumption with

minimal or no additional heat processing with 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 ready-to-eat foods, in a study of retail foods in Japan. Levels of *L. monocytogenes* were estimated to be less than 50 CFU/g.

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. Significant growth of *L. monocytogenes* on smoked salmon has been reported by Farber (1991a), Fletcher and Rogers (1991) and Rørvik and Yndestad (1991). Guyer and Jemmi (1991) reported that *L. monocytogenes* survives the brining and smoking stages that occurs 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 Rorvik et al. (1991). Jemmi and Keusch (1992) reported a 6 $\log_{10}$ reduction of *L. monocytogenes* during hot-smoking of trout when reaching an internal temperature of 65 °C during

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 to store hot-smoked fish at temperatures of 4 °C or below. Fish and shrimp samples inoculated with *L. monocytogenes* (10^3 of rinse buffer) and held in ice during 21 days, showed no increase in population (Harrison et al., 1991).

Weagant et al. (1988) tested samples of frozen seafood products from various countries looking for the presence of *L. monocytogenes* and other *Listeria* spp. They reported 35 out of 57 samples containing *Listeria* spp. with 15 samples 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 seafoods. 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%) and 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.

Hudson and Avery (1993) studied the growth of *L. monocytogenes*, *Aeromonas hydrophila* and *Yersinia enterocolitica* inoculated in samples of cooked mussel tissue at 5 and 10 °C under both aerobic and anaerobic conditions and found similar growth

rates under these conditions. A maximum population (10^8 CFU/g) was reached after 17 days at 5 °C and after 6 days at 10 °C. Little multiplication of *L. monocytogenes* inoculated in crawfish tail and incubated at 0 °C for 20 days was observed, where for incubation temperatures of 6 and 12 °C exponential growth was observed immediately and no lag phase detected (Dorsa et al., 1993).

Lachica (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 being food samples with listerial 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.

Brackett and Beuchat (1990) studied the pathogenicity of *L. monocytogenes* in relation with its growth in crab meat at 5 and 10 °C and found no significant effect of storage temperature in the pathogenicity of the microorganism.

E. USE OF BIOLOGICAL INDICATORS

The amount of bacteria in foods serves as a general indicator of hygiene; bacteria are important spoilers of many products, and many bacteria and their metabolites represent a health risk for the consumer (Gram, 1992).

The use of biological indicators to provide information concerning the adequacy of a thermal process 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 biological indicator for *L. monocytogenes*. They reported *Listeria 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 70 °C.

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 of process integrity was studied by Buchanan et al. (1992).

F. PREDICTION OF BACTERIAL GROWTH

Predictive microbiology is a tool that can be used to model microbial growth response with respect to the principal controlling factors; formulation, process and storage conditions (Gibson and Roberts, 1989). It offers an alternative to traditional microbiological assessment of food quality and safety (Maas, 1993). The concept is that a detailed knowledge of the microbial ecology of a food product can be expressed as a mathematical model to enable objective evaluation of the effect of processing, storage and distribution operations on microbial development (McMeekin et al.,

1992). One of the goals of predictive microbiology is to develop models which fit microbiological data as precisely as possible (Alber and Schaffner, 1993).

In one approach to predictive modeling, a kinetic model of the organism's response to its ecosystem is interpreted by sensors responding in the same way as the organism to changing environmental conditions. The sensor readings, provide a direct measure of the stage and extent of microbial growth, and specifications can be set from zero growth (lag phase not resolved) through any number of generations at which a public health or spoilage risk is perceived (McMeekin et al., 1992). The steps involved in the system are (McMeekin et al., 1992): (1) identify the organism of concern (spoiler or pathogen); (2) develop an understanding of the ecology of the organism to enable identification of the sources and likely level of contamination and the effect of post-harvest factors such as processing, distribution, or storage, on numbers; (3) compare the information obtained with present criteria to accept/reject the process or product or to indicate the remaining shelf life; and, (4) incorporate the information into monitoring devices that indicate the extent of microbial proliferation.

By using predictive modeling of bacterial growth, researchers can: (1) predict the microbiological stability of new food products that differ slightly from existing products which have an established database (Farber, 1986); (2) assess direct and interactive effects of new or different combinations of food preservatives (Farber, 1986); (3) estimate microbial behavior in foods containing inhibitory factors that could affect microbial growth (Baird-Parker and Kilsby, 1987); (4) help to estimate

the shelf life and safety of foods (Gibson et al., 1987; Labuza and Fu, 1993); and (5) aid in the evaluation of the potential health hazard of food that has been temperature abused (Farber, 1986; Gibson et al., 1987).

When bacteria are subjected to combined stress conditions, the resulting survival curves are often complex and multiphasic (Pruitt and Kamau, 1993). The concept of "hurdle technology" refers to the application of different factors (hurdles) that control or inhibit microbial growth, i.e., temperature, water activity (a_w), pH, and preservatives. When two or more hurdles are combined, the level of each needed to control or inhibit microbial growth is often less than it would be if used alone. An understanding of the interaction between hurdles, could allow for the inhibition of microbial growth, reduction of toxin production by pathogens, and for the prediction of the shelf life of food products. Whiting (1993) described two primary models capable of fitting survival/inactivation data that exhibit a lag or shoulder period followed by declining numbers of viable microorganisms. Deviations from a log-linear kinetics in the thermal inactivation of microorganisms, in particular *L. monocytogenes*, was demonstrated by Cole et al. (1993). Indeed, they proposed the use of a logistic function and log dose to more accurately describe the death kinetics of vegetative bacteria.

In traditional challenge and shelf-life studies, a food product is inoculated with the organism of concern, incubated under specific pre-determined conditions, and sampled at certain intervals. Estimation of a product's safety or shelf-life, usually

takes long periods of incubation (30-180 days). With a good predictive model, the effect of varying factors on microbial behavior could be readily calculated (Roberts, 1989). The steps involved in modeling include (Baranyi et al., 1993; Dodds, 1993): (1) planning, a well designed experiment is essential (Davies, 1993); (2) data collection (Miller, 1993); (3) model fitting and selection of the model which best describes the data; and (4) model validation using data not used to fit the model. If only two levels of a factor are studied, only a linear effect can be determined. If a surface response is desired, three or more levels are required. The predictive ability of any model is best within the range of conditions tested. Regression is the fitting technique used to quantify the relationship between variables when the value of one variable is affected by changes in the values of other variables. In its simplest expression, the relationship is linear and there is one dependent and one independent variable. If there is more than one independent variable, multiple linear regression can be used. Since physical and biological models often arise as solutions to differential equations, regression models that describe natural processes are often nonlinear in the model parameters (Ratkowsky, 1993). In a closed system, modeling of microbial growth is best represented by a sigmoidal curve. There are several choices of equations for sigmoidal curves (Zwietering et al., 1990). Logistic regression gives a symmetrical sigmoidal curve, so it is best for organisms growing under optimal conditions. The Stannard (1985) equation is also for a sigmoidal curve. The Gompertz equation (Gibson et al., 1987) is used when the relative growth is

believed to decrease exponentially with time. The Ratkowsky (Ratkowsky et al., 1982) equation is predictive for growth dependent on temperature. Jones and Walker (1993) developed a mathematical model that describes bacterial growth, survival and death.

Predictive models estimate parameters such as lag time, generation time (Pelczar and Chan, 1981), maximum growth rate, and maximum cell concentration of microorganisms under particular conditions (Skinner et al., 1994). Temperature is the prime factor controlling the rate of microbial proliferation in fish and crustaceans (McMeekin et al., 1992). Mathematical models have been developed for the prediction of bacterial growth at constant storage temperature (Buchanan et al., 1989b; Buchanan and Phillips, 1990). Among them is the utilization of surface response regression analysis coupled with the "Gompertz function" - found to qualitatively describe the growth kinetics. Buchanan and Phillips (1990) developed a mathematical model describing the effects of temperature (5 to 37 °C), pH (4.5 to 7.5), NaCl (5 to 45 g/l), $NaNO_2$x(0 to 1,000 g/ml) and atmosphere (aerobic or anaerobic) on the growth kinetics of *L. monocytogenes* Scott A in tryptone phosphate broth. Wijtzes et al. (1993) developed two equations to describe the growth rate of *L. monocytogenes* in relation to pH, temperature and water activity. Ratkowsky et al. (1991) discussed two types of kinetic models widely used in predictive microbiology; non linear Arrhenius (expresses dependent variable as ln *rate*) and Bělehrádek-type model (dependent variable is expressed as $\sqrt{rate}$), describing the effects of the form

of the model on the variance in rate and time. Zwietering et al. (1994b) proposed several models to predict microbial kinetics for bacterial growth with change in temperature. A procedure to statistically analyze non-isothermal microbial spoilage of refrigerated foods exposed to temperature abuse was described by Almonacid-Merino el al. (1993).

Zwietering et al. (1993) developed a method to combine qualitative and quantitative information for the prediction of microorganisms in foods. Microbial growth characteristics are coupled to food characteristics such as; pH, water activity, temperature and oxygen availability. A database with the kinetic parameters of microorganisms and a database with tree structure based on physical similarity of food products are built. Thus, information about a food product not listed can be estimated by comparison with similar products at the same level of the tree or the level above. A method to estimate microbial growth kinetics on the basis of models was developed. A similar tool, the microbial kinetics expert system (MKES), was developed by Agriculture Canada and Voyer and McKellar (1993) reported on its use for developing and assessing food production systems. In the United Kingdom, the Government commissioned a large program of work on predictive microbiology (UK Predictive Food Microbiology Program, UKPFMP). Among the aims of this program was the development of a set of mathematical models to predict the growth, survival and death of foodborne pathogens under conditions relevant to foods. Walker and Jones (1993) reported on the protocols and recommendations developed by the

UKPFMP for data generation for predictive microbiology. The establishment and operation of databases for storage of data and models, relevant to changes in populations of foodborne pathogens under given conditions, was described by Jones (1993).

1. Microbial Growth Curves

REGRESSION MODELS

Sigmoidal Functions

Sigmoidal curves such as the Gompertz and logistic functions (Gibson et al., 1987; Stannard et al., 1985) have been extensively used to model microbial numbers as a function of time. These type of functions have been chosen because they consist of four phases, similar to the microbial growth curve, including an initial stage of little change, a stage of accelerating change, a stage of decreasing change, and a stationary stage (Gibson et al., 1987).

The logistic function is symmetrical around the point of maximum growth rate, and is described by:

$$L(t) = A + \frac{C}{(1 + e^{(-B(t-M))})}$$

where L(t) = $\log_{10}$ of the bacterial count (cfu/ml) at any time t, [1]

A = asymptotic count as t decreases to zero (initial number of bacteria, N_0),

A + C = asymptotic number of microorganisms present as t increases indefinitely with C being the actual asymptotic amount of growth,

B = relative growth rate at M,

M = time where the absolute growth rate is maximum

The Gompertz function is not symmetrical around the point of maximum growth rate, and is described by:

$$L(t) = A + C(e^{(-e^{(-B(t-M))})})$$

where L(t) = $\log_{10}$ of the bacterial count (cfu/ml) at any time t, [2]

A = asymptotic count as t decreases to zero (initial number of bacteria, N_0),

A + C = asymptotic number of microorganisms present as t increases indefinitely with C being the actual asymptotic amount of growth,

B = relative growth rate at M,

M = time where the absolute growth rate is maximum

Stannard et al. (1985) used the following sigmoidal function to represent the growth curves of certain psychrotrophic food spoilage bacteria.

$$y = \frac{A}{(1 + e^{-\frac{(\lambda + K[X + 1])}{\theta}^{\theta}})}$$

where y = microbial growth [3]

X = time

A,λ,K, and θ = parameters of the curve

A sigmoidal equation is given by Zwietering (1990):

$$y = a\ (1 + \nu\ x\ e^{(\kappa(\tau - \chi))})^{-\frac{1}{\nu}}$$

where y = growth [4]

χ = time

a, ν, τ, and κ = equation variables

Sigmoidal functions are useful because actual microbial growth rates are not constant over the entire growth period. Growth increases to a maximum then decreases (Broughall and Brown, 1984; Gibson et al., 1987). The Gompertz function does not assume a constant growth rate. Although the same argument applies to the logistic function, it is symmetrical and therefore assumes identical acceleration and deceleration of growth rates. The Gompertz function also differs from the logistic function in that the former has a more rapid upward increase at the end of the lag phase, it also reaches a maximum rate of growth at lower growth level, and it has a

more gradual decrease in growth rate (Garthright, 1991). One drawback of the Gompertz and logistic functions is the large number of data points necessary to obtain good fit (Skinner et al., 1994).

Dual Stage Modeling Approaches

The use of a sigmoidal function to model the kinetics of microbiological behavior in foods has been expanded to combine non-linear regression and surface response methodology with a sigmoidal equation (Gibson et al., 1988; Bratchell et al., 1989, 1990; Buchanan, 1991). A multidimensional curve of bacterial growth as a function of a number (n) of growth factors represents situations in which all conditions on one side of a graphical curved surface support growth and all those on the other side inhibit growth. Extreme care must be taken if inferences are made outside the boundary conditions of the performed experiments because the range of test conditions defines the model's range of predictability (Farber, 1986). Modeling is carried out in a two-step process as performed by Broughall and Brown (1984). In the initial step, experimentally obtained data are fitted with a sigmoidal function, e.g., the Gompertz, to get estimates for model parameters such as B, M, A, and C. In step 2, polynomial equations describe the variations in sigmoidal function variables as a function of specific growth conditions. Estimates of growth rate, generation time, lag time, and time required for the microorganism to grow by a certain factor, may be obtained.

Buchanan (1991) and his colleagues used the two-step modeling approach, by which surface response curves are generated on the basis of microbiological growth data. They compiled data to predict the growth of food pathogens under various environmental conditions such as storage temperature, pH, a_w, and added ingredients such as nitrite and salt. In version 3.1 of the Pathogen Modeling Program (Buchanan, 1991; Buchanan, 1993), prediction of the microbiological parameters of six foodborne pathogens can be accomplished. These include *Salmonella*, *L. monocytogenes*, *Shigella flexeneri*, *S. aureus*, *Aeromonas hydrophila* and *Escherichia coli* O157:H7. Although models generated by this combination of techniques are well suited for "user friendly" computer software, because the program is based on surface response methodology, microbial behavior cannot be predicted beyond the experimental boundaries under which the data were obtained (Skinner et al., 1994).

2. Growth Rate Models

ARRHENIUS BASED MODELS

Simple Arrhenius Based Models

The Arrhenius equation was originally used to describe how the rate constant for a chemical reaction may change as a function of temperature (Ratkowsky et al., 1982). Equations on this group are based on an assumed linear relationship

between temperature and reaction rate (Davey, 1993). Ingraham (1958) used a modified form of the Arrhenius equation to model microbial growth:

$$k = A \; x \; e^{(-\frac{\mu}{RT})}$$

where k = specific growth rate [5]

A = constant

R = universal gas constant (1.987 cal mol^{-1} K^{-1})

T = absolute temperature (K)

μ = temperature characteristic substituted for the activation energy used to classify microorganisms into optimal growth ranges.

Nonlinear Arrhenius Models

Schoolfield et al. (1981) published a more complex model that also describes the effect of temperature on growth rate over the entire growth range:

$$\mu = \frac{\rho_{(25°C)} \frac{T}{298} e^{[\frac{H_A}{R}(\frac{1}{298}-\frac{1}{T})]}}{1+e^{[\frac{H_L}{R}(\frac{1}{T_{1/2L}}-\frac{1}{T})]}+e^{[\frac{H_H}{R}(\frac{1}{T_{1/2H}}-\frac{1}{T})]}}$$

where μ = growth rate (h^{-1}) or (generation $time^{-1}$) [6]

$\rho_{(25\,°C)}$ = growth rate at 25 °C

T = temperature (K)

H_A = enthalpy of activation of the reaction that is catalyzed by the growth limiting enzyme (cal mol^{-1})

R = universal gas constant (1.987 cal mol^{-1} K^{-1})

H_L & H_H = enthalpy of low and high temperature inactivation of growth

$T_{1/2L}$ & $T_{1/2H}$ = temperature at which the growth rates are reduced by 50% because of high or low temperature conditions

SQUARE ROOT OR BĚLEHRÁDEK TYPE MODELS

In this type of model (Ross, 1993), a linear relationship between the square root of the growth rate constant (μ) and the temperature (K) is assumed (Ratkowsky et al., 1982):

$$\sqrt{\mu} = b(T-T_{min}) \quad [7]$$

where μ = growth rate (h^{-1}) or (generation $time^{-1}$)

b = regression line slope

T = temperature (K)

T_{min} = conceptual temperature (K) below which the growth rate is zero and the lag time is infinite

Ratkowsky et al. (1983) proposed a modified version to cover the entire range of temperature:

$$\sqrt{\mu} = b(T - T_{min}) \; x \; (1 - e^{(c(T - T_{max}))})$$

where μ = growth rate constant [8]

T_{min} & T_{max} = minimum and maximum temperature at which the rate of growth is zero (K)

c = regression coefficient

Zwietering et al. (1991) proposed a further modification to the square root equation of Ratkowsky et al. (1983). The growth rate decline at temperatures near T_{max} in the new equation is described by an exponential function and not the square of an exponential function:

$$\mu = b(T - T_{min})^2(1 - e^{c(T - T_{max})})$$

[9]

Zwietering proposed another square root equation which disregarded the equation's minimum temperature portion, since at lower temperatures the asymptotic nature of the growth curves was not greatly affected (Skinner et al., 1994):

$$\sqrt{\mu} = b(1 - e^{(c(T - T_{Amax}))})$$

where T_{Amax} is defined as the maximum temperature at which growth is observed. [10]

New Model

Duh and Schaffner (1993) proposed a new empirical model, selected from a list in the curve fitting software TableCurve v. 3.01 (Jandel Scientific, California), which also describes the growth rate with respect to temperature:

$$\mu = \frac{(a+cx+ex^2)}{(1+bx+dx^2+fx^3)}$$

where μ = growth rate (h^{-1}) or (generation $time^{-1}$) [11]

x = temperature of incubation (K)

a,b,c,d,e,f = regression coefficients

3. Lag Time Models

Schoolfield and Square Root Models

These models can be used to predict lag time as well as growth rate. When used to predict lag time (λ), the term for growth rate (μ) is replaced by $1/\lambda$. The problem is that if the lag times are large, the inverted value of $1/\lambda$ will approach to zero. Those values close to zero, are weighed less during the regression procedure resulting in poor fit (Duh and Schaffner, 1993). Zwietering et al. (1991) modified the use of these equations by inverting the growth-rate equations prior to fitting the data. They also used a logarithmic transformation of the equations to limit this effect.

Hyperbolic Model

An hyperbolic relationship between the lag time and incubation temperature was proposed by Adair et al. (1989):

$$\ln(\lambda) = \frac{p}{(T-q)}$$

where p = measure of the decrease of the lag time when the temperature is increased [12]

q = temperature at which the lag time is infinite (i.e., no growth occurs)

New Model

Duh and Schaffner (1993) also proposed an empirical model selected by TableCurve v. 3.01 to describe the lag time in relation to incubation temperature:

$$\frac{1}{\mu} = \lambda = e^{\left[a+\left(\frac{b}{x^2}\right)\right]}$$

where λ = lag time (h) [13]

x = temperature of incubation (K)

a,b = regression coefficients

Comparative Studies

Saguy (1992) demonstrated that an Arrhenius type equation closely described the exponential growth rate and the lag duration time of *L. monocytogenes* in the 5 to

20 °C range, when simulating variable storage and distribution temperatures. Yousef et al. (1991) modeled the growth of *L. monocytogenes* Scott A in tryptose broth containing different concentrations of the antioxidants BHA and BHT. Growth data were fit using the logistic sigmoidal function, with a non-linear technique given by Gibson et al. (1987). Estimates of lag period, maximum growth level, and generation time were obtained for *L. monocytogenes* Scott A. Stannard et al. (1985) used a sigmoidal function to represent the growth curves of psychrotrophs *Citrobacter freundii*, *Alcaligenes viscous*, *Alteromonas putrefaciens*, *Serratia marcescens*, *Pseudomonas* spp., *Moraxella* spp., *Acitenobacter* spp., and *Brochothrix thermosphacta* at 2, 4, 6, 8, 12, and 15 °C in media. This model was satisfactory in fitting growth curves of pure and binary mixtures of the psychrotrophic food spoilage bacteria.

Duh and Schaffner (1993) compared several models which predict growth rate and lag time as a function of temperature. They used a strain of *L. monocytogenes* and a strain of *L. innocua* to determine growth and lag time of these organisms in brain heart infusion broth at temperatures in the range between 2 C° and 46 °C. A faster growth rate of *L. innocua,* compared to *L. monocytogenes,* was observed at temperatures below 42 °C, and the opposite situation was observed for temperatures above 42 °C. The lag phase of *L. monocytogenes* was longer than that of *L. innocua* at temperatures below 8 °C. Based on the r^2 and F values they reported a better fit by the 4-parameter growth rate models than by the 6-parameters models. To predict

growth rate, the 4-parameter Zwietering Square Root Model, was the best model among those tested. To predict lag phase, the 2-parameter models were better than the models containing more parameter, and among those tested the 2-parameter Square Root Model gave the best fit when using a traditional method of determination. When using the Solberg method of determination for lag phase, the 2-parameter New Model gave the best fit.

Stannard et al. (1985) compared the simple Arrhenius equation (Eq. [5]) to the square root equation (Eq.[7]), using growth data for pure culture cultures and binary mixtures of psychrotrophic food spoilage bacteria. At chill temperatures (2-15 °C), the square root equation described microbial growth as a function of temperature better than the Arrhenius function. Similar results were reported by Phillips and Griffiths (1987) when comparing these equations to describe the growth of psychrotrophic food spoilage organisms in various pasteurized milks and creams. The square root equation accurately predicted effects of temperature on microbial growth in various dairy products regardless of the growth medium.

The performance of a linear equation, equations where the growth rate is zero and constant, and the equations of Schoolfield (Eq.[6]), Ratkowsky square root (Eq.[7] and [8]), and Zwietering (Eq.[9] and [10]) were compared by Zwietering et al. (1991). Maximum bacterial growth, lag time, and specific growth rate were used to evaluate which model would be more useful in predicting the effect of temperature on the safety of refrigerated foods. Thirty-eight sets of *L. plantarum* growth data at

18 different temperatures were compared. Comparisons were made by using an F ratio test. Initial F tests led to the conclusion that the Schoolfield equation (six parameters), and both Ratkowsky equations (four parameters) adequately fit the curvature of the growth-temperature relationship. However, of the equations accurately describing growth data, Zwietering et al. (1991, 1994a) felt their own equation (Eq.[9]) was most adequate for describing growth rate as a function of temperature because it had the lowest residual sum of squares. This equation predicted an exponential decrease in growth rate at high temperatures and did not predict positive growth rates above the actual maximum growth temperatures.

Complexity of the many models developed to predict bacterial behavior varies, and the choice of which one to use depends on the user's requirements. If there are concerns about the potential safety of a food, the model used should be conservative; it should overestimate the microbial growth rate, underestimate the number of microorganisms needed to initiate growth, and underestimate the time a product would be safe under a given set of conditions. In evaluating the microbial safety of foods, the conditions under which the model was produced must be appropriate for the foods of interest. The actual translation of how results of a study will apply to a food must be carefully analyzed. Under certain conditions organisms grow better in foods than in media. Experimental approaches and methodologies vary among laboratories, making comparisons difficult and causing data to be valid only for those conditions under which the study was carried.

An ideal predictive model would have a true physicochemical or kinetic basis, with known thermophysical or biological assumptions, and would not be simply statistically based. It would predict microbial behavior in foods or in the environment on the basis of actual chemical or physical responses to specific conditions. Under the kinetics of how microorganisms behave when exposed to given product or storage conditions would increase the accuracy of predictive models about food safety.

If a certain amount of microbial growth is tolerated in a food, the ability to estimate the amount of microbial growth with respect to the time and temperature of storage conditions of real food systems would be advantageous to the food industry (Gibson et al., 1988). For this reason, growth rate models are preferred for pathogens such as *S. aureus* which must experience a substantial increase in numbers to reach an infectious or toxic level (Baker and Genigeorgis, 1990). If the food safety concern involves inhibition of an organism under certain set of condition, knowing the lag time becomes important. Baker and Genigeorgis (1990) feel that models based on lag times are most appropriate for pathogens subject to a zero tolerance in foods, such as *Salmonella*, *L. monocytogenes*, or *C. botulinum*.

G. BACTERIAL COMPETITION AGAINST L. MONOCYTOGENES

Tran et al. (1990) suggested that competition with *L. monocytogenes* may be due to specific bacteria rather than bacterial numbers. Known to be antagonistic to *Listeria* are *Enterococcus faecalis* (Arihara et al., 1991; Dallas and Hitchins, 1993),

Enterococcus faecium (McKay, 1990), *Corynebacteria* (Valdes-Stauber et al., 1991) and certain acid bacteria (Harris et al., 1989); in particular *pediococci* (Yousef et al., 1989), *Leuconostoc gelidium* (Harding and Shaw, 1990) and *Lactococcus lactis* (Wenzel and Marth, 1990). An antagonistic, non-acid effect by species of *pediococci* against *L. monocytogenes* was reported by Hoover et al. (1989). Distinct inhibitory reaction was observed in 28% of the pairings studied with a resistant sub-population arising after prolonged exposure. Farrag and Marth (1989) reported *P. fluorescens* as having a negative effect on growth of *L. monocytogenes* Scott A and retardation in the growth of *L. monocytogenes* California and *P. aeruginosa* with a negative effect on survival of *L. monocytogenes* California but no effect on strain Scott A. Bacteriocins such as nisin from *Lactococcus lactis* and pediocin A from *Pediococcus pentosaceus*, will inhibit *L. monocytogenes* (McKay, 1990). A bacteriocin (hieracin S) produced by *Enterococcus hirae,* isolated from bovine intestin, was shown to have inhibitory effect upon *L. monocytogenes* and other *Listeria* spp. (Siragusa, 1992). The inhibitory action of a bacteriocin, pediocin JD, produced by *Pediococcus acidilactici* JD1-23 against *L. monocytogenes* is believed to be directed at the cytoplasmic membrane causing collapse of one or both of the individual components of the proton motive force (Christensen and Hutkins, 1992). This same mechanism is proposed for the action of nisin and lactococcal bacteriocins (Christensen and Hutkins, 1992).

Marshall et al. (1992) reported stimulation of growth of *L. monocytogenes* in chicken nuggets when incubated at 3 °C in the presence of *Pseudomonas fluorescens*

in air and in a modified atmosphere (76% CO_2: 13.3% N_2: 10.7% O_2). Prior work has shown stimulated growth of *L. monocytogenes* by *Pseudomonas* spp. in milk (Marshall and Schmidt, 1988, 1991) and in beef (Gouet et al., 1978). Farrag and Marth (1991) reported enhanced growth of *L. monocytogenes* when in the presence of *flavobacteria* under certain conditions.

Rørvik et al. (1991) reported slightly faster growth of *L. monocytogenes* in smoked salmon with better hygienic quality, when comparing samples of different hygienic quality, artificially inoculated with low (6 CFU/g) and high (600 CFU/g) levels of a mixture of three strains of *L. monocytogenes*.

Competition between *L. innocua* and *L. monocytogenes* has been suggested; however, a study done by Petran and Swanson (1993) indicates no significant difference in the growth of both species in non-selective and food systems and a higher population of *L. innocua* when using selective media. As a result of an interlaboratory study, Curiale and Lewus (1994) reported better recovery of *L. innocua* than of *L. monocytogenes* from samples containing both organisms. They also determined the generation times of *L. monocytogenes* and *L. innocua* in both selective and non-selective medium and reported a faster growth of *L. innocua* in both cases, strongly suggesting that *L. innocua* has a selective advantage over *L. monocytogenes* during normal enrichment culturing.

Zaika and Kim (1993) reported inhibition in the growth of *L. monocytogenes* by high molecular weight sodium polyphosphates specially at low temperatures and when combined with NaCl.

A bacteriocin produced by *Enterococcus faecium* has been reported (Parente and Hill, 1992) to have a rapid bactericidal effect upon *Listeria* in buffer systems, broth and milk.

H. THERMAL INACTIVATION STUDIES

In designing *L. monocytogenes* thermal inactivation studies, factors such as: type and number of strains of *L. monocytogenes* to use as inoculum; methods for production, enumeration and standardization of the inoculum; size of inoculum; method of inoculation; packaging product; sample size; number of samples to test; heating of products; calculation of D and z values; enumeration and detection media; and product composition should be considered (Brown, 1991). In food microbiology, the thermal resistance of bacteria is usually described by two parameters, D and z. The D value, at a particular temperature, is the time needed for the number of surviving cells to be reduced by a factor of 10. The z value is the increase in temperature required to reduce the D value 10-fold. Miles and Mackey (1994) developed two mathematical methods for determining D and z values from viable count data obtained at linearly rising temperatures.

Harrison and Huang (1990) determined thermal death times for *L. monocytogenes* (Scott A) in blue crab meat. D-values of 40.43, 12 and 2.61 min at 50, 55 and 60 °C respectively (z-value of 8.4 °C), were found when Trypticase Soy agar (TSA) was used to enumerate colonies. Using modified Vogel-Johnson agar as the plating medium, D-values of 34.48, 9.18 and 1.31 min at each respective temperature, with a z-value of 6.99 °C, were determined. The current pasteurization process for crab meat in steel, aluminum cans, plastic containers, and flexible films, is the equivalent of 32 min at 185°F (85 °C) with a z-value of 16°F (8.89 °C), which would provide a D-process of several hundred fold. Budu-Amoako et al. (1992b) determined a z value of 5.0 °C for *L. monocytogenes* in lobster meat with D values ranging from 97.0 min at 51.6 °C to 1.06 min at 62.7 °C.

I. CONTROL/REDUCTION OF *L. MONOCYTOGENES* IN FOOD PROCESSING

L. monocytogenes is particularly difficult to control in food-processing plants, specially in refrigerated food plants where the refrigerated, moist environment, coupled with soil deposition, allows for the survival and growth of the microorganism. The organism can adhere to food contact surfaces and form a 'biofilm' or coating (Doyle, 1991) which impedes removal and decreases the effectiveness of sanitation procedures. *L. monocytogenes* is also a frequent contaminant of raw materials utilized in plants; therefore, there is constant re-

introduction of the organism into the plant environment. Use of Hazard Analysis Critical Control Point (HACCP) and/or other process control strategies is currently considered the most effective method to control *L. monocytogenes* in the processing environment (Microbiology and Food Safety Committee of the National Food Processors Association, 1992; National Advisory Committee on Microbiological Criteria for Foods, 1990, 1991, 1992; Rippen and Hackney, 1992). The goal of these systems is the prevention of problems from occurrence.

Tompkin et al. (1992) concluded that with the current technology available, *Listeria* cannot be eliminated from the finished environment in processing plants of meats and poultry product.

J. PASTEURIZATION OF CRAB MEAT

1. Conventional method

There is no uniform, mandatory requirement for pasteurization of ready-to-eat crab meat. The recommendation of the Committee (National Advisory Committee on Microbiological Criteria for Foods, 1990) is a minimum thermal process of $F_{185} \geq 31$ minutes to be required for a product to be labeled pasteurized. The conventional pasteurization of crab meat, heating 401X301 tinplate cans of crab meat in a 88 °C water bath until the cold point temperature reaches 85 °C and holding for 1 min, results in an extension in the shelf life from 6-10 days for fresh crab meat to 6-18 months for properly pasteurized crab meat. There is no target organism for the

pasteurization process, this being based on historical data that resulted in the desired shelf-life. A z-value of 8.9 °C has been arbitrarily chosen (Rippen and Hackney, 1992; Ward et al., 1982, 1984).

2. Microwaves

Most authors have concluded that inactivation of microorganisms during microwave process occurs solely due to generation of heat (Decareau, 1985). This is confirmed by Mertens and Knorr (1992) who reported on the inability, at this point, to demonstrate the non-thermal usage of microwaves as a preservation method.

The principal reason to consider the use of microwaves for pasteurization of foods is the ability of microwaves to penetrate many packaging materials with almost no energy loss (Schiffmann, 1989). The design of microwave processes requires the knowledge of both, thermal and electrical properties of foods (Mudgett, 1986; Heddleson and Doores, 1994). Mudgett et al. (1977) proposed a general physical-chemical model for high-frequency dielectric behavior of solid foods based on observed mechanisms of interactions between water and the biochemical constituents of foods.

Ockerman et al. (1976) compared the efficiency in bacterial reduction of oven versus microwave cooking of artificially inoculated (*B. subtilis*, *L. mesenteroides* and *P. putrefaciens*) pork meat of different quality at various temperatures and reported no influence of meat quality in bacterial destruction. *B. subtilis* was found to be the

most heat tolerant and *P. putrefaciens* the least, and temperature had a significant effect on bacterial reduction with oven cooking being more efficient than microwaves in the reduction of survivors. These results agreed with prior studies by Crespo and Ockerman (1977) and Crespo et al. (1977) who reported low temperature (149 $\pm$ 6 °C) oven cooking in ground beef to be more effective than microwave cooking for the thermal destruction of microorganisms. Baldwin et al. (1971) studied the adequacy of microwave heating for destruction of Salmonellae in fish. They reported that exposure of 270 g portions of carp to microwaves (2450 MHz) for 195 sec was not adequate for complete destruction of *Salmonella typhimurium* inoculated on the surface of the fish. Coote et al. (1991) used conventional heating to expose *L. monocytogenes* cells, either in broth or on chicken skin, to mean times and temperatures that are achieved during a 28-min period of microwave cooking of a whole chicken. After cooking for the full time, a mean temperature of 85 °C and no surviving listerias were observed. They concluded that when a temperature of 70 °C is reached and maintained for at least 2 min throughout a food, there is a substantial reduction in the numbers of *L. monocytogenes*. Fruin and Guthertz (1982) studied the reduction in bacterial numbers (*E. coli*, *C. perfringens*, *S. faecalis* and *S. aureus*) of artificially inoculated meat loaf when cooked by microwave oven, conventional oven and slow cooker. They reported the greatest variation in internal temperature within the microwaved loaf and the smallest for the slow cooked; however, no significant difference was observed between cooking methods, at the 0.05 level of significance,

for bacterial destruction. Sawyer et al. (1983) determined the microbiological quality of reheated food products when comparing conduction, convection and microwave reheating of beef loaf, instant mashed potatoes and frozen peas. They reported that although reheated products were similar microbiologically, internal end temperatures did not meet FDA recommended standards ($\geq$7.4 °C) for reheated products in up to 83% of the cases studied, representing a potential for foodborne illness in cook/chill food services. Sawyer (1985) reported a more often post-processing rise in temperature of products heat-processed by microwave than by hot-air ovens. Conventional oven cooking was more effective than microwave cooking in the reduction of the numbers of aerobic microorganisms and *Clostridium perfringens* in ground beef patties when the meat was heated to approximately the same internal temperature (Wright-Rudolph et al., 1986).

K. PRINCIPLES OF MICROWAVE HEATING

Among the several components known to play an important role in the uniformity of microwave heating of a food, the following can be mentioned: moisture and ionic content of the food, the specific heat of the various food constituents, product density, shape and load volume. The ionic content of a food is important as it is well established that dissolved salts decrease the penetration depth of incident microwave energy (Heddleson et al., 1993; Lentz, 1980). Temperature gradients are

formed within the product, with lower temperatures at greater depths, which could result in survival of any bacterial pathogen present.

Microwave energy is a form of non-ionizing radiation with energy in the region of 1.2×10^{-5} eV, well below the energy necessary to break chemical bonds (5.2 eV to break hydrogen bonds) (Rosen, 1972). The role of the electric field is to heat by promoting rotation of polar molecules, resulting in heat generated by molecular friction, thus the absorbing system dissipates energy as heat (Curnutte, 1980). Microwaves travel at the speed of light, and their ability to heat is largely determined by the parameters of the product being heated. The dielectric constant (k'), dielectric loss (k") and tangential loss (∂) play a critical role (George, 1993). The dielectric constant describes a material's capacity to store electrical energy, whereas the dielectric loss, describes a material's ability to dissipate electrical energy as heat. The dielectric constant and loss factor determine the quantity of energy that will be reflected, transmitted or absorbed by a material (Tinga, 1970). The loss tangent is related to a material's capacity to be penetrated by an electrical field and subsequently dissipate electrical energy as heat, and is expressed as the ratio of k" to k' (Mudgett, 1986). Swami and Mudgett (1981) reported on the importance of moisture and ionic content in modeling heating patterns, and found that these two parameters could be estimated by moisture and conductivity measurements. Water comprises 50 to 90% of most foods (Ohlsson, 1983). High water contents generally results in greater microwave absorption, and decreased penetration depth. Products

with high moisture content, will heat more efficiently due to the larger dielectric loss factor (Schiffmann, 1986). However, products with lower moisture may also heat well due to lower specific heat capacity and because as moisture content decreases, wave penetration will increase (Mudgett, 1982).

The depth of microwaves penetration in foods is largely determined by the wave's frequency (Mudgett, 1986; Ohlsson, 1983). In the United States, commercial microwave ovens generally use 2,450 MHz, although in some industries and in several countries outside the U.S., 915 MHz is used. The wavelength (λ) resulting from these frequencies are 0.12 and 0.33 m, respectively (Annis, 1980). As wavelength increases (or processing frequency decreases), penetration depth increases. Frequency also has a great impact on the dielectric loss factor. For aqueous solutions, a lower frequency will result in a smaller dielectric loss, although in the case of free ions in solution, the loss factor is larger at lower frequencies (Schiffmann, 1990). Frequency also influences the heating pattern by microwaves. In general, surface heating effects (the surface achieves higher temperatures than the geometric center of the product) are of concern when using ovens at 2,450 MHz. Opposite are the center heating effects, depending on product shape, size and salt content, and more prominent in ovens operating at frequencies of 915 MHz.

The relationship between mass of the food and its influence on heating, can be described by the "coupling" effect. By this phenomenon, larger objects usually absorb more microwave energy than smaller ones. However, larger masses will

usually take longer to heat in a microwave oven, as more time will be necessary for conduction to cause the temperature gradient to equilibrate and decrease. Density affects microwave heating; the relationship is nearly linear between density and the dielectric constant. Air within a product makes it a good insulator (Schiffmann, 1990), and also decreases the dielectric constant. Compared to conventional heating, the deeper penetration of microwaves results in decreased baking times. Size and shape of products will affect microwave heating. Objects with corners (90° edges, etc.) have a tendency to show localized heating due to the multi-directional distribution of microwave energy. As penetration depth decreases, these corner effects have larger impact. The size of the object being heated is important in relation to wavelength and penetration depth. Copson (1962) described a focussing (center heating) effect on microwave heating. Rounder shapes reduce corner heating but are susceptible to focussing effects. These effects have only been noted in cylinders and spheres and are dependent on the diameter and conductivity of the product, as well as the processing frequency.

Specific heat is the amount of heat gained or lost by a unit weight of a product to accomplish a desired change in temperature without a change in state. The specific heat of a food component may allow a component that has a poor intrinsic ability to increase in temperature to heat well when exposed to a field. Thermal conductivity is another factor that helps in promoting uniform heating throughout masses during (and potentially after) microwave heating. The dielectric properties of a food will be

affected by the temperature achieved after a determined heating time. As temperature increases, the moisture level in the food will decrease due to the evaporative effect.

1. Lethality mechanisms of microwaves

Numerous studies indicate that microwaves reduce bacterial numbers in foods through the effects of heat; potentially irreversible heat denaturation of proteins, enzymes, nucleic-acids or other cellular constituents vital to cell metabolism or reproduction, with the subsequent cellular death. Leakage of metabolites and cofactors required for cell function through membranes damaged by heat, would have a similar final result (Heddleson and Doores, 1994). A second school of thought proposes and supports an "athermal" mechanism of lethality with an effect attributable only to the intrinsic nature of microwaves, and unrelated to lethality caused by heat (Chipley, 1980). The calculation of microbial destruction for a microwave heating process is much more complicated to do than for a conventional thermal process. The difficulty in maintaining a constant temperature within a microwave oven, makes the determination of D-values, the time in minutes required at a constant temperature to reduce a microbial population by 90%, difficult to achieve. Quantification of total bacterial destruction (or destruction of biological compounds) during microwave heating has been described by (Heddleson and Doores, 1994):

$$k_1 = k_2^{E_a(T-T_2/RTT_2)}$$

where; k_1 = thermal inactivation rate at temperature T (min^{-1}),

k_2 = thermal inactivation rate at temperature T_2,

E_a = activation energy (cal/mol),

T = product temperature (K),

T_2 = reference temperature (K)

R = the universal gas constant (1.987 cal mol^{-1} K^{-1})

In a simulation of high-temperature, short-time (HTST) and low-temperature, long-time (LTLT) milk pasteurization using microwaves, Knutson et al. (1988) found that the non-uniform distribution of heat in the milk resulted in recovery of viable *Salmonella typhimurium*, initially inoculated at 10^3-10^4 CFU/ml. Although microwave pasteurization is supposedly more costly than traditional pasteurization processes, the advantage of reduced processing time with the subsequent cost reduction makes microwave pasteurization/ sterilization processes an attractive and promising industrial application (Rosenberg and Bogl, 1987). However, most studies on the effectiveness of microwave heating in eliminating pathogens in food products have concluded that although large decreases (up to 7-8 $\log_{10}$ cycles) in bacterial populations may be observed (Cunningham, 1980) complete elimination of pathogens is more difficult to obtain by microwave heating (Alexio et al., 1985).

2. Destruction of *L. monocytogenes* by microwave heating

The thermotolerance of *L. monocytogenes* Scott A and V7, suspended in nonfat dry milk and heated by microwaves, was studied by Galuska et al. (1988). They concluded that at conventional pasteurization process temperatures, microwaves could be as effective as conventional heating in destroying *L. monocytogenes*. Lund et al. (1989) inoculated the surface and stuffing of chickens with approximately 10^7 CFU/g of *L. monocytogenes*. After following the recommended cooking and standing protocol they found a reduction in the number of viable cells by a factor of 10^6. Even though temperatures of 72 to 85 °C were recorded within the stuffing and on the chicken, in occasions enrichment procedures allowed for the recovery of *L. monocytogenes*, highlighting the lack of uniformity in heating within microwaved-cooked foods. Allowing standing time for temperature equilibration within the food is essential for microbiological safety.

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SECTION II: *LISTERIA MONOCYTOGENES* OCCURRENCE AND GROWTH AT REFRIGERATION TEMPERATURES IN FRESH BLUE CRAB (*CALLINECTES SAPIDUS*) MEAT.

(Paper accepted for publication in the Journal of Food Protection)

ABSTRACT

The ability of listeriae to grow over a wide temperature range, including refrigeration temperatures, is of great concern to the food industry. For seafood processors in particular, it is of most importance to understand the growth characteristics of this pathogen under normal commercial storage conditions. In this study, 126 samples of fresh blue crab (*Callinectes sapidus*) meat collected from different processing facilities were analyzed for the presence of *Listeria spp.* Thirteen samples (10%) were positive for *Listeria*, with 10 samples positive for *L. monocytogenes* and 3 samples positive for *L. innocua*. Fraser broth was used in a 5-tube most probable number (MPN) enumeration, in duplicate, of *Listeria* in 25 g samples incubated at 36 °C for 24 hours and plated in Modified Oxford Agar and Blood Agar with API strip confirmation. The levels of *Listeria* found in fresh blue crab meat were in all but one sample less than 100 /g, for this the MPN index was 1,100 /g. A *L. monocytogenes* 4b strain (168) isolated from fresh blue crab meat was inoculated (less than 50 cfu/g) into pasteurized crab meat and incubated at 1.1, 2.2 and 5 °C for 21 days. Growth curves were obtained by analyzing, 25 g samples at

intervals of 0, 8, 10, 12, 14, 16, 19 and 21 days. In the absence of competing microflora, *L. monocytogenes* had an increased growth rate as the storage temperature increased, with approximately a 7 $\log_{10}$ increase in population at 5 °C and only a 2.5 $\log_{10}$ increase in population at 1.1 °C after the 21 days of incubation.

INTRODUCTION

The United States department of Agriculture (USDA), Centers for Disease Control (CDC) and the Food and Drug Administration (FDA) agreed on a zero tolerance in foods not intended for further heat treatment (11) due to the ability of *Listeria monocytogenes* to grow at refrigeration temperatures and because the infective dose for pregnant women and the immunocompromised are unknown. However, the "zero tolerance" policy is being challenged by facts such as that in a population as large as that of the U.S., every year there are only few cases of listeriosis, the majority of these cases involve immunocompromised people; the ubiquitousness of the organism; and that outbreaks and sporadic cases implicate certain foods such as soft cheeses, undercooked chicken and poorly reheated hot dogs. Therefore, the FDA is presently considering possible changes to the current policy. According to Madden (7) these changes would include: "stratification of products by known, established risks; maintenance of a strict standard for those products which have received a listericidal process or which would support the reproduction of the microbe to higher numbers; and, the allowance for low numbers of *L. monocytogenes* in foods demonstrated to be listericidal, but in which the microbe is found in low numbers due to the unavoidable presence of the microbe in the raw food". The presence of *Listeria* spp. in the processing environment has been widely reported (1,5,15,16). An in-line survey of establishments processing crab meat conducted by Wentz et al. (18) showed the finished product microbial quality correlates very well with post cooking

handling practices. Ryu et al. (14) isolated *L. monocytogenes* in 6.1% of the samples of fish and fish products, including ready-to-eat foods, in a study of retail foods in Japan. Levels of *L. monocytogenes* were estimated to be less than 50 cfu/g.

Listeria can grow and reproduce at 1.1 °C and thus it is considered a psychrotrophic organism (12). In a study by Wilkins et al. (18), *L. monocytogenes* grew at temperatures between 3 and 45 °C. The duration of the lag phase increased with decreasing temperature and 3.3 °C was estimated as the minimum temperature for growth. Junttila et al. (6) reported the minimum growth temperature for *L. monocytogenes* to be 1.1 °C, and also observed that *L. monocytogenes* pathogenic strains grew at about 0.6 °C lower than non-pathogenic strains. To limit the growth of organisms of public health concern the Food Code 1993 - Chapter 3 subpart 501 - specifies that "potentially hazardous food shall be maintained at 5 °C (41°F) or below" (3).

In this study, the occurrence of *Listeria* in freshly cooked and picked blue crab (*Callinectes sapidus*) meat was determined and the growth and behavior of *L. monocytogenes* in pasteurized crab meat stored at refrigeration temperature was observed.

MATERIALS AND METHODS

Enumeration study

One hundred twenty six samples of fresh (cooked for 10 min at 121 °C, 15 psi) blue crab (*Callinectes sapidus*) meat were collected from different processing facilities in the Chesapeake Bay region. The samples were maintained in ice and shipped overnight to the Department of Food Science and Technology at Virginia Polytechnic Institute & State University, where they were analyzed for the presence of *Listeria* spp. upon arrival. The analysis was based on the USDA method for isolation of *L. monocytogenes* developed by McClain and Lee (8,9,10) and described by Pusch (13). Twenty-five gram samples were diluted in 225 ml of Fraser broth (FB) (Difco 0219 + 0211, Detroit, MI). Enumeration was accomplished on duplicate samples from this dilution using FB in a 5-tube most-probable-number (MPN) method; 10 ml of the 1:10, 1:100 and 1:1000 dilutions were incubated for 24 and 48 hours at 36 °C. After incubation, the MPN index was determined by counting the number of positive tubes, those with black color due to the hydrolysis of esculin by *Listeria* spp. (4). From each positive tube, a loop of inoculum was streaked onto Modified Oxford agar (MOX) (Difco 0225 + 0218) and incubated for 24 - 48 h at 36 °C. From each MOX plate, suspected colonies (black colonies), were identified and confirmed using blood agar (Blood agar base Nº2 - Difco 0696, and 6% defibrinated sheep blood) and biochemical tests (API *Listeria* kits, BioMerieux Vitek, Inc., St. Louis, MO).

Isolated pure cultures were maintained at 4 °C in slants of Tryptic Soy Agar (Difco 0369) + 0.6% Yeast Extract (Difco 0127).

Growth of *L. monocytogenes* at refrigeration temperatures

A *L. monocytogenes* 4b strain 168 isolated from fresh blue crab meat was inoculated (less than 50 cfu/g) into commercially pasteurized crab meat and incubated at 1.1, 2.2 and 5 °C for 21 days. The storage temperatures were chosen to comply with what has been established by the FDA as the maximum temperature to store refrigerated products (5 °C) and according to the minimum temperature required for growth of *L. monocytogenes* (1.1 °C) (6). For each storage temperature 200 grams of pasteurized crab meat was inoculated with 2 ml of a 10^{-6} dilution of an 18 h culture (36 °C) of *L. monocytogenes* strain 168. The samples were placed in a 24 oz., puncture proof Whirl-pak® (Nasco, Fort Atkinson, WI) plastic bag, homogenized in a stomacher (Lab blender 400, Tekmar, Cincinnati, OH) for 2 minutes and stored at the incubation temperatures. At pre-determined intervals of 0, 8, 10, 12, 14, 16, 18, and 21 days, one 25 g sample was taken from each package for each storage temperature and analyzed in duplicate for *L. monocytogenes*. Enumeration of *L. monocytogenes* was done with blood agar (Blood agar base Nº2 - Difco 0696 and 6% defibrinated sheep blood) containing colistin sulfate (10 mg/L) and moxalactam (20 mg/L). Each experiment was repeated six times.

RESULTS AND DISCUSSION

Listeria was found in thirteen (10%) of the 126 samples of freshly cooked and picked blue crab meat analyzed to determine the occurrence of *Listeria* spp. (Table 1). Ten samples were positive for *L. monocytogenes* and 3 samples positive for *L. innocua*. The levels of *Listeria* were in all but one sample less than 100 /g, for the one exception the MPN index was 1,100 /g.

Since the average level of *L. monocytogenes* in positive samples was approximately 50 /g and the reported levels in which it is found in seafood (14) is low, the inoculum of *L. monocytogenes* was prepared to obtain less than 50 cfu/g. In the absence of competing microflora, *L. monocytogenes* grew more rapidly as the storage temperature increased (Figure 1). The generation time (13) was 68.7, 31.4, and 21.8 hours at 1.1, 2.2 and 5 °C, respectively. The ability of *L. monocytogenes* to grow at refrigeration temperature has been well documented (6,17) and the results of this study confirm such behavior. The results of this study suggest that fresh blue crab meat be maintained at or below a temperatures of 1.1 °C if growth of *L. monocytogenes* is to be suppressed. This being the temperature at which minimum growth was observed during the 15 days maximum anticipated shelf life) (2). The next logical step in understanding the behavior of *L. monocytogenes* under normal commercial storage conditions would be a study to determine the growth characteristics of the organism in fresh blue crab meat in the presence of naturally occurring microflora.

Table 1. Natural occurrence of *Listeria* in fresh blue crab (*Callinectes sapidus*) meat.

Sample Nº	MPN/g(*)	*Listeria* spp.
1	39	*L. monocytogenes*
2	93	*L. monocytogenes*
3	43	*L. monocytogenes*
4	23	*L. monocytogenes*
5	9	*L. monocytogenes*
6	93	*L. monocytogenes*
7	93	*L. monocytogenes*
8	43	*L. monocytogenes*
9	1,100	*L. monocytogenes*
10	23	*L. monocytogenes*
11	93	*L.innocua*
12	43	*L.innocua*
13	93	*L.innocua*

(*) Values represent the average of MPN/g index from duplicate analysis.

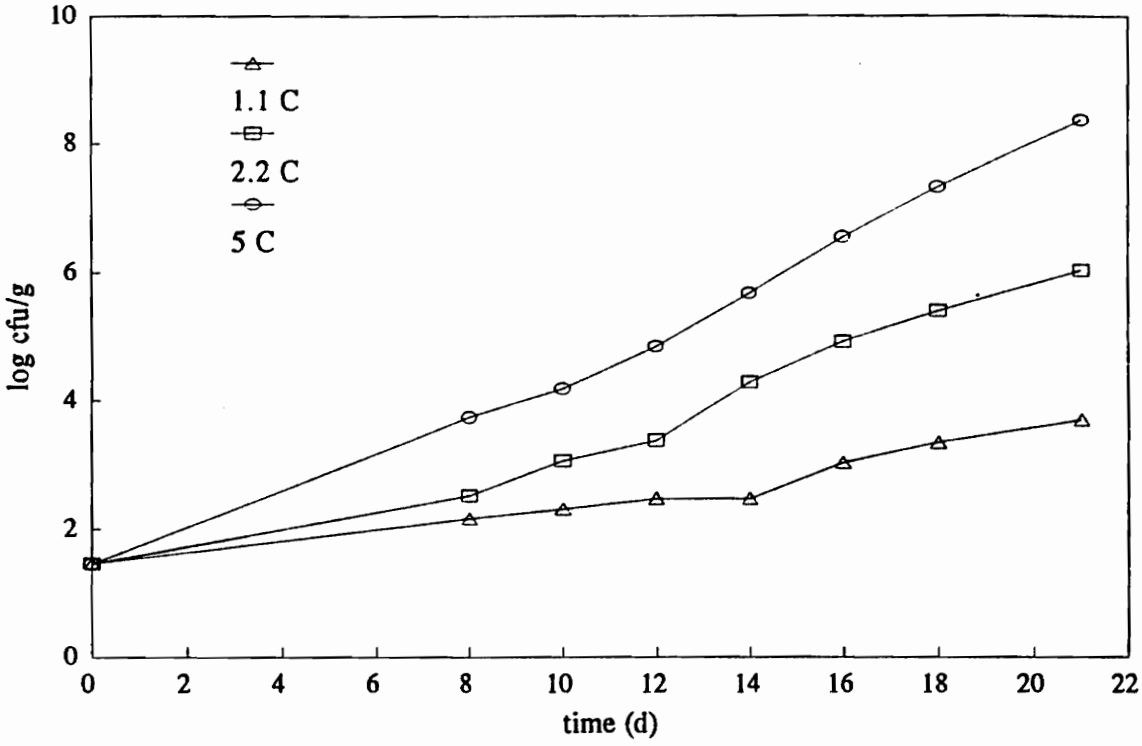

Figure 1. Growth of *L. monocytogenes* in pasteurized blue crab meat at refrigeration temperature; 1.1°C (-△-), 2.2°C (-□-) and 5°C (- o -). Each point represents the geometric mean of 6 determinations.

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APPENDIX

Linear regression was determined for each growth curve. R^2 of 0.95, 0.96 and 0.99 were obtained for curves at 1.1, 2.2 and 5 °C, respectively. Generation time from the regression lines were calculated according to (13):

$$G = \frac{t}{3.3\log(\frac{b}{B})}$$

where G = generation time,

t = time interval between measurements of the number of cells in the population at one moment in the log phase (B) and then again at a later point in time (b),

B = initial population,

b = population after time t

SECTION III: SIMULTANEOUS GROWTH OF *LISTERIA MONOCYTOGENES* AND *LISTERIA INNOCUA* IN BLUE CRAB (*CALLINECTES SAPIDUS*) MEAT.

(Paper accepted for publication in the Journal of Food Protection)

ABSTRACT

In most instances, when *Listeria monocytogenes* has been isolated in blue crab (*Callinectes sapidus*) processing plants, *Listeria innocua* has also been detected. This has led to the belief among blue crab processors that there is some association in the growth of these two *Listeria* spp., suggesting suppression of *L. monocytogenes* by *L. innocua*. In this study the growth of a *L. monocytogenes* (serotype 4b) and a *L. innocua* strain, both isolated from crab meat, was observed by inoculating pasteurized crab meat with different proportions of standardized inoculum of the two microorganisms, individually and simultaneously. Parallel samples inoculated with each microorganism, and with a mixture of both in different proportions, were incubated at two different temperatures (4 °C and 36 °C) for up to 15 days. Mixture ratios of *L. monocytogenes* to *L. innocua* of 1:1, 9:1 and 1:9 were used to inoculate the crab meat. At each growth interval (0, 24, 36, 48, 72, 96 hrs and 7, 10, and 15 days), 25 grams samples were taken and enumerated for each microorganism. Blood agar, with colistin sulfate (10 mg/L) and moxalactam (20 mg/L), was used to enumerate and differentiate between the two species. No significant differences

($p > 0.05$) in the growth of either microorganism separately or when in presence of the other, at any inoculation level and temperature, was observed.

INTRODUCTION

Over the past years the impact of *Listeria monocytogenes* on the food industry has been greater than any other food-borne pathogen (3). *Listeria* is widely distributed in the environment and can be isolated from soil, sewage, plants and water. In the food industry it is usually found in cool, damp environments on both food-contact and non-food-contact surfaces, and has been isolated from conveyors, floors and drains (13). The incidence of *Listeria spp.* in seafood has been reported to be approximately 28% (1), having been found in commodities such as raw and cooked shrimp, crab meat (6,7), lobster tails (7), squid, finfish, smoked fish, surimi analogs (9), cooked or smoked mussels (8) and convenience foods (4). *Listeria spp.* have also been reported in frozen seafood samples (14). In this study, 35 out of 57 frozen seafood samples contained *Listeria* spp., and of those, 15 contained *L. monocytogenes*. *L. monocytogenes* was found in samples of raw, cooked and peeled shrimp, cooked crab meat, raw lobster tails, langostinos, scallops, squid, and surimi-based imitation seafoods. Ryu et al. (12), in a study of retail foods in Japan, isolated *L. monocytogenes* in 6.1% of the samples of fish and fish products, including ready-to-eat foods. Levels of *L. monocytogenes* were estimated to be less than 50 cfu/g. Competition between *L. innocua* and *L. monocytogenes* has been suggested; however, a study performed by Petran and Swanson (11) indicates no significant difference in the growth of both species in non-selective media and food systems, and a higher population of *L. innocua* when using selective media. *Listeria innocua* has been

found more frequently than *L. monocytogenes* in blue crab (*Callinectes sapidus*) processing plants (4). This has resulted in many processors believing that there is some association between the growth of these two organisms. It is generally believed by the processors, that *L. innocua* can suppress the growth of *L. monocytogenes*. Consequently, many processors are reluctant to develop and implement comprehensive cleaning and sanitation programs since control over *L. innocua* may result in a lack of control over *L. monocytogenes*. Therefore, it is of interest to determine the growth characteristics of these two organisms when present in the same food system. This research project studied the growth and population maxima of these two *Listeria* species when grown separately and simultaneously in crab meat inoculated at different levels.

MATERIALS & METHODS

The inoculum was prepared from a strain of *L. monocytogenes* (serotype 4b) and of *L. innocua,* both isolated from fresh blue crab (*Callinectes sapidus*) meat. Pure cultures of each organism were maintained at 4 °C in slants of Tryptic soy agar (Difco 0369, Detroit, MI) + 0.6% yeast extract (Difco 0127) (TSA+0.6% YE). An 18 h culture of *Listeria* in Tryptic soy broth (TSB) (Difco 0370), incubated at 36 °C, yielded 10^9 cfu/ml. Crab meat was inoculated at two levels: between 1,000 and 10,000 cfu/g; and less than 50 cfu/g. For the inoculation level between 1,000 and 10,000 cfu/g, 2.5 ml of inoculum from the 10^{-4} dilution of the pure cultures were

used, and when used in the mixture, the volume (2.5 ml) also contained the desired proportion of each organism. The inoculation level of less than 50 cfu/g was obtained by using 2.5 ml of the 10^{-6} dilution of the pure cultures and mixture. For each experiment, three samples (250 g each) of commercially pasteurized blue crab meat were inoculated in parallel. One of the samples received pure *L. monocytogenes*, another pure *L. innocua* and the third, the required amount of each organism to obtain the desired proportion in the meat. The inoculated samples were stomached for 2 minutes and incubated at 4 °C for up to 10 days or 36 °C for up to 4 days. Studies of the simultaneous growth of these organisms utilized a mixture of *L. monocytogenes* to *L. innocua* with ratios of approximately 1:1, 1:9 and 9:1. At growth intervals of 0, 24, 36, 48, 72, 96 hours and 7 and 10 days, 25 gram samples were analyzed in duplicate to determine the quantity of each microorganism. However, when the inoculum was less than 50 cfu/g, the incubation time was extended to 15 days. Blood agar prepared from Blood agar base N^0 2 (Difco 0696) and 6% defibrinated sheep blood, with colistin sulfate (10 mg/L) and moxalactam (20 mg/L), was used to enumerate and differentiate the two species (5). Based in their hemolytic behavior, *L. monocytogenes* produces hemolysis whereas *L. innocua* does not, differentiation was possible in mixed cultures. Each experiment was repeated three times. A two sample t-test was used to statistically analyze for significant differences between the geometric means of the population maxima of the two species when grown in the presence of each other and individually (10).

RESULTS

At both incubation temperatures (4 °C and 36 °C) no significant differences ($p > 0.05$) in the population maxima of either microorganism grown individually and in the presence of the other was observed. No difference was observed for inoculation levels between 1,000 and 10,000 cfu/g and less than 50 cfu/g, and for the mixture ratios of 1:1; 1:9 and 9:1. For inoculation levels between 1,000 and 10,000 cfu/g incubated at 36 °C, a peak concentration of 6 to 7 $\log_{10}$ cfu/g was reached after 24 hrs of incubation. Figure 1 shows the growth curves for each organism individually and in mixture when the ratio of *L. innocua* to *L. monocytogenes* was 9:1. When *L. innocua* was inoculated in the same (1:1 ratio) proportion as *L. monocytogenes*, a population maxima between 6 and 8 $\log_{10}$ cfu/g was reached after 24 hrs of incubation (Figure 2). Simultaneous inoculation of crab meat with *L. monocytogenes* in excess of *L. innocua* in a 9:1 ratio, resulted in a population maxima between 6 and 9 $\log_{10}$ and no significant difference was observed between the growth of each organism separately or in the presence of the other. At 4 °C, an approximate 1 $\log_{10}$ increase in growth occurred for both organisms during the first 100 hours of incubation with a 2 $\log_{10}$ increase in growth throughout the remainder of the test period (10 days) (Figure 3).

For inoculation levels of less than 50 cfu/g, a population maxima between 8 and 9 $\log_{10}$ cfu/g was reached after 48 hours of incubation at 36 °C (Figure 4). At 4 °C, no growth was observed during the first 7 days of incubation, therefore the samples were incubated up to 15 days, the anticipated maximum shelf life of fresh blue crab meat (2).

After 7 days at 4 °C, *Listeria monocytogenes* was detected before *Listeria innocua,* even when the initial inoculum contained an excess of *L. innocua* (9:1 proportion). Even though the growth rate was depressed, the population increased approximately 4 $\log_{10}$ after 15 days of incubation (Figure 5). No significant difference ($p > 0.05$) in the growth of either organism grown in the presence of the other was observed for any of the treatments. There was no significant variation ($p > 0.05$) in the populations of either *L. monocytogenes* or *L. innocua* when grown individually and simultaneously during all the incubation times.

DISCUSSION

No significant differences in the growth of either *L. monocytogenes* or *L. innocua* were observed when these organisms were inoculated individually and simultaneously in pasteurized crab meat at inocula levels above 1,000 cfu/g and incubated at either 4 °C or 36 °C for up to 10 days. When lower inocula levels were used, less than 50 cfu/g, no significant differences were observed at 36 °C. At 4 °C, no growth was detected for either organism during the first 7 days of incubation, but a 2 to 4 $\log_{10}$ increase in growth was observed after 15 days. At 4 °C, *L. monocytogenes* was observed to have a faster growth rate than *L. innocua* but the difference in growth was not significant ($p > 0.05$). The results of this study do not support the belief among blue crab processors that *L. monocytogenes* is suppressed by *L. innocua.* Therefore, the importance of an appropriate cleaning and sanitation program should be emphasized in the industry. According to the

results obtained in this study, there was no significant association between the growth of these two organisms when present simultaneously in crab meat.

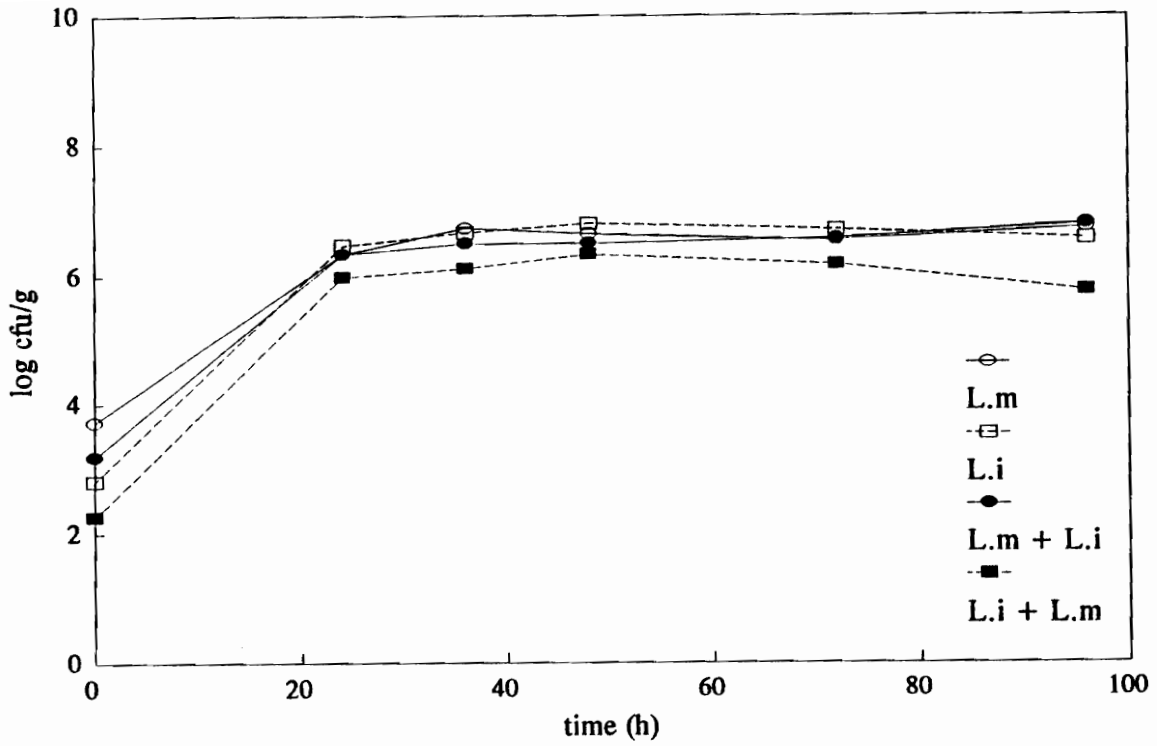

Figure 1. Growth at 36 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with 10^3 to 10^4 cfu/g. *L. innocua* in excess of *L. monocytogenes* in a 9:1 proportion. Each point represents the geometric mean of three repetitions.

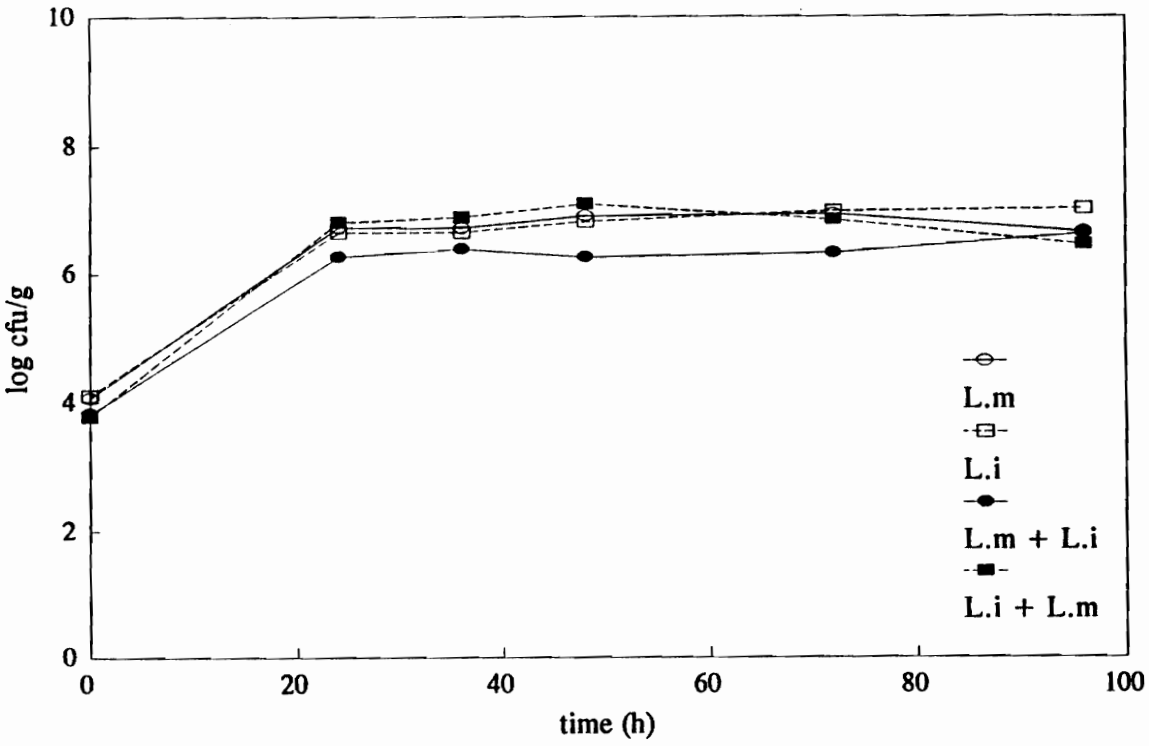

Figure 2. Growth at 36 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with 10^3 to 10^4 cfu/g. *L. innocua* in same proportion as *L. monocytogenes* (1:1 ratio). Each point represents the geometric mean of three repetitions.

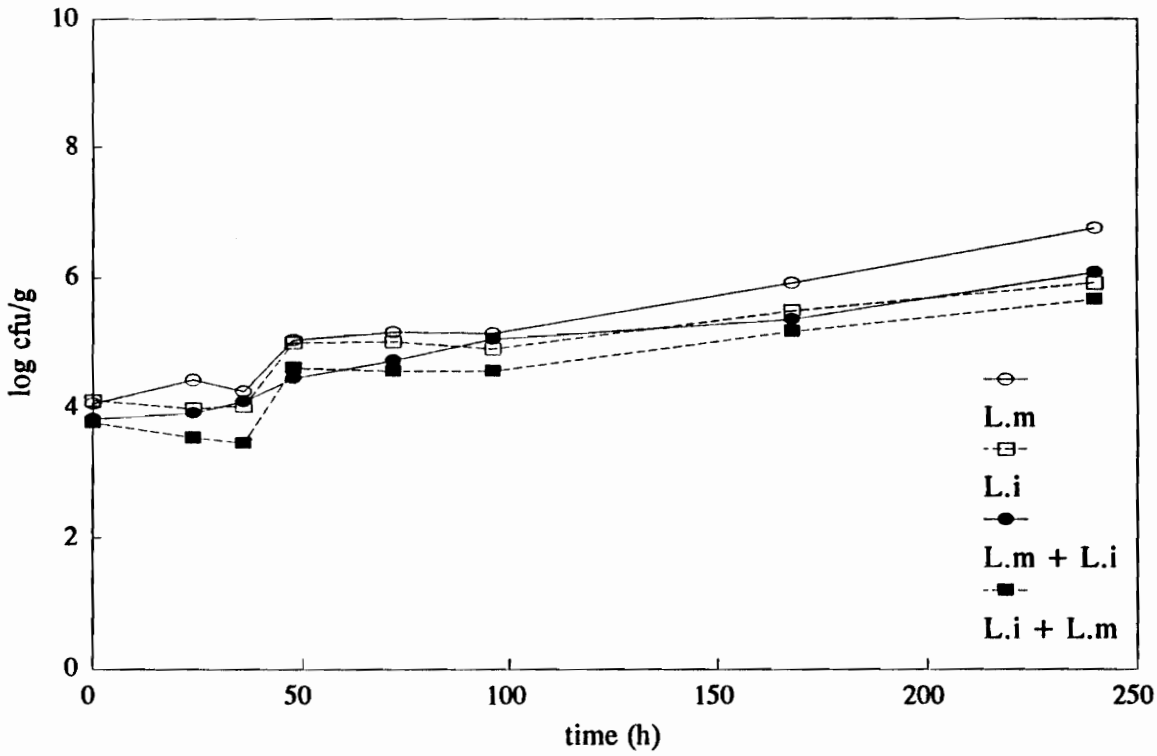

Figure 3. Growth at 4 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with 10^3 to 10^4 cfu/g with same proportion of both microorganisms (1:1 mixture). Each point represents the geometric mean of three repetitions.

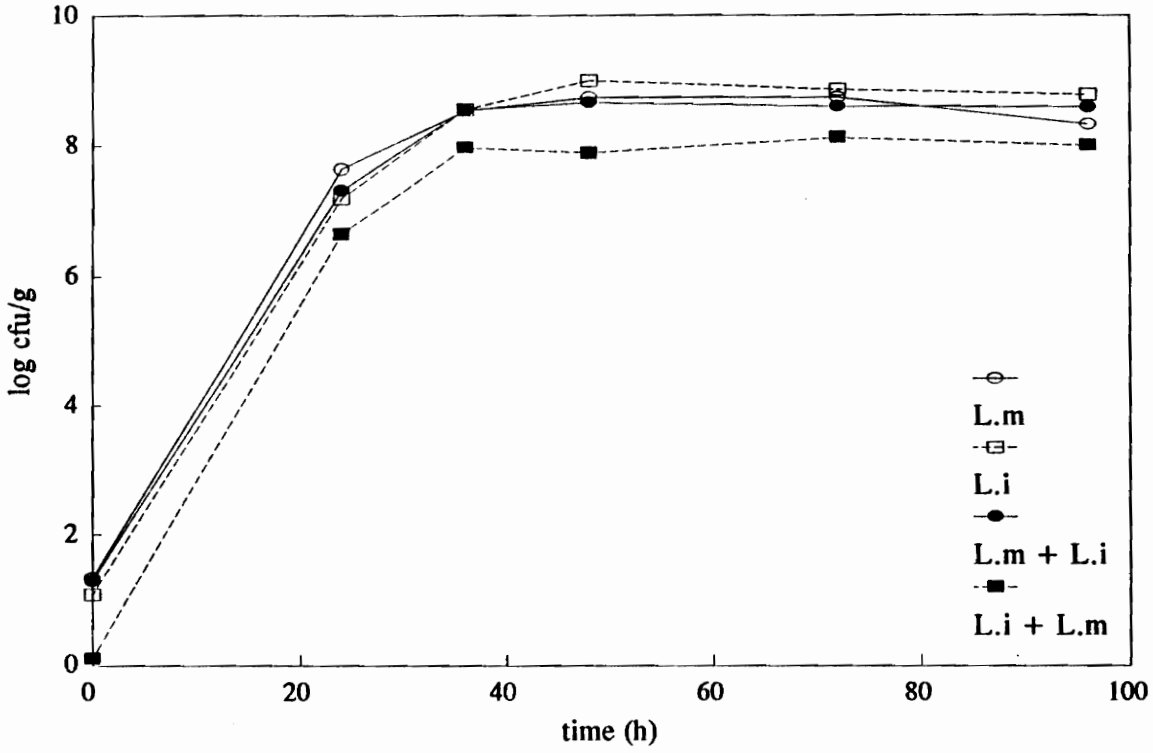

Figure 4. Growth at 36 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with less than 50 cfu/g. *L. monocytogenes* in excess of *L. innocua* in a 9:1 proportion. Each point represents the geometric mean of three repetitions.

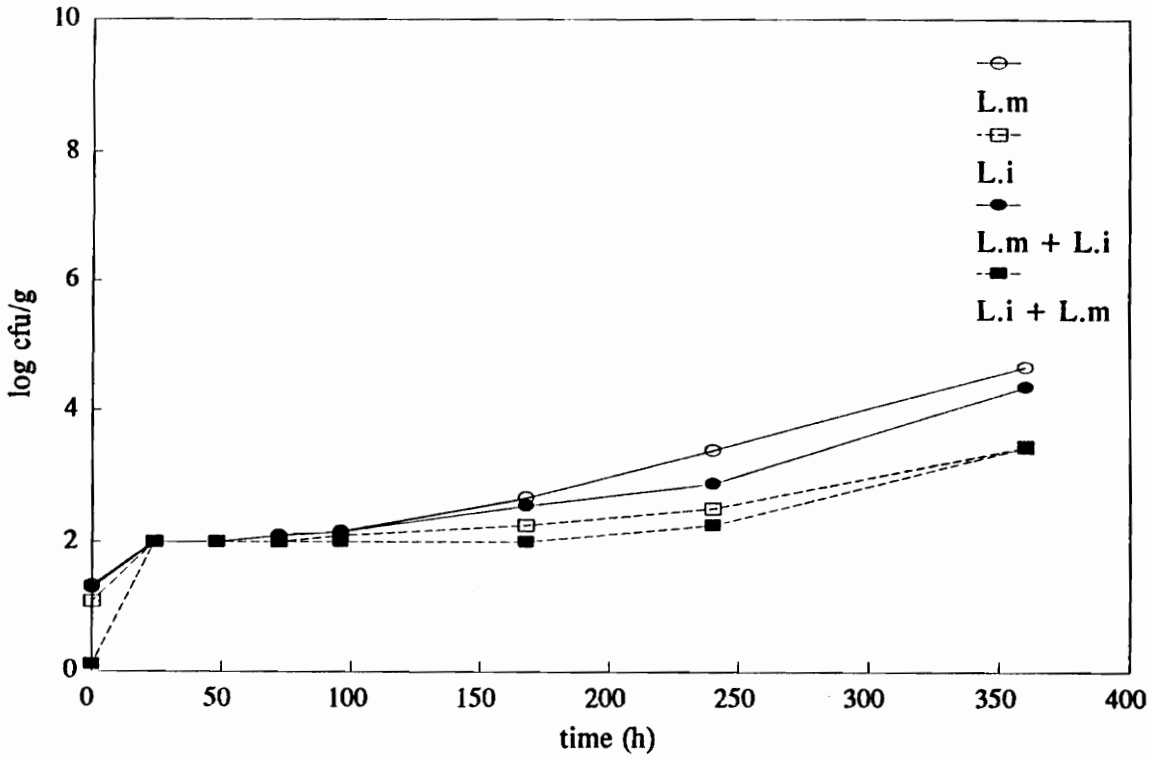

Figure 5. Growth at 4 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with less than 50 cfu/g. *L.monocytogenes* in excess of *L.innocua* in a 9:1 proportion. Each point represents the geometric mean of three repetitions.

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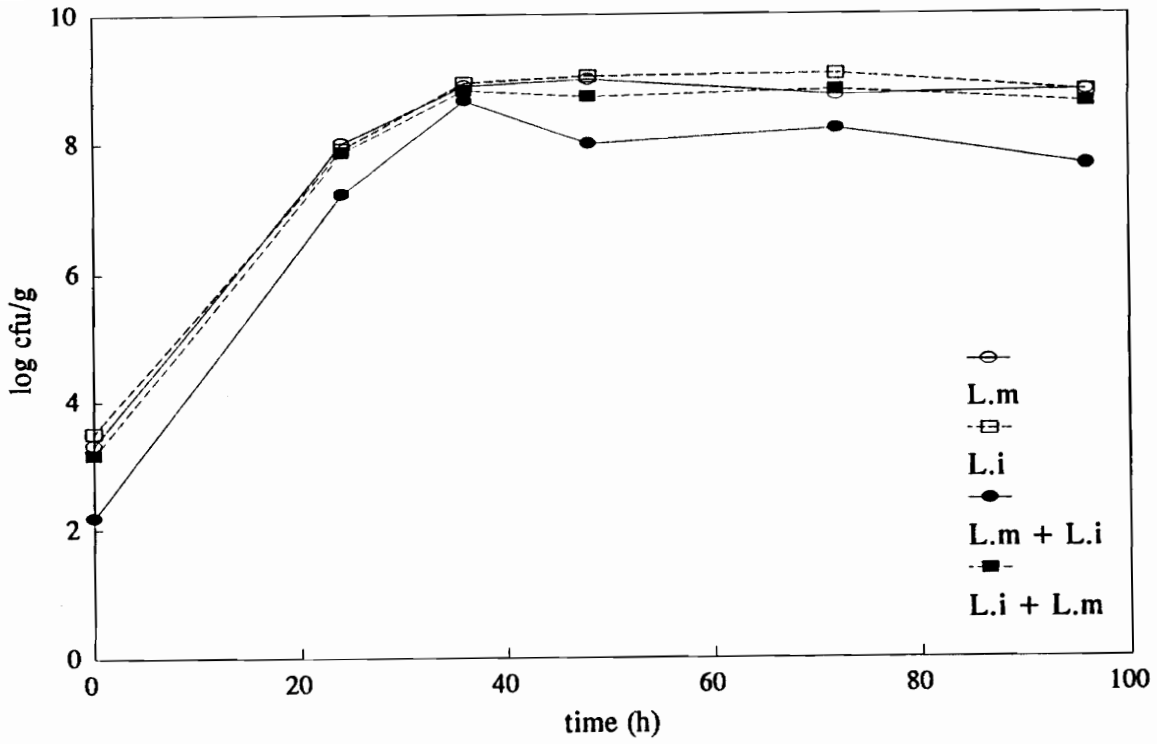
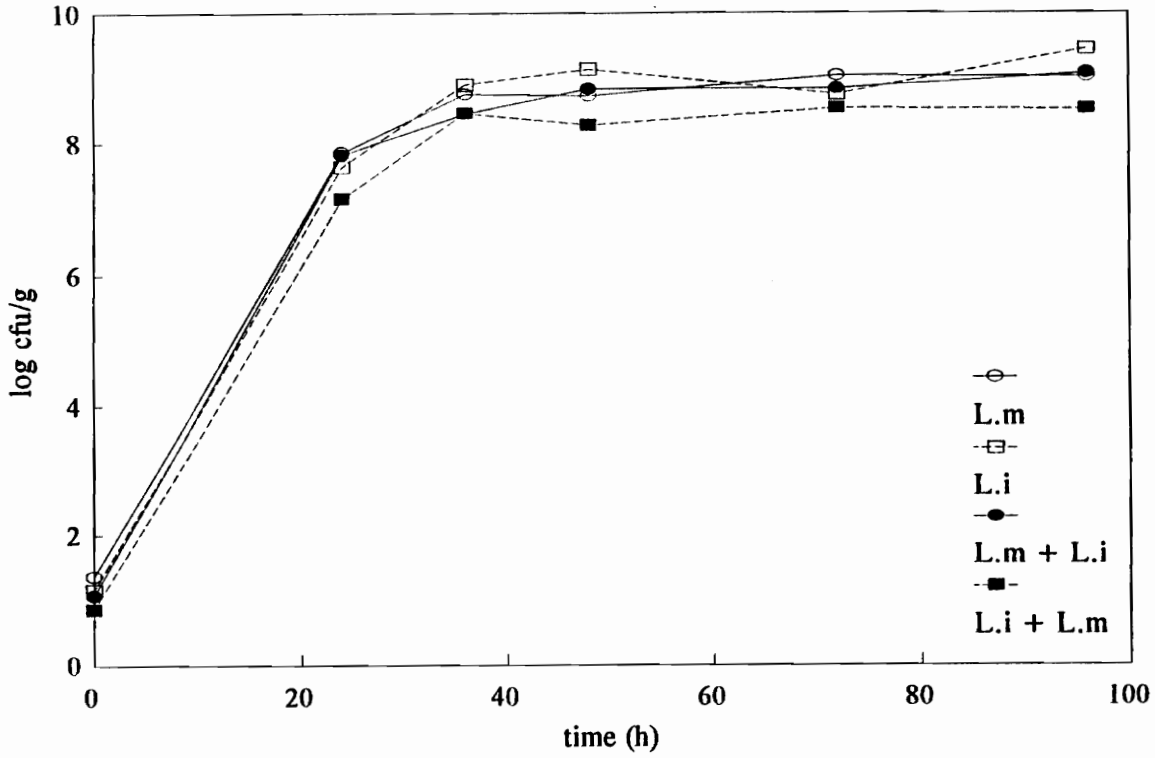
APPENDICES

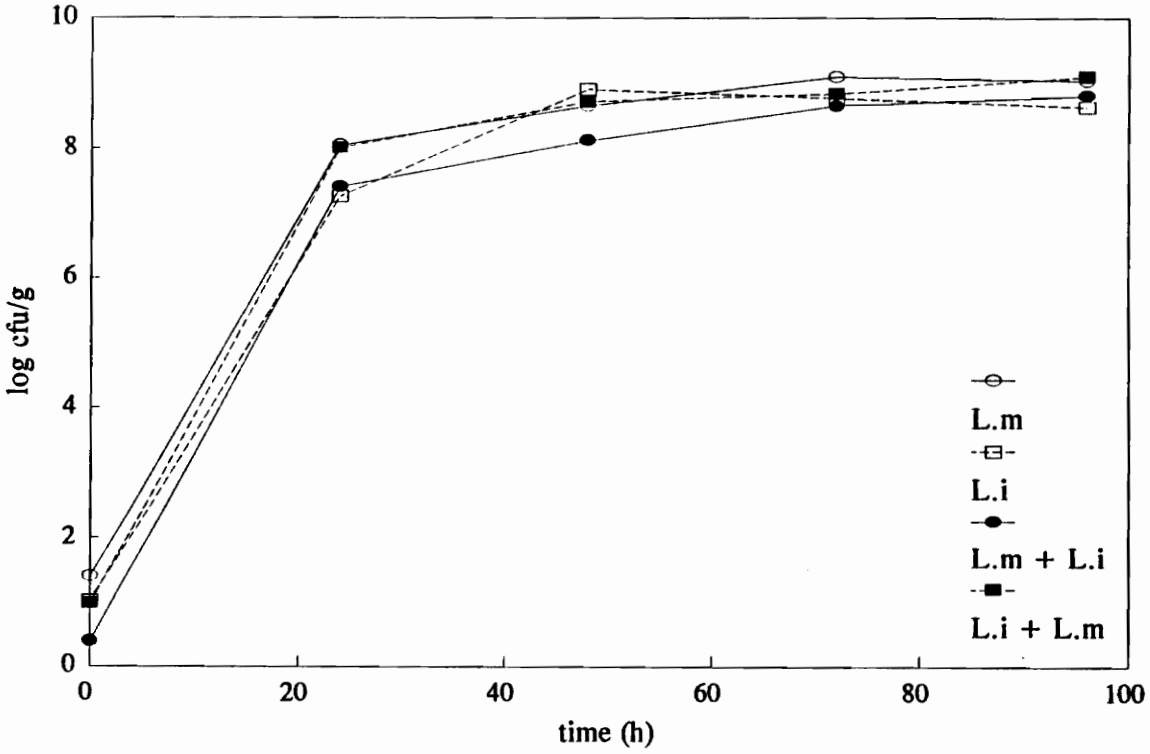
Appendix A through E shows graphs for the simultaneous growth of *Listeria monocytogenes* and *Listeria innocua*, other than those included in the manuscript.



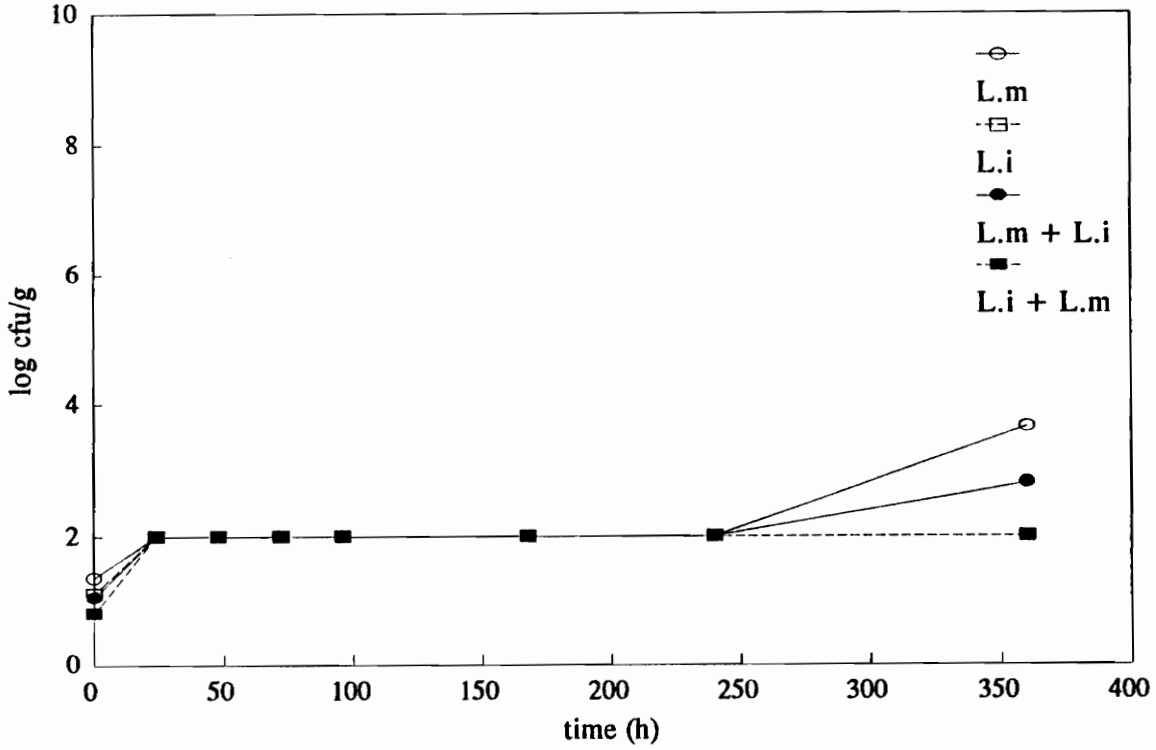
Appendix A. Growth at 36 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with 10^3 to 10^4 cfu/g. *L. innocua* in excess of *L. monocytogenes* in a 9:1 proportion. Each point represents the geometric mean of three repetitions.



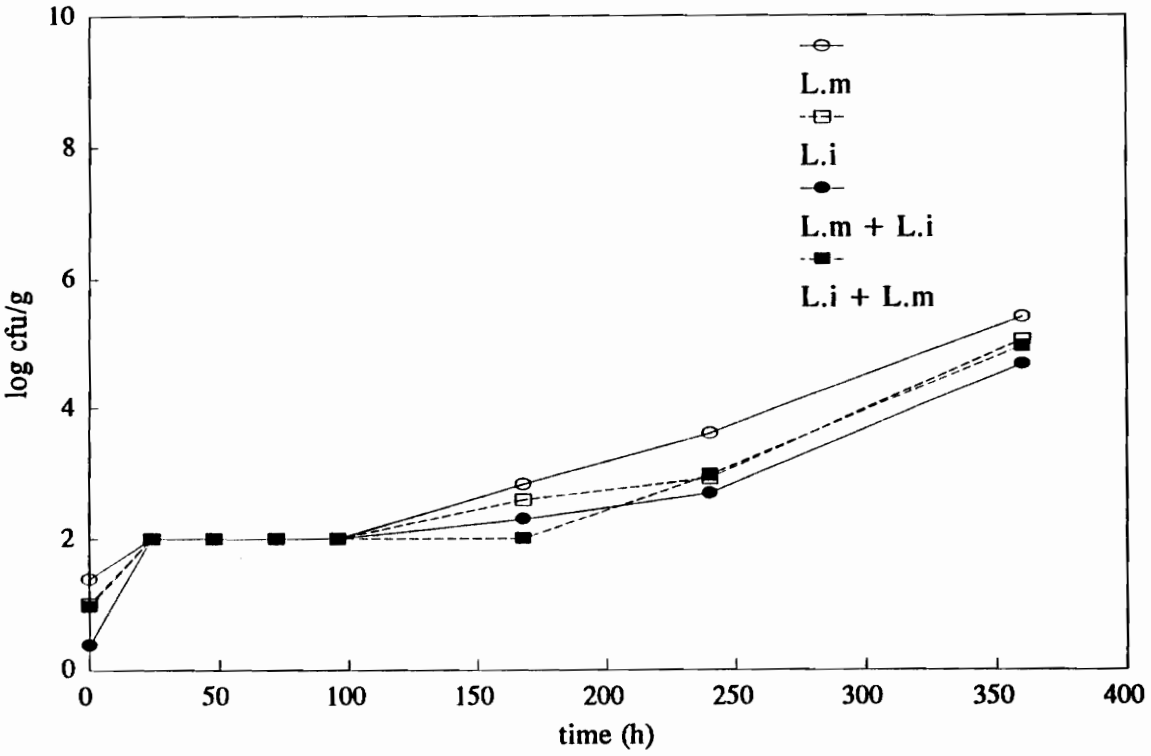
Appendix B. Growth at 36 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with less than 50 cfu/g. *L. innocua* in same proportion as *L. monocytogenes* (1:1 ratio). Each point represents the geometric mean of three repetitions.



Appendix C. Growth at 36 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with less than 50 cfu/g. *L. innocua* in excess of *L. monocytogenes* in a 9:1 proportion. Each point represents the geometric mean of three repetitions.



Appendix D. Growth at 4 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with less than 50 cfu/g with same proportion of both microorganisms (1:1 mixture). Each point represents the geometric mean of three repetitions.



Appendix E. Growth at 4 °C of *L. monocytogenes* (L.m) and *L. innocua* (L.i) individually and simultaneously (L.m+L.i, *L. monocytogenes* in presence of *L. innocua*; L.i+L.m, *L. innocua* in presence of *L. monocytogenes*) inoculated in crab meat with less than 50 cfu/g. *L. innocua* in excess of *L. monocytogenes* in a 9:1 proportion. Each point represents the geometric mean of three repetitions.

SECTION IV: GROWTH OF *LISTERIA MONOCYTOGENES* IN FRESH BLUE CRAB (*CALLINECTES SAPIDUS*) MEAT IN THE PRESENCE OF NATURALLY OCCURRING MICROFLORA DURING REFRIGERATED STORAGE

(Paper formatted for submission to the Journal of Food Science)

ABSTRACT

The seafood industry faces a challenge regarding the zero tolerance for *Listeria monocytogenes* in ready-to-eat products. *Listeria* is ubiquitous in nature and its presence can be expected in the processing environment. The growth of *L. monocytogenes* in the presence of naturally occurring levels of microflora was studied in freshly cooked and picked blue crab meat during refrigerated storage. Fresh meat was inoculated with *L. monocytogenes* at less than 50 cfu/g and incubated at 1.1, 2.2 and 5 °C for 21 days. Aerobic, anaerobic, psychrotrophic, and lactic acid bacteria and *L. monocytogenes* counts were done after 0, 1, 2, 5, 7, 10, 13, 15, 17, 19 and 21 days. The crab meat was classified according to the initial aerobic plate count (APC) as $<15{,}000$ cfu/g, between 15,000 - 60,000 cfu/g and between 60,000 - 100,000 /g. Growth curves were obtained for each group with three replications per storage temperature. Regardless of the initial APC, and for all storage temperatures, psychrotrophic counts were the highest, reaching a population of 10^9 to 10^{10} /g after 7 - 10 days of storage. Aerobic and anaerobic counts reached a maximum of after 10^8

to 10^9 /g after 7 -10 days of storage. Lactic acid bacteria had a decreased growth rate, reaching 10^7 to 10^8 /g in population maxima after 10 - 13 days of storage. *L. monocytogenes* increased to 10^2 to 10^3 /g and remained constant throughout the storage period at 1.1 °C and 2.2 °C with a slight increase in population maxima (10^4 to 10^5 /g) at the 5 °C storage temperature. The growth rate of *L. monocytogenes* was significantly suppressed in the presence of the natural crab microflora as the refrigeration temperature decreased.

INTRODUCTION

The psychrotrophic nature of *L. monocytogenes* has been well established (Junttila et al., 1988; National Advisory Committee on Microbiological Criteria for Foods, 1991; Walker and Stringer, 1987). Its presence is widespread in nature and in the processing environments (McCarthy, 1990; Slade, 1992; Tiwari and Aldenrath, 1990; Weis and Seeliger, 1975). Although most individuals frequently ingest the bacterium without experiencing ill effects (Farber, 1991), mortality in predisposed groups may exceed 25 percent (Health and Welfare Canada, 1988). Therefore, as the infective dose for pregnant women and the immunocompromised are unknown, the U.S. Food and Drug Administration (F.D.A), the United States Department of Agriculture (U.S.D.A) and the Center for Disease Control (C.D.C) have agreed on a "zero tolerance" for *L. monocytogenes* in foods that will not undergo further heat treatment (McNamara, 1994). Commodities found to contain *L. monocytogenes* include raw and cooked shrimp, crab meat, lobster tails, squid, finfish, and surimi analogs (Farber, 1991; National Advisory Committee on Microbiological Criteria for Foods, 1991). Although, estimated to be present in low levels (less than 50 cfu/g), *L. monocytogenes* has been isolated in 6.1% of samples of fish and fish products, including ready-to-eat foods (Ryu et al., 1992). We have observed an incidence of approximately 8% and low numbers (average 50 cfu/g) of *L. monocytogenes* in freshly cooked and picked blue crab meat (unpublished data). Other authors have reported the incidence of *Listeria* spp. in seafoods at the retail level to be

approximately 28% (Buchanan et al., 1989), and it has been detected in 61% of samples of frozen seafoods (Weagant et al., 1988). Therefore, the "zero tolerance" policy affects and may have a great impact on the seafood industry.

Competition with *L. monocytogenes* by specific bacteria rather than total bacterial numbers has been suggested by Tran et al.(1990). Bacteria known to be antagonistic to *Listeria* include *Enterococcus faecalis* (Arihara et al., 1991; Dallas and Hitchins, 1993), *Enterococcus faecium* (McKay, 1990); *Corynebacteria* (Valdes-Stauber et al., 1991); and certain acid bacteria (Harris et al., 1989); pediococci (Yousef et al., 1991), *Leuconostoc gelidium* (Harding and Shaw, 1990) and *Lactococcus lactis* (Wenzel and marth, 1990). Rørvik et al. (1991) compared samples of smoked salmon of different hygienic quality and found a slightly faster growth of *L. monocytogenes* in smoked salmon with better hygienic quality when artificially inoculated with low (6 cfu/g) and high (600 cfu/g) levels of a mixture of three strains of *L. monocytogenes*.

The growth of *L. monocytogenes* in the presence of naturally occurring levels of microflora was studied in freshly cooked and picked crab meat during refrigerated storage.

MATERIALS AND METHODS

Inoculum preparation

L. monocytogenes 4b was isolated (Rawles et al., 1995) from fresh blue crab (*Callinectes sapidus*) meat and maintained at 4 °C in slants of Tryptic Soy Agar (Difco 0369, Detroit, MI) + 0.6% Yeast Extract (Difco 0127) (TSA + 0.6% YE). An 18 h (36 °C) culture of this organism in Tryptic Soy Broth (TSB) (Difco 0370) yielded approximately 10^9 cfu/ml. Freshly cooked (121.1 °C for 10 min at 15 psi) and picked crab meat was inoculated with less than 50 cfu/g of *L. monocytogenes*, to simulate the low, naturally occurring levels of this pathogen in seafood (Ryu et al., 1992). Three-hundred grams of fresh blue crab meat were placed in a sterile 24 oz., puncture proof Whirl-pak® (Nasco, Fort atkinson, WI) bag and inoculated with 3 ml of a 10^{-6} dilution of the *L. monocytogenes* culture. The inoculated samples were homogenized for 2 minutes in a stomacher (Lab blender 400, Tekmar, Cincinnati, OH). A similar procedure was used to inoculate *L. monocytogenes* in pasteurized crab meat. This growth curve was the control data for the growth of *L. monocytogenes*.

Sample preparation

Freshly cooked and picked blue crab meat was obtained from processing facilities in the Chesapeake Bay region. The samples were maintained in ice and shipped overnight to the Department of Food Science and Technology at Virginia

Polytechnic Institute and State University. Upon arrival, the samples were analyzed for the presence of *Listeria* spp., and aerobic, anaerobic, psychrotrophic, and lactic acid bacteria counts were obtained. These were considered to be the initial counts (time 0). Three sets of 300 g each of crab meat were inoculated with the 18 h culture of *L. monocytogenes* as described above, and incubated at 1.1, 2.2 and 5 °C during a 21 day period in the same plastic bags the samples were homogenized. These temperatures were chosen to comply with recommendations for storage of refrigerated food products (Food Code, 1993) and to fall within the minimum growth range of *L. monocytogenes* (Junttila et al., 1988; National Advisory Committee on Microbiological Criteria for Foods, 1991; Wilkins et al., 1972). At 0, 1, 2, 5, 7, 10, 13, 15, 17, 19, and 21 days, 25 g sub-samples were analyzed, in duplicate, to determine the numbers of *L. monocytogenes*, aerobic, anaerobic, psychrotrophic and lactic acid bacteria, present at each storage temperature. According to the initial aerobic plate counts (APCs), the experiments were assigned to one of three categories: APC $< 15{,}000$ cfu/g, APC between 15,000 and 60,000 cfu/g, and APC between 60,000 and 100,000 cfu/g. The higher bracket was determined to be in compliance with the action levels established by the Virginia Health Department for fresh crab meat. The experiment was replicated to obtain three measurements for each set of conditions. Growth curves were obtained by plotting the geometric mean of the three experiments for each set of conditions.

Microflora enumeration

Upon arrival, the freshly cooked and picked blue crab meat was analyzed for the presence of *Listeria* spp. by a 5-tube most probable number (MPN) procedure (Pusch, 1989). Twenty-five grams of sample were diluted in 225 ml of Fraser Broth (FB) (Difco 0219 + Difco 0211). Ten ml of the 10^{-1}, 10^{-2}, and 10^{-3} dilution were incubated in duplicate, for 24 and 48 h at 36 °C. Samples positive for *Listeria* spp. were discarded. For the study, initially (time 0) and at each time interval, 25 g sub-samples were diluted in 225 ml of Peptone water 0.1% (Difco 0118). From this dilution, *L. monocytogenes*, aerobic, anaerobic, psychrotrophic, and lactic acid bacteria plate counts were performed. *L. monocytogenes* was enumerated by surface plating dilutions in blood agar (Blood Agar Base Nº2 - Difco 0696 and 6% defibrinated sheep blood) containing colistin sulfate (10 mg/L) and moxalactam (20 mg/L) and incubating these plates for 24 - 48 h at 36 °C. *L. monocytogenes* was identified by the hemolytic reaction in blood agar and further confirmed in Modified Oxford Agar (MOX) (Difco 0225 + Difco 0218) and by biochemical tests (API *Listeria* kits, BioMerieux Vitek, Inc., St. Louis, MO). Aerobic plate counts (APCs) were obtained by surface plating the appropriate sample dilution on Tryptic Soy Agar (Difco 0369) and incubating for 48 h at 36 °C. Anaerobic plate counts (AB) were obtained by surface plating the adequate sample dilution on TSA and incubating anaerobically for 48 h. Psychrotrophic bacteria (PB) were enumerated by surface plating on TSA and incubation at 21 °C for 2 and 6 days. Enumeration of lactic acid bacteria (LA) was

performed anaerobically with surface plates of MRS agar (BBL 95617, Becton Dickinson & Co., Franklin Lake, NJ) which were incubated at 36 °C for 48 h. Anaerobiosis was obtained by using the BBL Gaspak 100 Jar System (jar and GasPak plus disposable generator envelopes - BBL 71040).

Statistical Analysis

A two-way Analysis of Variance (ANOVA) with a 5% level of confidence was used to determine significance in the observed differences (Norman and Streiner, 1994).

RESULTS

Regardless of the initial aerobic plate count (APC) and at all the sample incubation temperatures examined (1.1, 2.2 and 5 °C), the psychrotrophic bacteria had the highest growth rate. Growth levels of 10^9 - 10^{10} /g were reached within 7 - 10 days of storage (Figures 1 - 4). The growth pattern of aerobic and anaerobic bacteria was very similar, reaching a maximum in population between 10^8 - 10^9 /g within 7 - 10 days of storage. Lactic acid bacteria had a slower growth rate, reaching a maximum in population between 10^6 and 10^7 /g within 10 - 13 days of storage. There was very little growth of *L. monocytogenes* throughout the 21 days of storage at 1.1 °C and 2.2 °C (approx. 1 $\log_{10}$ cycle increase). Similar results were reported for precooked crawfish tail meat stored at 0 °C in which an increase of less than 1

$\log_{10}$ in the population of *L. monocytogenes* was observed during 20 d of storage (Dorsa et al., 1993). Harrison et al. (1991) reported no increase in the population of *L. monocytogenes* on fish and shrimp samples held on ice for 21 days. During storage at 5 °C (Figures 2 and 4), a higher growth rate in the population of *L. monocytogenes* was observed, reaching a population of 10^4 to 10^5 /g within 10 days of storage. Wang and Shelef (1992) reported an increase, after remaining practically unchanged for 10 days, in the numbers of *L. monocytogenes* inoculated onto raw fish and stored at 5 °C.

A significant ($p \leq 0.05$) effect of the microflora in the growth of *L. monocytogenes* was observed after 13 (2.2 and 5 °C) and 17 (1.1 °C) days of storage (Figures 5 - 7). During the first 13 days of storage, at all refrigeration temperatures, there were no significant ($p > 0.05$) differences in the growth of *L. monocytogenes* in the presence of naturally occurring microflora when compared to the control growth curves. However, after 13 days of storage, the levels of bacterial growth were at their maximum in population and the meat was inedible. The initial APCs level had no significant ($p > 0.05$) effect in the growth of *L. monocytogenes* during 21 days of refrigerated storage.

Storage temperature had a significant ($p \leq 0.05$) effect on the growth of *L. monocytogenes*. This effect was largest at the lower level of microflora tested (< 15,000 cgu/g APC) and became nonsignificant ($p > 0.05$) when the initial microflora level was between 60,000 and 100,000 cfu/g APC (Figures 8 - 10).

DISCUSSION

Hudson and Avery (1994) reported an increase of approximately 4.5 $\log_{10}$ cycles for *L. monocytogenes* inoculated on pasteurized mussel tissue and stored at 5 °C for 17 days. We observed an increase of approximately 2.5 $\log_{10}$, 4 $\log_{10}$ and 7 $\log_{10}$ in the growth of *L. monocytogenes* inoculated on pasteurized blue crab meat and stored during 21 days at 1.1, 2.2 and 5 °C respectively. Comparing the growth of *L. monocytogenes* in fresh and pasteurized crab meat, a significant ($p \leq 0.05$) suppression in growth, by the naturally occurring microflora, is observed within 13 and 21 days of refrigerated storage. At 5 °C, *L. monocytogenes* had an increase in population of 3 $\log_{10}$ (when initial APC was $< 15{,}000$ cfu/g) and of 2.5 $\log_{10}$ (when initial APC was between 60,000 and 100,000 cfu/g). However, after 13 days of storage the levels of bacterial spoilage were such that the crab meat was rendered inedible. Within initial levels of aerobic plate counts (less than 100,000 cfu/g) at which the crab meat is acceptable under the action levels applied by the Virginia Health Department, there was no significant ($p > 0.05$) difference in terms of suppression in the growth of *L. monocytogenes*. The results obtained in this study highlight the importance of maintaining the storage temperatures for fresh blue crab meat at or below 1.1 °C to suppress the possible growth of the pathogen *L. monocytogenes*.

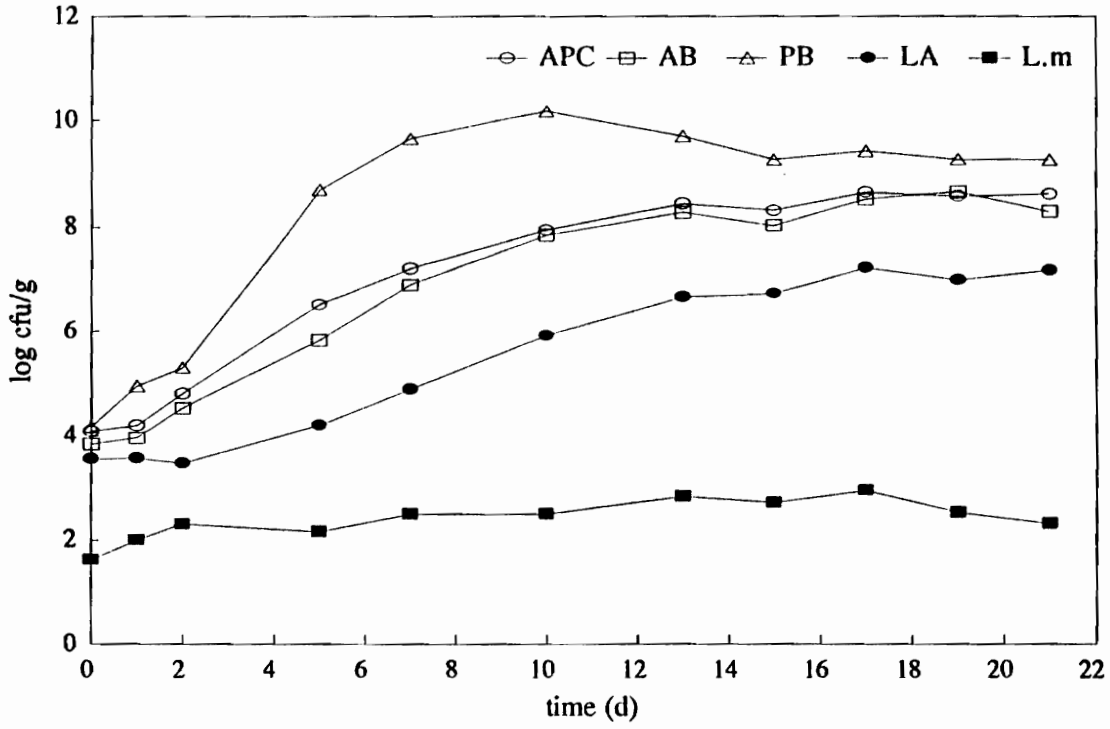


Figure 1. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 1.1 °C. The initial aerobic plate count was less than 15,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).

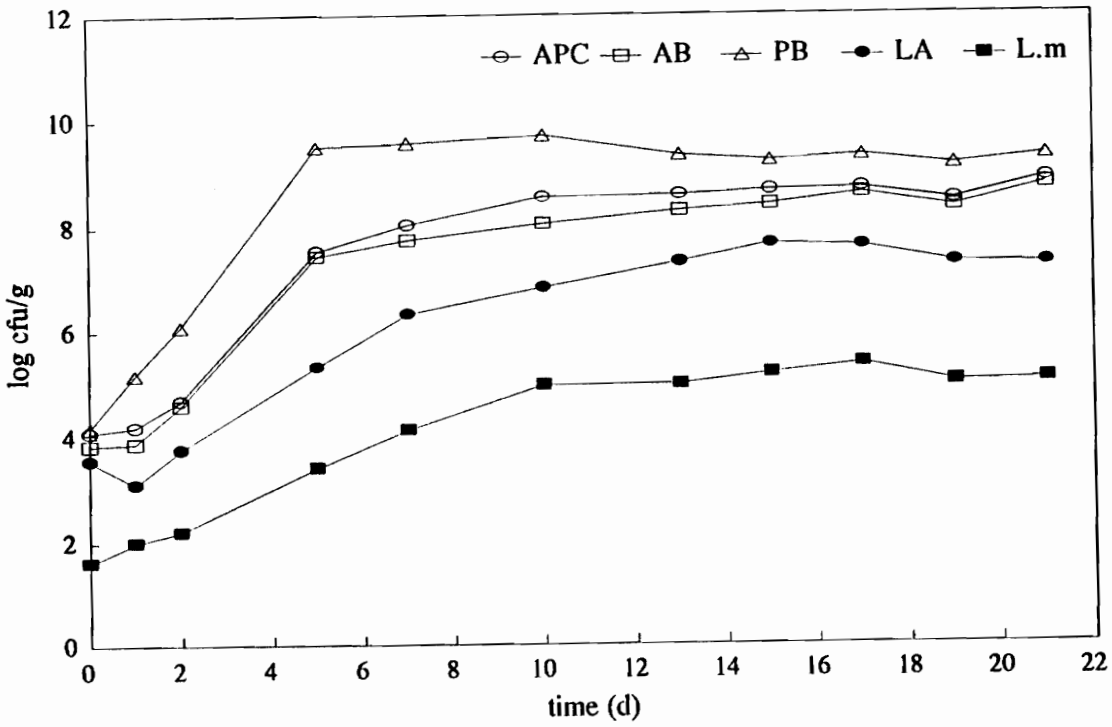


Figure 2. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 5 °C. The initial aerobic plate count was less than 15,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).

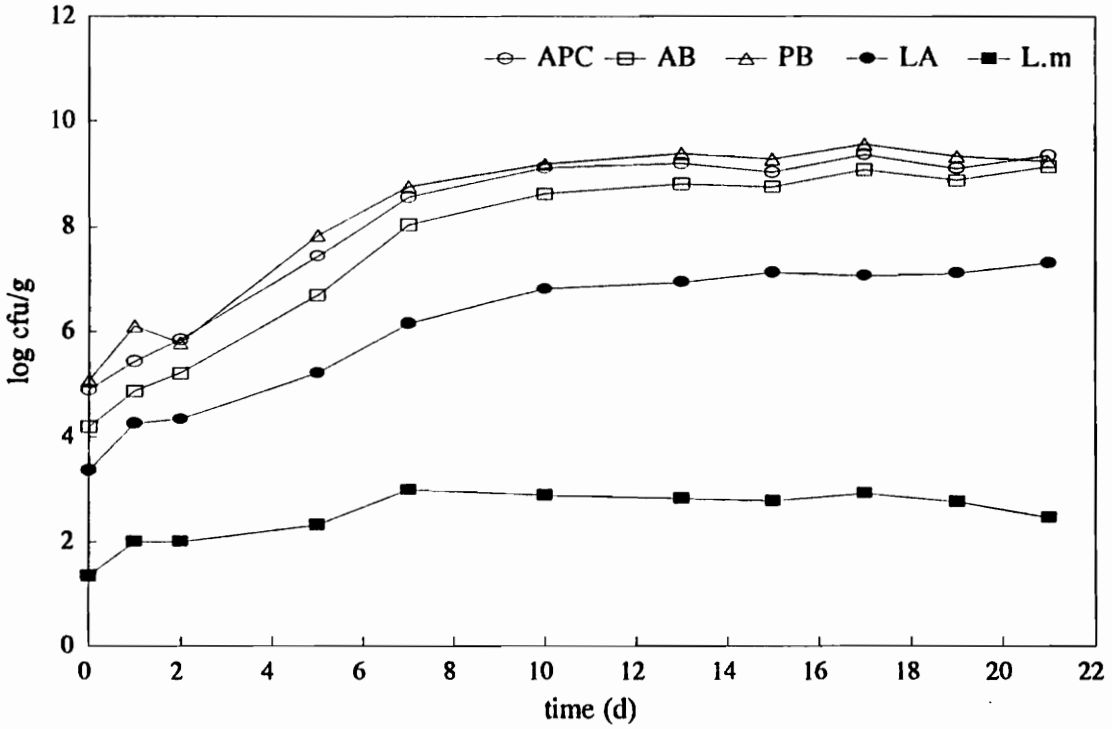


Figure 3. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 1.1 °C. The initial aerobic plate count was between 60,000 and 100,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).

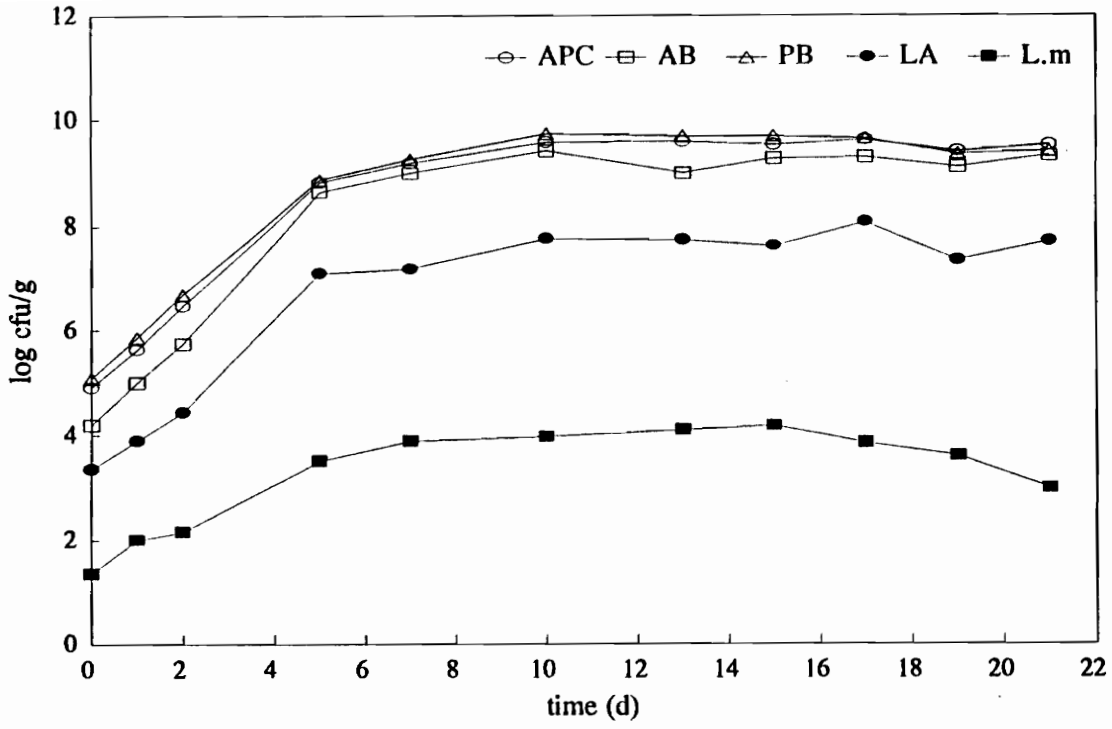


Figure 4. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 5 °C. The initial aerobic plate count was between 60,000 cfu/g and 100,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).

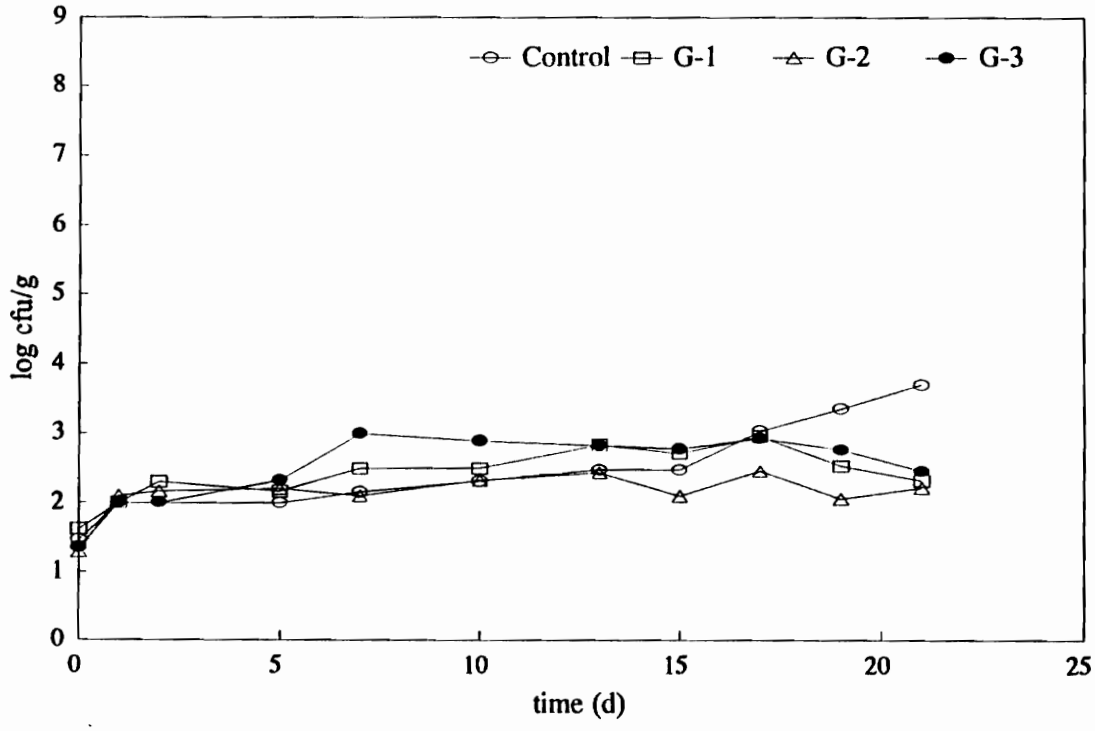


Figure 5. Growth of *Listeria monocytogenes*, inoculated with less than 50 cfu/g, in pasteurized crab meat (- o -) and in the presence of naturally occurring microflora in fresh blue crab meat during storage at 1.1 °C. The initial aerobic plate count was less than 15,000 cfu/g (-□-), between 15,000 and 60,000 cfu/g (-▵-), and between 60,000 cfu/g and 100,000 cfu/g (-•-).

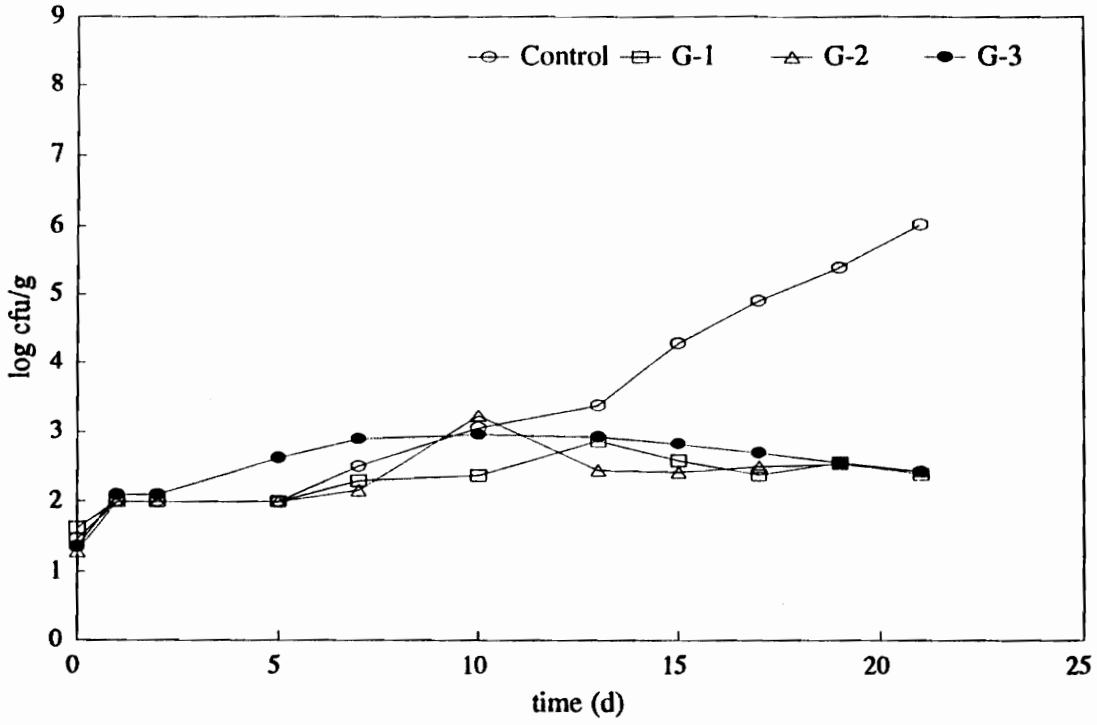


Figure 6. Growth of *Listeria monocytogenes*, inoculated with less than 50 cfu/g, in pasteurized crab meat (- o -) and in the presence of naturally occurring microflora in fresh blue crab meat during storage at 2.2 °C. The initial aerobic plate count was less than 15,000 cfu/g (-□-), between 15,000 and 60,000 cfu/g (-▵-), and between 60,000 cfu/g and 100,000 cfu/g (-•-).

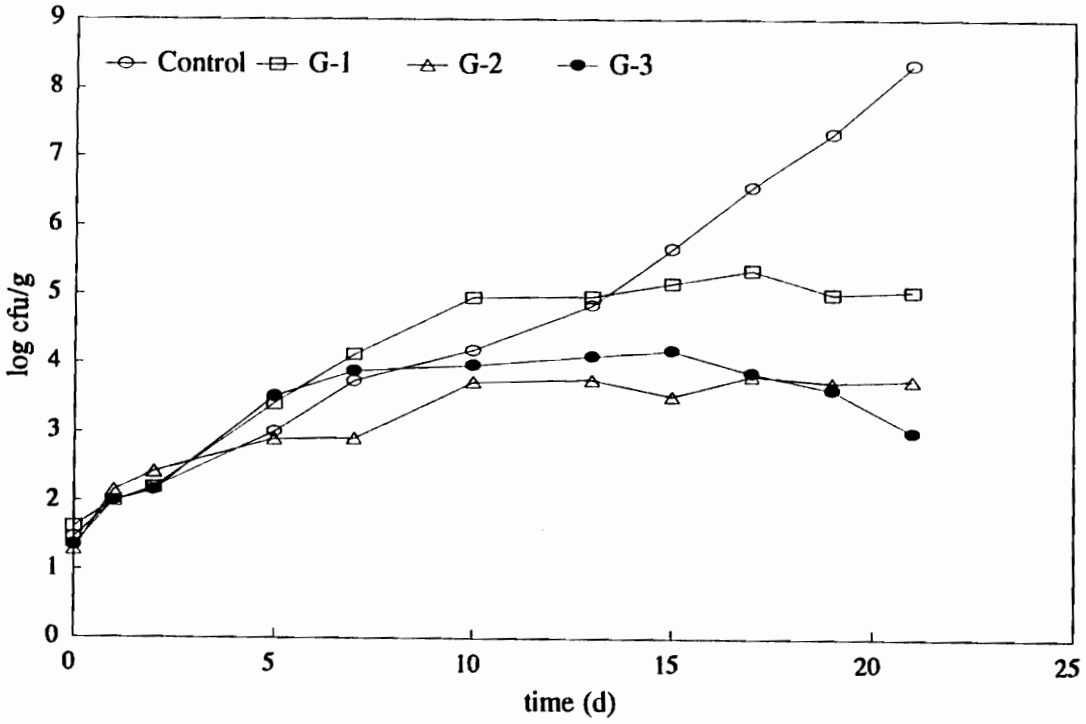


Figure 7. Growth of *Listeria monocytogenes*, inoculated with less than 50 cfu/g, in pasteurized crab meat (- o -) and in the presence of naturally occurring microflora in fresh blue crab meat during storage at 5 °C. The initial aerobic plate count was less than 15,000 cfu/g (-□-), between 15,000 and 60,000 cfu/g (-△-), and between 60,000 cfu/g and 100,000 cfu/g (-•-).

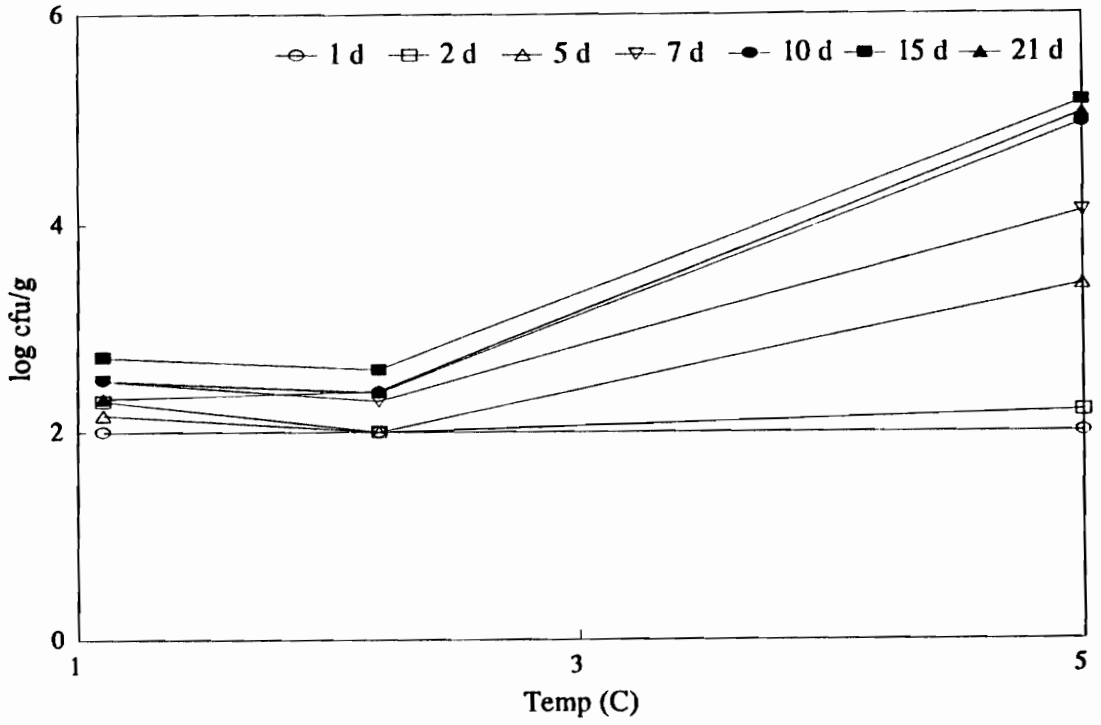


Figure 8. Effect of various storage temperatures on the growth of *Listeria monocytogenes*, inoculated with less than 50 cfu/g, in fresh blue crab meat with initial aerobic plate count of less than 15,000 cfu/g. Growth observed at 1 d (- o -), 2 d (-□-), 5 d (-▵-), 7 d (-▽-), 10 d (-•-), 15 d (-■-), and 21 d (-▲-).

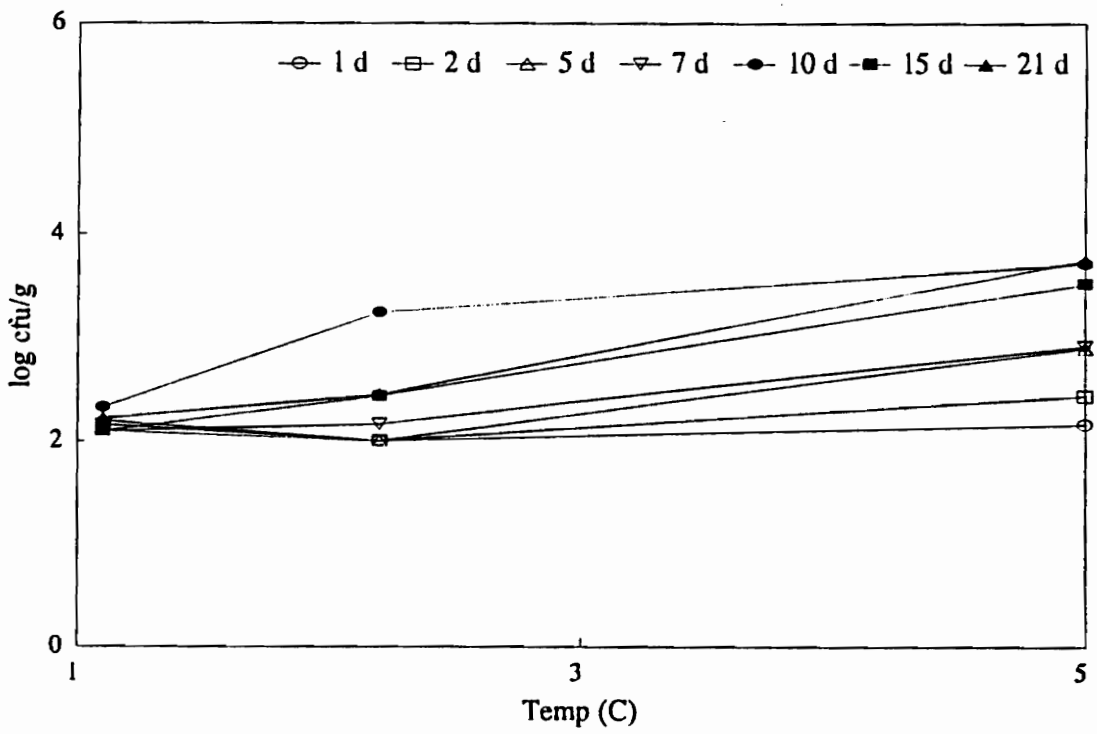


Figure 9. Effect of various storage temperatures on the growth of *Listeria monocytogenes*, inoculated with less than 50 cfu/g, in fresh blue crab meat with initial aerobic plate count between 15,000 and 60,000 cfu/g. Growth observed at 1 d (-o-), 2 d (-□-), 5 d (-△-), 7 d (-▽-), 10 d (-•-), 15 d (-■-), and 21 d (-▲-).

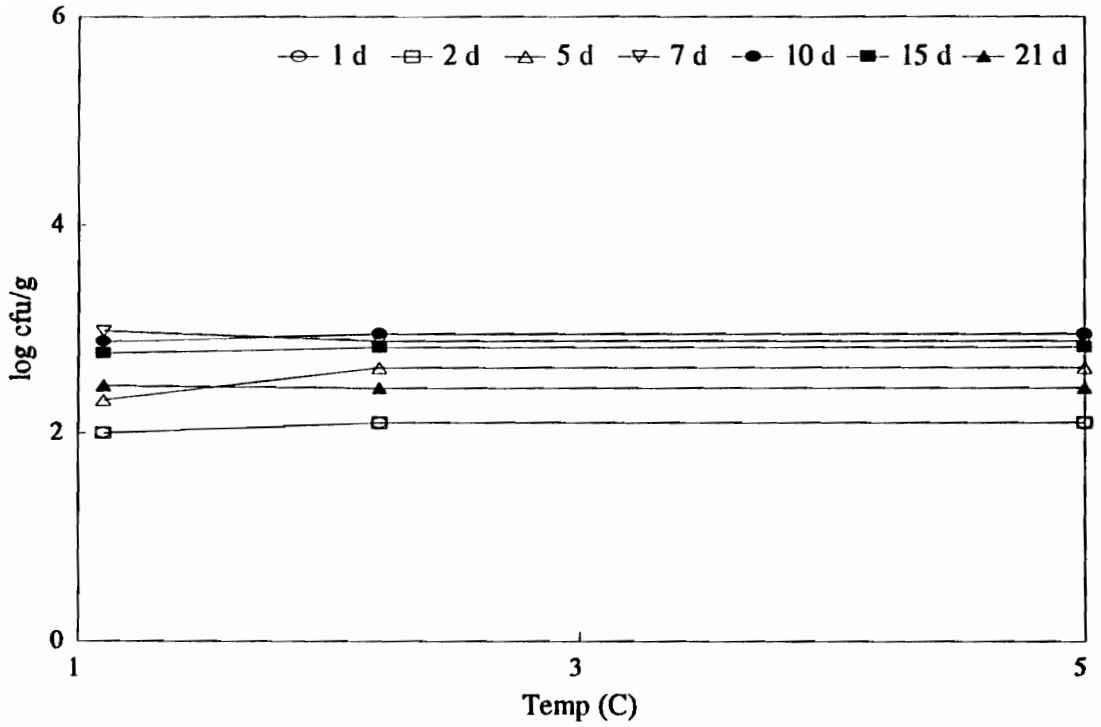


Figure 10. Effect of various storage temperatures on the growth of *Listeria monocytogenes*, inoculated with less than 50 cfu/g, in fresh blue crab meat with initial aerobic plate count between 60,000 and 100,000 cfu/g. Growth observed at 1 d (-o-), 2 d (-□-), 5 d (-▵-), 7 d (-▽-), 10 d (-•-), 15 d (-■-), and 21 d (-▲-).

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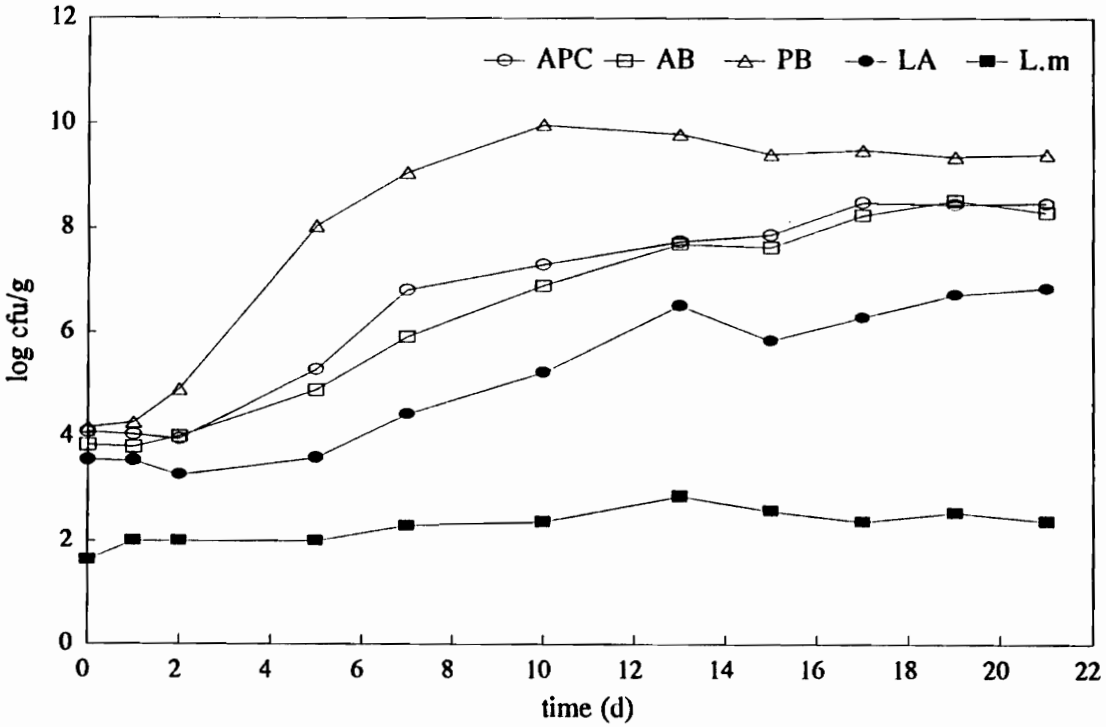
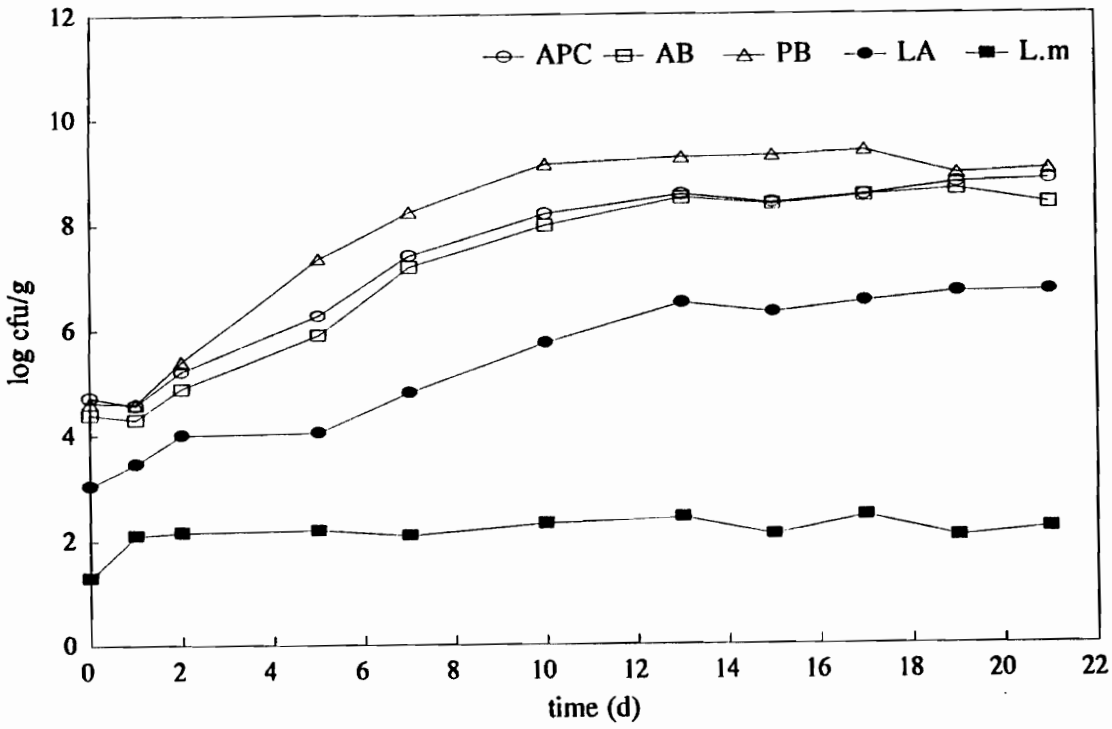
APPENDICES

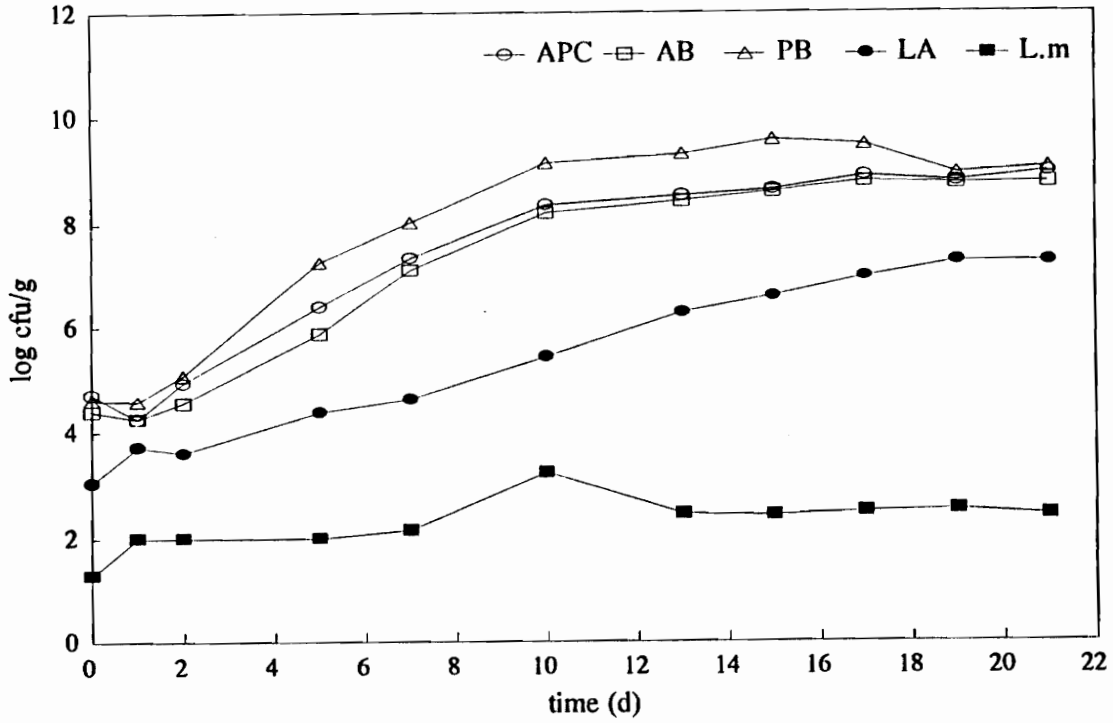
Appendices A through E show the growth of *Listeria monocytogenes* in the presence of naturally occurring microflora in freshly cooked and picked blue crab meat for those conditions not included in the manuscript.



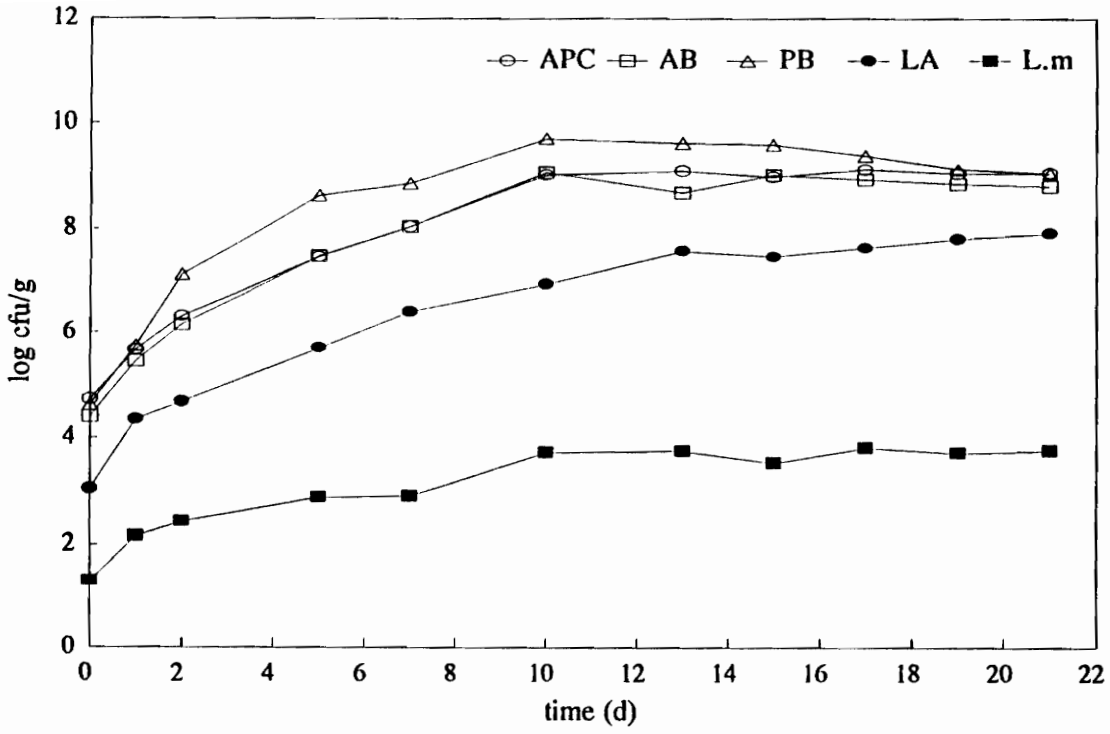
APPENDIX A. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 2.2 °C. The initial aerobic plate count was less than 15,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).



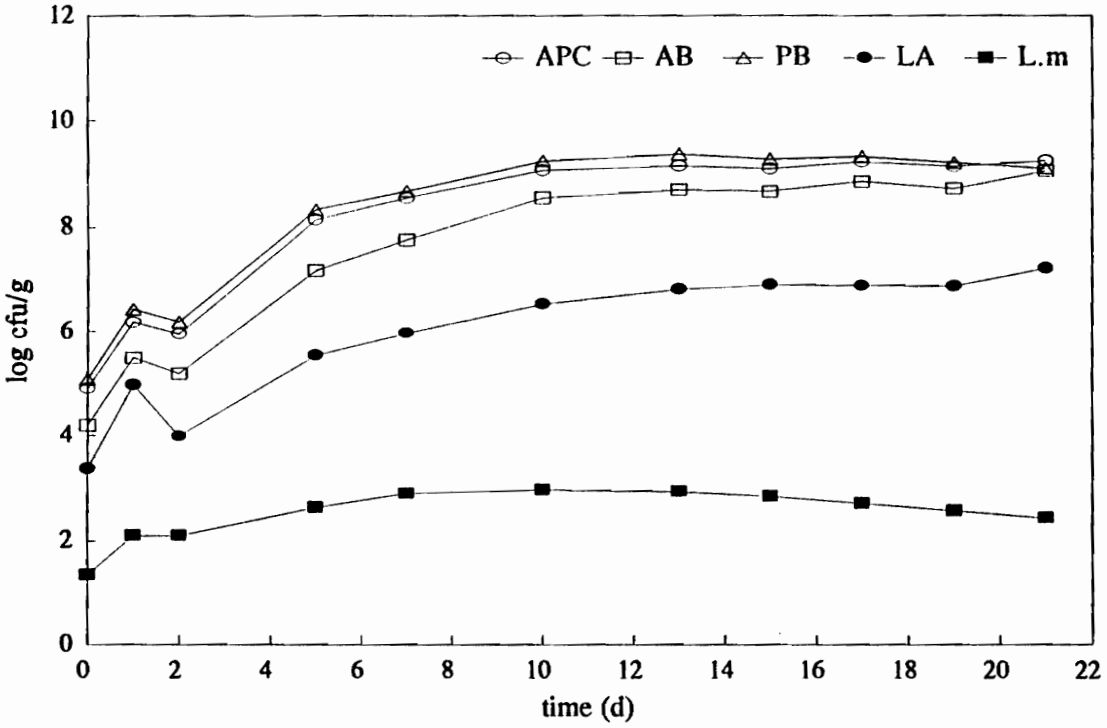
APPENDIX B. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 1.1 °C. The initial aerobic plate count was between 15,000 cfu/g and 60,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).



APPENDIX C. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 2.2 °C. The initial aerobic plate count was between 15,000 cfu/g and 60,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).



APPENDIX D. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 5 °C. The initial aerobic plate count was between 15,000 cfu/g and 60,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).



APPENDIX E. Growth of *Listeria monocytogenes* (-■-), inoculated with less than 50 cfu/g, in the presence of naturally occurring microflora in fresh blue crab meat during storage at 2.2 °C. The initial aerobic plate count was between 60,000 cfu/g and 100,000 cfu/g. Aerobic plate count (- o -), Anaerobic plate count (-□-), Psychrotrophic plate count (-▵-), and Lactic acid bacteria (-•-).

SECTION V: USE OF MICROWAVE ENERGY TO DESTROY OR INACTIVATE *LISTERIA MONOCYTOGENES* IN BLUE CRAB (*CALLINECTES SAPIDUS*) MEAT

ABSTRACT

The ubiquitousness of *Listeria monocytogenes* ensures that the organism will be found in most seafood processing environments. Therefore, seafood processors are facing an enormous challenge in having to deal with this pathogen and complying with the federal regulation for a "zero tolerance" in ready-to-eat fish and shellfish products. Fresh processed crab meat presents a potential risk, since it can be consumed without further heat treatment. In this study, a listericidal process using microwave energy was developed as a potential, low-cost process to decrease, if not eliminate the presence of *L. monocytogenes* from blue crab meat. The infective dose of *L. monocytogenes* is unknown, but it has been well established that it only targets certain segments of the population. Therefore, if *L. monocytogenes* can be reduced beyond the naturally occurring levels in fresh crab meat, an alternative to the zero tolerance policy may be considered. Machine picked blue crab (*Callinectes sapidus*) meat was inoculated with *L. monocytogenes* to obtain a concentration of less than 50 cfu/g. Using a commercial microwave oven, wave frequency 2,450 MHz, four hundred fifty four grams of the inoculated crab meat contained in a polypropylene with snap-on-lid tub (11 cm top diameter, 9 cm bottom diameter, 7 cm height) or in a

polyethylene bag (18 x 20 cm) was microwaved for 3 min. The temperature reached after microwave exposure was determined at 6 positions throughout the container and monitored for 3 and 5 min. The product was placed on ice and samples from three spots were taken from each container after 3 h and 5 d of 0 °C of storage. The recovery method used 250 ml bottles of *Listeria* Enrichment Broth (LEB) containing cysteine (0.5 g/L), resazurin (0.25 g/L) and flushed with N_2 gas to create an anaerobic environment. After incubation of LEB at 35 °C for 48, 72, and 96 h *L. monocytogenes* was not detected in any of the samples analyzed at either 3h or 5 days after microwave application. Therefore, microwave energy has commercial potential to destroy *L. monocytogenes* in the occurring levels encountered in freshly cooked and picked blue crab meat.

INTRODUCTION

Listeria monocytogenes is particularly difficult to control in food processing plants, specially in refrigerated food plants where the refrigerated, moist environment, coupled with soil deposition, allows for the survival and growth of the microorganism. The bacterium can adhere to food contact surfaces and form a 'biofilm' or coating (Doyle, 1991), which impedes removal and decreases the effectiveness of sanitation procedures. *L. monocytogenes* is also a frequent contaminant of raw materials utilized in plants; therefore, there is constant re-introduction of the organism into the plant environment.

There is no uniform, mandatory requirement for pasteurization of ready-to-eat crab meat. The recommendations of the National Advisory Committee on Microbiological Criteria for Foods (1990) is a minimum thermal process of $F_{85} \geq 31$ minutes to be required for a product to be labeled pasteurized. The conventional pasteurization of crab meat, heating 401x301 tinplate cans of crab meat in a 88 °C water bath until the the cold point temperature reaches 85 °C and holding for 1 min, results in an extension in the shelf life from 6-10 days to 6 to 18 months. There is no target organism for the pasteurization process, since the process basis was shelf-life extension rather than pathogen destruction or inactivation. A z-value of 8.9 °C has been arbitrarily chosen (Rippen and Hackney, 1992; Ward et al., 1984).

Microwave energy is a form of non-ionizing radiation with energy in the region of 1.2×10^{-5} eV, well below the energy necessary to break chemical bonds (5.2

eV to break hydrogen bonds) (Rosen, 1972). Microwaves are electromagnetic waves. The heat production is mainly due to rotation of polar molecules, resulting in heat generated by molecular friction, thus the absorbing system dissipates energy as heat (Curnutte, 1980). Microwaves travel at the speed of light, and their ability to heat is largely determined by the parameters of the product being heated (George, 1993; Mudgett, 1982, 1986; Ohlsson, 1983; Schiffmann, 1986, 1990; Swami and Mudgett, 1981; Tinga, 1970). The depth of microwaves penetration in foods is largely determined by the wave's frequency (Mudgett, 1986; Ohlsson, 1983). In the United States, commercial microwave ovens generally use frequencies of 2,450 MHz, although in some industries and in several countries outside the U.S., 915 MHz is used. The wavelength (λ) resulting from these frequencies are 0.12 and 0.33 m, respectively (Annis, 1980; Perry, 1986). As wavelength increases (or processing frequency decreases), penetration depth increases. In general, surface heating effects (the surface achieves higher temperatures than the geometric center of the product) are of concern when using ovens at 2,450 MHz. Opposite are the center heating effects, depending on product shape, size and salt content, and more prominent in ovens operating at frequencies of 915 MHz. Size and shape of products will affect microwave heating (Copson, 1962). Objects with corners (90° edges, etc.) have a tendency to show localized heating due to the multi-directional distribution of microwave energy. As penetration depth decreases, these corner effects have larger impact. Size of the objects being heated is important in relation to wavelength and

penetration depth. Rounder shapes reduce corner heating but are susceptible to focussing effects. These effects have only been noted in cylinders and spheres and are dependent on the diameter and conductivity of the product, as well as the processing frequency.

Most authors have concluded that inactivation of microorganisms during a microwave process occurs solely due to generation of heat (Decareau, 1985; Heddleson and Doores, 1994). This is confirmed by Mertens and Knorr (1992) who reported on the inability to demonstrate the non-thermal usage of microwaves as a preservation method. An opposite school of thought, proposes and supports an athermal mechanism of lethality with an effect attributable only to the intrinsic nature of microwaves, and unrelated to lethality caused by heat (Chipley, 1980). The principal reason to consider the use of microwaves for pasteurization of foods is the ability of microwaves to penetrate many packaging materials with almost no energy loss (Schiffmann, 1989). Coote et al. (1991) used conventional heating to expose *L. monocytogenes* cells, either in broth or in chicken skin, to mean times and temperatures that are achieved during a 28 min period of microwave cooking of a whole chicken. After cooking for the full time, a mean temperature of 85 °C and no surviving listerias were observed. They concluded that when a temperature of 70 °C is reached and maintained for at least 2 min throughout a food, there is a substantial reduction in the numbers of *L. monocytogenes*. Galuska et al. (1988) studied the thermotolerance of *L. monocytogenes* Scott A and V7 suspended in nonfat dry milk

and heated by microwaves. They concluded that at conventional pasteurization process temperatures, microwaves could be as effective as conventional heating in destroying *L. monocytogenes*.

The calculation of lethality for a microwave heating process is much more complicated than for a conventional thermal process. The difficulty in maintaining a constant temperature within a microwave makes the determination of D-values, the time in minutes required at a constant temperature to reduce a microbial population by 90%, difficult to achieve. Although microwave pasteurization is supposedly more costly than traditional pasteurization processes, the advantage of reduced processing time with the subsequent cost reduction, makes microwave pasteurization/sterilization processes an attractive and promising industrial application (Rosenberg and Bogl, 1987). However, most studies on the effectiveness of microwave heating in eliminating pathogens in food products have concluded that although large decreases (up to 7-8 $\log_{10}$ cycles) in bacterial populations may be observed (Cunningham, 1980), complete elimination of pathogens is more difficult to obtain by microwave heating (Alexio et al., 1985).

In this study the use of microwave energy to destroy or reduce *L. monocytogenes* artificially inoculated in fresh blue crab (*Callinectes sapidus*) meat was explored.

MATERIALS AND METHODS

Microwave characteristics and preliminary determinations

A commercial microwave oven (Amana Radarange, Model RC 14 SE, Amana, IA) was used to process the inoculated crab meat. The oven characteristics are: wave frequency 2,450 MHz, with "instant on" operation (heating time does not include warm-up sequence); 203 Volts; 1,500 Watts of microwave power delivered to the oven cavity through a waveguide. Machine picked blue crab (*Callinectes sapidus*) meat was obtained from a processing facility in the Chesapeake Bay region and delivered overnight to the Department of Food Science and Technology at Virginia Tech. Upon arrival the meat was stored at 0 °C. The microwave pasteurization experiment was designed as follows: crab meat (454 g) was placed in either a polypropylene tub with snap-on-lid (11 cm top diameter, 9 cm bottom diameter, 7 cm height) or in a polyethylene bag (18 x 20 cm) (Ziploc®, DowBrands L.P., Indianapolis, IN) and heated to reach approximately 85 °C at the cold spot. Prior to microwaving, 6 thermocouples (hypodermic probes) (Omega, Stamford, CT) were positioned throughout the container. When the plastic tub was used, a lid with perforations in the desired positions was used to measure temperatures, then the regular lid was placed on the tub (the lid was left loose to avoid collapsing of the container), and microwave energy was applied. Inmediately after the microwave cycle was completed, the container was removed and the lid was replaced by the one with the perforations and temperature was measured at the 6 pre-determined points

and labeled. When the polyethylene bag was used, the bag itself was labeled for the thermocouple positions. To avoid excessive pressure build-up during microwaving, a 2.5 cm opening in the seam was maintained. Following this procedure, preliminary studies were performed to establish optimum conditions for the study. This included determining appropriate thermocouple depths and processing times. Thermocouples 1,2, and 3 were type T (Omega industrial thermocouple probe model HYP-3 with T calibration: copper-constantan elements, grounded junction) and thermocouples 4,5, and 6 were type K (Omega industrial thermocouple probe model HYP-3 with K calibration: chromel-alumel elements, grounded junction).

Listericidal process

Based on the preliminary results, it was obvious that a pasteurization process based on $F_{85°C}$=31 min could not be achieved without destruction (overcooking) of the crab meat. Hence, a different approach for the process was considered and a decision made to study the listericidal efficiency of a microwave process applied for 3 min to 454 g of crab meat, in a plastic tub and in a polyethylene bag, after inoculation with less than 50 cfu/g of *L. monocytogenes*. Microwave time was established at three min, since this interval was the lowest setting that resulted in an acceptable cooking of the crab meat surface while still reaching temperatures throughout the meat that achieved destruction or reduction of *L. monocytogenes*. To attain higher temperatures in the center of the meat in the plastic tub, the crab meat was left at room temperature

prior to microwaving to reach ambient temperature (for less than 2 h), then inoculated and microwaved. The crab meat in the polyethylene bag was kept in ice. Higher temperatures at the center of the container were achieved by placing the container on top of an inverted cardboard plate. The thermocouple positions are those described in Figures 1a and 1b, and the penetration depths are given in Table 1.

Inoculated pack study

Once optimum processing conditions were obtained, crab meat was inoculated with *L. monocytogenes* at initial levels of less than 50 cfu/g to simulate the occurring levels of this pathogen in fresh crab meat (Rawles et al., 1995). A *L. monocytogenes* strain previously isolated from freshly picked crab meat has been maintained as stock strain. The organism is kept in slants of Tryptic Soy Agar + 0.6% Yeast Extract (Difco 0369 + 0127) (TSA + 0.6%YE) with periodic transfers and biochemical pattern confirmation using API *Listeria* kits (Bio Merieux Vitek, Inc., St. Louis, MO). An 18 h (36 °C) culture of this organism in Tryptic Soy Broth (TSB, Difco 0370) yields approximately 10^9 cfu/ml. Therefore, 454 g of crab meat was inoculated with 4.5 ml (10^{-6} dilution) of the overnight culture. The crab meat was placed in a Whirl-pak® bag (Nasco, Fort Atkinson, WI), inoculated and shaken by hand for 1 min to distribute the inoculum and homogenize the meat. The inoculated crab meat was placed in the appropriate container and kept in ice (polyethylene bags) or at ambient temperature (plastic tub). After microwave processing, the thermocouples were

inserted and held in place for 3 min (polyethylene bag) and 5 min (plastic tub). The product was placed in ice and tested for survival of *L. monocytogenes* after 3 h and 5 days of storage at 0 °C. Each experiment was replicated four times. According to the observed temperature profiles in each case, three 25 g samples were taken from the colder spots (3) in each package. Following the procedure developed by Knabel and Thielen (1995) to recover heat-injured *L. monocytogenes*, 25 g of the microwaved crab meat was placed in 225 ml of *Listeria* Enrichment Broth (BBL 12333, Becton Dickinson, Franklin Lake, NJ)) containing 0.5 g/L cysteine (Sigma Chemical Co., St. Louis, MO) and 0.25 g/L resazurin (Sigma). To decrease the headspace, the samples were placed in 250 ml bottles, the media added and purged with N_2 gas for approximately 10 s before applying the screw cap. The bottles were incubated at 35 °C and 0.1 ml portions were subsequently inoculated in tubes containing Fraser Broth (FB, Difco 0219 + 0211) at 48, 72, and 96 h. The FB tubes were incubated at 35 °C and inocula streaked onto Modified Oxford Agar (MOX, Difco 0225 + 0218) plates at 24 and 48 h. Identification was performed according to colony characteristics (convex, shining-blue, positive for esculin hydrolysis) and hemolysis in Blood Agar (Difco 0696 + 6% difibrinated sheep blood).

RESULTS

The first approach was to develop a 85 °C pasteurization process at the coldest spot. In developing the process several impediments were experienced. The cold

spot in the plastic tub was at a depth of 3.75 cm, and when starting from refrigerated storage at 0 °C, only an average temperature of 37.7 °C was achieved after 8 min of microwave processing. If held at ambient temperature, the post processing temperature throughout the container approached equilibrium, heating in the center and cooling off on the sides. However, the meat was overcooked, had a dark color and unacceptable texture and the container was deformed. A package thickness of 0.5 inches was obtained in the polyethylene bags. The temperature was difficult to measure since the cold spot continuosly shifted and after 4 min of microwaving, the meat was overcooked and the cold spot(s) had reached an average temperature of 79.4 °C. Nine hundred eight grams of crab meat were placed in the polyethylene bag and microwaved to determine the effect of increasing mass on product temperature. The thickness of the packed meat was 5 cm. However, the cold spot varied and the meat reached approximately 52.9 °C after 4 min of microwaving. The main problem observed, was the large variation in temperature throughout the containers and the lack of reproducibility between experiments.

Microwave energy applied for 3 min to the 454 g of 0 °C crab meat inoculated with *L. monocytogenes* (7 cfu/g) contained in a polyethylene bag, resulted in an initial cold spot located at the center (0.6 cm depth) of the container. However, during the post process holding time of 3 min at ambient temperature, the temperature at this point (1 in Fig. 1a) remained constant, where the temperatures at the outside of the pouch rapidly decreased (Figure 2). This observation can be explained by the

larger surface area at these outside points. For the recovery of *L. monocytogenes*, samples were extracted from positions 1, 4 and 5 (Figure 1a). The largest variance between replications was for the thermocouple in position 3 (Table 2) during the holding time at ambient temperature.

The crab meat processed in the plastic tub had an initial count of *L. monocytogenes* of 50 cfu/g. Starting from ambient temperature (20 °C) and after 3 min of microwave application, the cold spot was located at the geometric center (1.5" deep). During the holding time of 5 min at ambient temperature, the temperature of the colder spots increased as the temperature of the hoter spots decreased (Figure 3). The cold spot had the largest variation in temperature between replications during the holding time (Table 3). Samples were taken from positions 1, 2, and 4 (Fig. 1b) to detect the survival of *L. monocytogenes*.

L. monocytogenes was not detected in either the treated crab meat stored in the polyethylene bag or in the polypropylene tub after 3 h or 5 days of microwave application.

The equivalent heat treatment (Reference temperature = 85 °C, z = 8.9°C) obtained during the holding time after microwave application was 1.13 min for the polypropylene tub (5 min holding) and 0.41 min for the polyethylene bag (3 min holding). The equivalent F-values for the individual thermocouples are shown in Table 4.

DISCUSSION

A commercial pasteurization process for crab meat, based on the criteria of a minimum of 85 °C at the cold spot holding for 1 min, was not attainable using a microwave oven with 2,450 MHz wave frequency, 1,500 watts power, for either 454 g of crab meat packed in a polypropylene tub (11 cm top diameter, 9 cm bottom diameter, 7 cm height) or packed in a polyethylene bag (18 x 20 cm). Difficulties in achieving adequate pasteurization temperatures without cooking the surface of the products were encountered. These surface heating effects of microwave heating at 2,450 MHz have been described since early application of microwave heating to foods (Copson, 1962).

Since *L. monocytogenes* was not detected in the microwaved crab meat after 3h or 5 days from the process time, the potential use of microwave energy to destroy or reduce *L. monocytogenes* numbers in fresh blue crab meat can be further explored. Improvements in the heat transfer throughout the meat could be obtained by, for example, using microwaves at a frequency of 915 MHz instead of 2,450 MHz. The larger wavelength would allow improved penetration in the meat. The results obtained in this study, although applicable to the low occurring levels of *L. monocytogenes* in fresh blue crab meat may not necessarily be reproducible for higher numbers of the bacteria which could be obtained if temperature abuse during storage or unsanitary processing conditions occur. Still, it is encouraging to have an

alternative process that could enable crab meat processors to diminish the chances of *L. monocytogenes* in their products.

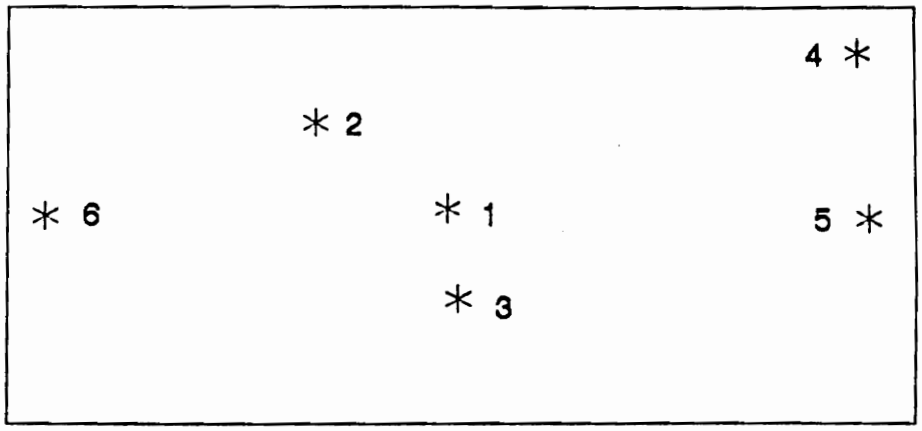


Figure 1a: Thermocouple distribution throughout 454 g of crab meat in a polyethylene bag (18 x 20 cm)

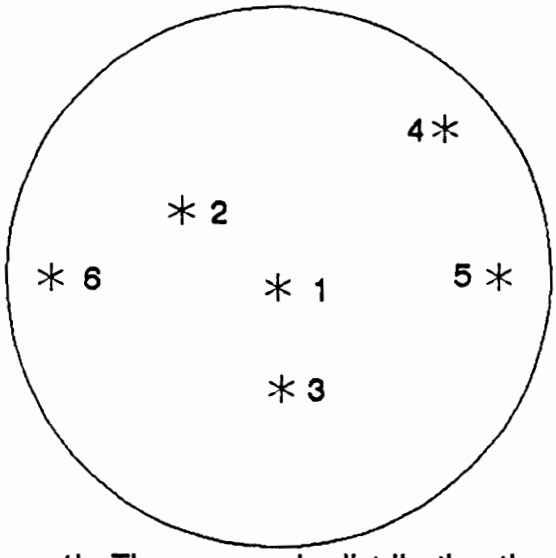


Figure 1b: Thermocouple distribution throughout 454 g of crab meat in a polypropylene tub (11 cm top diameter, 9 cm bottom diameter, 7 cm height)

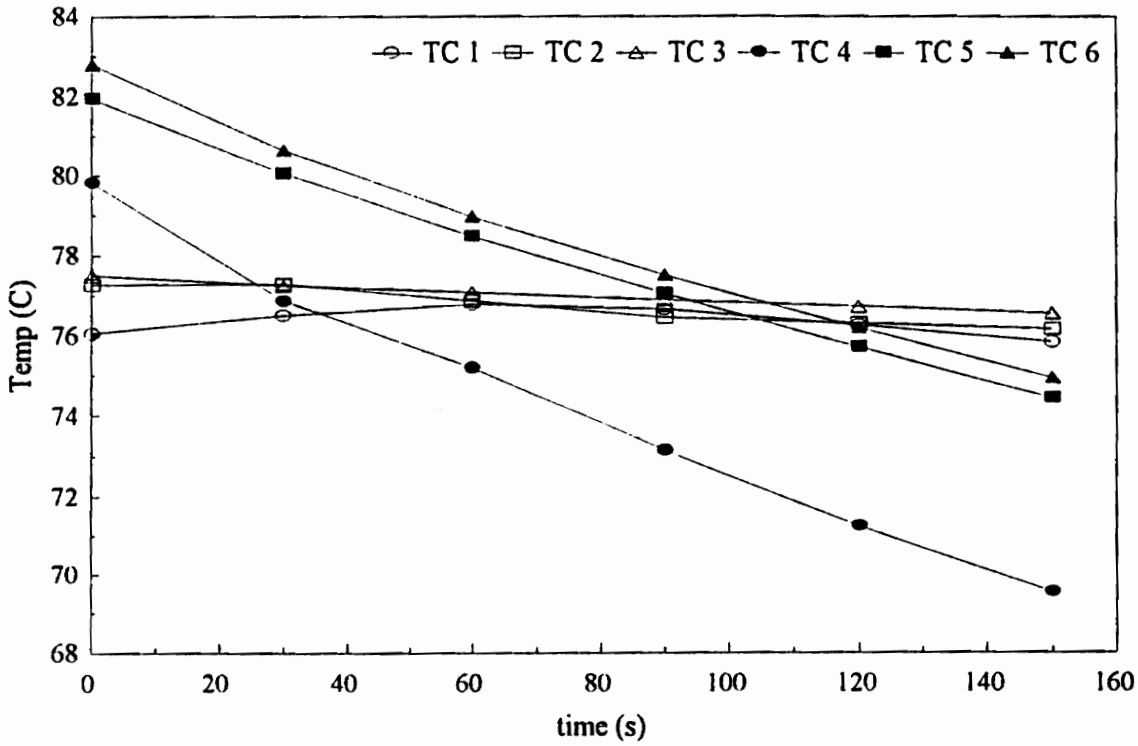


Figure 2. Temperature profile throughout 454 g of crab meat in a polyethylene bag (18 x 20 cm) after microwave application during 3 min. Temperature measured with thermocouples at 6 different points for 3 min.

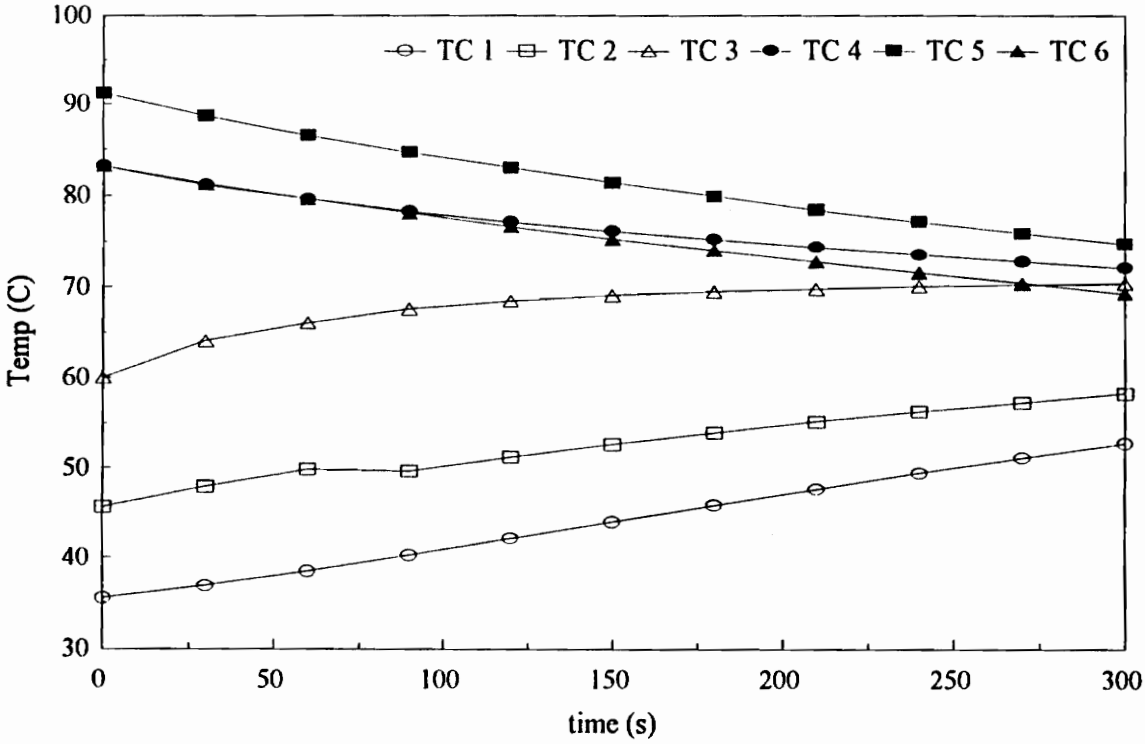


Figure 3. Temperature profile throughout 454 g of crab meat in a polypropylene tub (11 cm top diameter, 9 cm bottom diameter, 7 cm height) after microwave application during 3 min. Temperature measured with thermocouples at 6 different points for 5 min.

Table 1. Thermocouple insertion depth (cm) to measure temperature in 454 g of crab meat contained in a polypropylene tub and in a Ziploc bag.

Thermocouple Nº	plastic tub (7.6 cm depth)	Ziploc bag (1.3 cm depth)
1	3.81	0.64
2	2.54	0.64
3	2.54	1.27
4	1.27	1.27
5	3.81	1.27
6	1.27	0.64

Table 2. Standard deviation (°C) between 4 replications of temperature measurement at 6 positions throughout 454 g of crab meat packed in a polyethylene bag, microwaved for 3 min, and held at ambient temperature for 3 min.

	Thermocouple Nº					
time (s)	1	2	3	4	5	6
0	15.97	12.60	15.42	15.11	15.85	13.77
30	15.10	13.49	15.91	14.75	15.94	14.01
60	15.35	13.66	16.30	14.48	16.02	14.24
90	15.55	13.71	16.47	14.48	16.02	14.48
120	15.73	13.95	16.64	14.42	15.98	14.63
150	15.83	14.26	16.75	14.46	15.99	14.77

Table 3. Standard deviation (°C) between 4 replications of temperature measurement at 6 positions throughout 454 g of crab meat packed in a polypropylene tub, microwaved for 3 min, and held at ambient temperature for 5 min.

	Thermocouple Nº					
time (s)	1	2	3	4	5	6
0	2.91	10.22	4.88	15.64	15.55	14.64
30	3.12	10.72	7.16	15.75	15.21	15.28
60	3.55	11.44	8.11	15.94	14.89	15.68
90	4.03	13.27	8.94	15.98	14.69	15.94
120	4.52	13.31	9.49	16.04	14.56	16.14
150	5.06	13.38	9.99	16.05	14.44	16.22
180	5.60	13.38	10.42	16.02	14.35	16.28
210	6.15	13.44	10.77	16.02	14.27	16.32
240	6.64	13.45	11.09	15.99	14.25	16.28
270	7.16	13.48	11.39	16.00	14.14	16.23
300	7.59	13.50	11.65	15.94	14.13	16.15

Table 4. Equivalent $F_{85°C}$, (z=8.9°C) achieved at various locations in 454 g of crab meat following microwave application of 3 min and 3 min ambient hold for polyethylene bags and 5 min ambient hold for polypropylene tub.

Thermocouple #	Polyethylene bag (18 x 20 cm)	Polypropylene tub (11 cm top diameter, 9 cm bottom diameter, 7 cm height)
1	0.2726 min	0.0001 min
2	0.2947 min	0.0009 min
3	0.3151 min	0.514 min
4	0.2081 min	0.7601 min
5	0.4703 min	4.5353 min
6	0.5504 min	0.7132 min

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SECTION VI: MODELING THE GROWTH OF *LISTERIA MONOCYTOGENES* IN FRESH BLUE CRAB (*CALLINECTES SAPIDUS*) MEAT DURING REFRIGERATED STORAGE

ABSTRACT

Predictive microbiology offers an alternative to traditional microbiological assessment of food safety and quality. In this study, mathematical modeling was directed towards the growth prediction of *Listeria monocytogenes* in fresh blue crab (*Callinectes sapidus*) meat during refrigerated storage. Growth curves were fit using a logistic function, the Gompertz equation, a developed non-linear regression with a modified Gompertz equation to include temperature effect, and a developed extended non-linear modified Gompertz equation to cover the range of storage temperature between 1.1 and 5 °C. The best fit was obtained with the developed modified Gompertz equation including the effect of storage temperature on the growth of *L. monocytogenes*. The prediction was more accurate if growth during only the first 10 days of storage was modeled, instead of the total storage period of 21 days, considering that after 10 days of storage the bacteriological quality of the crab meat was such that it was inedible. The extended modified Gompertz model combined the effects of temperature throughout the range of refrigerated storage, between 1.1 and 5 °C, and was able to estimate the growth of *L. monocytogenes* with correlation coefficients between 0.84 and 0.95.

INTRODUCTION

Predictive microbiology offers a powerful tool to explain and estimate the behavior of microorganisms when subjected to factors that influence their growth (Gibson and Roberts, 1989). A detailed knowledge of the microbial ecology of a food product can be expressed as a mathematical model allowing for objective evaluation of the effect of processing, storage and distribution operations on microbial development (McMeekin et al., 1992). Predictive microbiology can be used as an alternative to traditional microbiology to assess food quality and safety (Maas, 1993), with the advantage that with a good predictive model, the effect of varying factors on microbial behavior could be easily calculated (Roberts, 1989). Among the goals of predictive microbiology is to develop models which fit microbiological data as precisely as possible (Alber and Schaffner, 1993).

The steps involved in modeling include (Baranyi et al., 1993; Dodds, 1993): (1) planning, a well designed experiment is essential (Davies, 1993), (2) data collection (Miller, 1993), (3) model fitting and selection of the model which best describes the data, (4) model validation using data not used to fit the model. It is important to keep in mind that the predictive ability of any model is best within the range of conditions tested. Since physical and biological models often arise as solutions to differential equations, regression models that describe natural processes are often non-linear in the model parameters (Ratkowsky, 1993). In a closed system, modeling of microbial growth is best described by a sigmoidal curve. There are

several choices of equations for sigmoidal curves (Stannard, 1985; Zwietering et al., 1990). The logistic regression gives a symmetrical sigmoidal curve (Buchanan, 1993), so it is best for organisms growing under optimal conditions. The Gompertz equation (Buchanan, 1993: Gibson et al., 1987) is used when the relative growth is believed to decrease exponentially with time. The Ratkowsky equation (Ratkowsky et al., 1985) equation is predictive for growth dependent on temperature, the prime factor controlling the rate of microbial proliferation in fish and crustaceans (McMeekin et al., 1992).

In this study, two of the most commonly used sigmoidal functions, the Gompertz and logistic functions, were fitted to model the growth of *Listeria monocytogenes* in fresh blue crab meat during refrigerated storage. A modified Gompertz equation to include the effect of temperature upon the growth of *L. monocytogenes* as well as an extended modified Gompertz equation to predict growth in the range of customary storage temperatures were developed.

MATERIALS AND METHODS

L. monocytogenes growth data

Data for the growth of *L. monocytogenes* in freshly cooked and picked blue crab (*Callinectes sapidus*) meat was collected by replicating each experiment 3 times. Freshly cooked and picked blue crab meat was obtained from processing facilities in the Chesapeake Bay region and sent overnight, in ice, to the Department of Food

Science and Technology at Virginia Tech. Upon arrival, the crab meat was divided in 3 samples of 300 g each. Each sample was inoculated with *L. monocytogenes*, strain 168, previously isolated from fresh blue crab meat. The inoculum was to obtain an initial level of less than 50 cfu/g, according to the occurring levels previously found in fresh crab meat (Rawles et al., 1995). An 18 h culture (36 °C) of *L. monocytogenes* in Tryptic Soy Broth (TSB, Difco 0370, Detroit, MI) yielded approximately 10^9 cfu/ml. Each sample was placed in a 24 oz., puncture proof sterile Whirl-pak® (Nasco Inc., Fort Atkinson, WI) plastic bag, inoculated with 3 ml of a 10^{-6} dilution of the *L. monocytogenes* culture and homogenized for 2 minutes in a stomacher (Lab Blender 400, Tekmar, Cincinnati, OH). The samples were respectively stored at 1.1, 2.2, and 5 °C for 21 days. A 25 g sub-sample was evaluated after 0, 1, 2, 5, 7, 10, 13, 15, 17, 19, and 21 storage days and aerobic plate counts (APCs) and *L. monocytogenes* counts were obtained. According to the initial APCs, the fresh crab meat was classified into three groups. Group I was for crab meat with an initial APC less than 15,000 cfu/g; Group II for crab meat with initial APCs between 15,000 and 60,000 cfu/g, and Group III for crab meat with initial APCs between 60,000 and 100,000 cfu/g. The experiment was replicated 3 times for each APC group and storage temperature.

Bacteria enumeration

Before the samples were inoculated with *L. monocytogenes,* the crab meat was analyzed for the presence of occurring *Listeria* spp. by placing 25 g of sample in 225 ml of Fraser broth (FB, Difco 0219 + 0211). Following a 5-tube most probable number (MPN) procedure (Pusch, 1989), 10 ml of the 10^{-1}, 10^{-2}, and 10^{-3} dilution were incubated in duplicate, for 24 and 48 h, at 36 °C. Samples positive for *Listeria* spp. were discarded. After inoculation with *L. monocytogenes*, a 25 g sub-sample was taken from each storage temperature at the predetermined storage time and placed into 225 ml of Peptone water 0.1% (Difco 0118). From this dilution, surface plate counts for *L. monocytogenes* and aerobic bacteria were obtained. *L. monocytogenes* was enumerated in blood agar (Blood Agar Base Nº2, Difco 0696 and 6% difibrinated sheep blood) containing colistin sulfate (10 mg/L) and moxalactam (20 mg/L) after 24 -48 h incubation at 36 °C. The use of antibiotics in the media permitted listeria enumeration without interference from the existing microflora. Identification of *L. monocytogenes* was based on the colonies characteristic, hemolytic activity in the medium and further confirmed by streaking suspect colonies onto Modified Oxford Agar (Difco 0225 + 0218) and by biochemical tests (API Listeria kits, BioMerieux Vitek, Inc., St. Louis, MO) when definite confirmation was required. Aerobic plate counts were obtained by surface plating the appropriate sample dilution on Tryptic Soy Agar (Difco 0639) and incubating for 48 h at 36 °C.

Statistical Analysis

A two-way analysis of variance (ANOVA) with a 5% level of confidence was used to determine the significance of the effects of temperature and initial microflora level on the growth of *L. monocytogenes* (Norman and Streiner, 1994). A correlation analysis in the fitted curves determined which model correlated best to the experimental data (Norman and Streiner, 1994) . All statistical analyses were conducted with Quattro Pro for Windows v.5.0 (Borland International, Inc.) and JUMP (statistical package for Apple MacIntoch developed by SAS).

Mathematical Modeling

Once the experimental growth curves for *L. monocytogenes* were obtained for each set of conditions, the data were fitted to the logistic and Gompertz functions and to a Gompertz type function modified to include the effect of temperature. The logistic and Gompertz equations are described by:

logistic equation [1]:

$$L(t)=A+\frac{C}{(1+e^{(-\beta(t-M))})}$$

Gompertz equation [2]:

$$L(t)=A+C(e^{-e^{(-\beta(t-M))}})$$

In these equations:

L(t) = Log_{10} count of the number of bacteria at time (t) in hours, [Log_{10}(cfu/g)]

A = Asymptotic log_{10} count of bacteria as time decreases indefinitely (i.e., initial level of bacteria [Log_{10}(cfu/g)]

C = Asymptotic amount of growth thar occurs as t increases indefinetely (i.e., number of log cycles of growth) [Log_{10}(cfu/g)]

M = Time at which the absolute growth rate is maximum [h]

B = Relative growth rate at M [(log_{10}[cfu/g])/h]

The parameters used in the logistic and Gompertz functions were derived from the growth curves of *L. monocytogenes* in blue crab meat (Figure 1) according to the definitions given by Linton (1994). The logistic and Gompertz equations were then evaluated for these parameters and compared to the experimental curves obtained for each data set.

Modified Gompertz

A modified Gompertz function, to include the effect of temperature in the growth of *L. monocytogenes* in blue crab meat during refrigerated storage, was also

developed. Using the non-linear regression modeling program JUMP (SAS), the Gompertz function was fitted to each data set and the parameters for the equation obtained. The model fitted was [3]:

$$L(t)=(CTemp)e^{-e^{-((ATemp)+(BTemp)t)}}$$

The equation was then extended to predict growth of *L. monocytogenes* in the range of refrigeration temperatures used. The extended equation was [4]:

$$L(t)=(C_0+C_1Temp)e^{-e^{-((A_0+A_1Temp)+(B_0+B_1Temp)t)}}$$

where, L(t) = estimate of the bacterial growth [$\log_{10}$(cfu/g)]

A, B, C = model constants

Temp = storage temperature (°C)

t = time (d)

The parameters estimates from each individual data set were compared to the parameters obtained from the combined model.

RESULTS

A non-significant effect ($p>0.05$) of the initial microflora level upon the growth of *L. monocytogenes* was observed during the first 10 days of storage at any of the refrigeration temperatures used. After 10 days of storage the naturally occurring microflora had reached levels between 9 to 10 $\log_{10}$. Storage temperature

had a significant ($p \leq 0.05$) effect on the growth of *L. monocytogenes*, which was larger at lower levels of initial microflora.

From each growth curve, the parameters for the Gompertz [1] and logistic [2] functions were determined (Table 1) and used to obtain the predicted growth curves. The nonlinear fitting of the Gompertz function [3] including the effect of temperature resulted in three constants, A, B, and C (Table 2) when each set was analyzed in separate and in six constants A_0, A_1, B_0, B_1, C_0, and C_1 (Table 3) when the sets were combined and the extended model [4] fitted. The logistic and Gompertz functions gave a similar fit to the experimental data being always better at 5 °C than at lower growth temperatures. This can be due to the small increase in growth over the initial inoculum level at 1.1 and 2.2 °C. Both functions had increased correlation with the experimental data as the initial microflora decreased (Table 4). The modified Gompertz equation [3] provided the best fit to the experimental data when compared to the logistic [1] and Gompertz [2] functions (Figures 2-11). Correlation of the estimates for the growth of *L. monocytogenes* obtained from the modified Gompertz equation [3] is between 0.78 and 0.99 (Table 4). The extended modified Gompertz equation [4] had correlation coefficients between 0.84 and 0.95 (Table 4) with better correlation as the initial microflora decreased. Most deviations occurred for the predicted values after 10 days of storage at which time the crab meat was rendered inedible through microbial decomposition. When the modified Gompertz equation [3]

was fitted to the experimental data obtained during the first 10 days of storage, the correlation coefficients improved significantly (Table 4).

DISCUSSION

Storage temperature had a significant effect upon the growth of artificially inoculated *Listeria monocytogenes* in fresh crab meat, whereas the effect of different levels of naturally occurring microflora was only significant after it reached levels that rendered the meat inedible. The best fit to the experimental growth curve, was obtained from the modified Gompertz equation including the effect of storage temperature. The correlation of the estimates was improved when the model was fitted to the data corresponding to the first 10 days of storage, while the meat was edible. Using an extended modified Gompertz equation, to include the combined effect of temperature throughout the storage temperature range, a model was developed that allows for growth prediction of *L. monocytogenes* in fresh blue crab meat stored between 1.1 and 5 °C. As the initial microflora level decreases, the prediction becomes more accurate. The logistic and Gompertz equation can be used to obtain reasonable first estimates of the growth of *L. monocytogenes* for storage temperatures of 5 °C, since the growth curve becomes more sigmoidal producing a better fit.

Table 1. Gompertz and logistic function parameters for the growth of *Listeria monocytogenes* in fresh blue crab meat with varying levels of initial microflora during refrigerated storage.

	Initial APC < 15,000 /g			Initial APC 15,000-60,000 /g			Initial APC 60,000-100,000/g		
Parameter	1.1°C	2.2°C	5°C	1.1°C	2.2°C	5°C	1.1°C	2.2°C	5°C
A [log(cfu/g)]	1.62	1.62	1.62	1.29	1.29	1.29	1.35	1.35	1.35
C [log(cfu/g)]	1.33	0.97	3.73	1.14	1.16	2.43	1.58	1.58	2.82
B [log(cfu/g)/h]	0.006	0.012	0.011	0.005	0.025	0.007	0.014	0.011	0.014
M [h]	216	216	144	240	168	120	120	120	96

Table 2. Parameter estimates for the Gompertz function modified to include the effect of temperature upon the growth of *Listeria monocytogenes* in fresh blue crab meat with varying levels of initial microflora during refrigerated storage.

	Initial APC < 15,000 /g			Initial APC 15,000-60,000 /g			Initial APC 60,000-100,000/g		
Parameter	1.1°C	2.2°C	5°C	1.1°C	2.2°C	5°C	1.1°C	2.2°C	5°C
A	0.87	0.91	-0.24	0.60	0.61	0.14	0.40	0.40	-0.10
C	2.64	2.56	5.27	2.23	2.55	3.76	2.79	2.75	3.82
B	0.29	0.20	0.24	2.10	0.33	0.25	0.43	0.62	0.46

Table 3. Parameter estimates for the extended Gompertz function modified to include the combined effect of temperature upon the growth of *Listeria monocytogenes* in fresh blue crab meat with varying levels of initial microflora during refrigerated storage.

	Initial APC <15,000 /g	Initial APC 15,000-60,000 /g	Initial APC 60,000-100,000/g
A_0	1.26	0.87	0.64
C_0	1.77	1.77	2.33
B_1	0.04	-0.13	-0.003
C_1	0.66	0.38	0.28
A_1	-0.29	-0.15	-0.15
B_0	0.07	0.92	0.49

Table 4. Correlation of the estimates of the growth (log cfu/g) of *Listeria monocytogenes* to the experimental growth observed in fresh blue crab meat in the presence of varying levels of initial microflora during 21 days of refrigerated storage. Estimates of the growth (log cfu/g) of *L. monocytogenes* were obtained by 4 models.

Model	Correlation of estimates								
	Initial APC < 15,000 /g			Initial APC 15,000-60,000 /g			Initial APC 60,000-100,000/g		
	1.1°C	2.2°C	5°C	1.1°C	2.2°C	5°C	1.1°C	2.2°C	5°C
logistic	0.73	0.82	0.97	0.49	0.76	0.93	0.84	0.74	0.86
Gompertz	0.72	0.79	0.97	0.47	0.75	0.93	0.84	0.74	0.87
Modified Gompertz	0.85	0.87	0.99	0.91	0.78	0.97	0.92	0.83	0.88
Modified Gompertz (first 10 days only)	0.89	0.90	0.99	0.98	0.82	0.94	0.95	0.96	0.98
Extended Modified Gompertz	0.95			0.93			0.84		

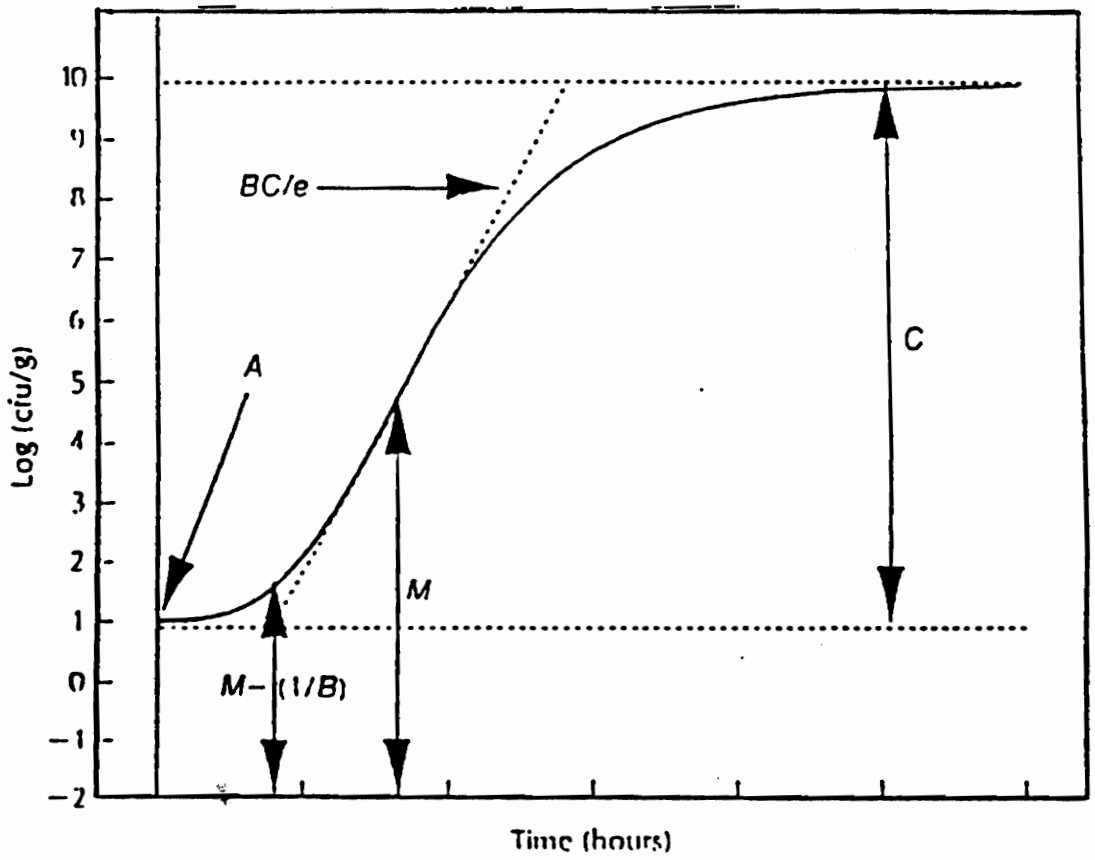

Figure 1. Parameters associated with the Gompertz function.

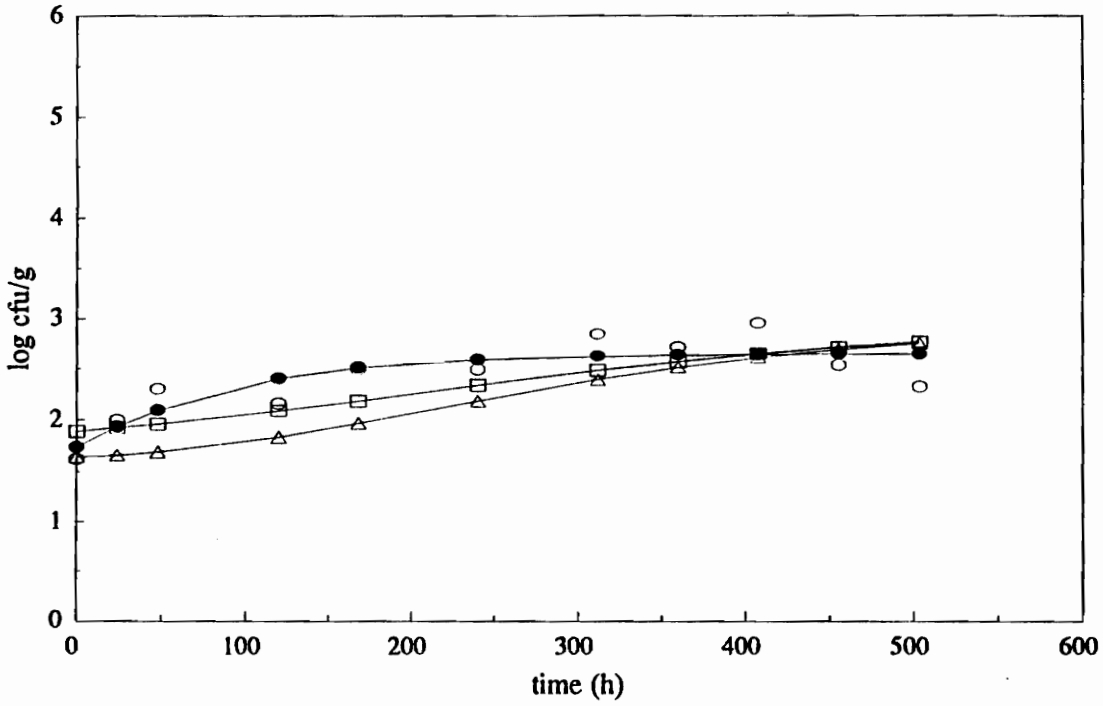

Figure 2. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 1.1°C and initial aerobic plate count less than 15,000 cfu/g.

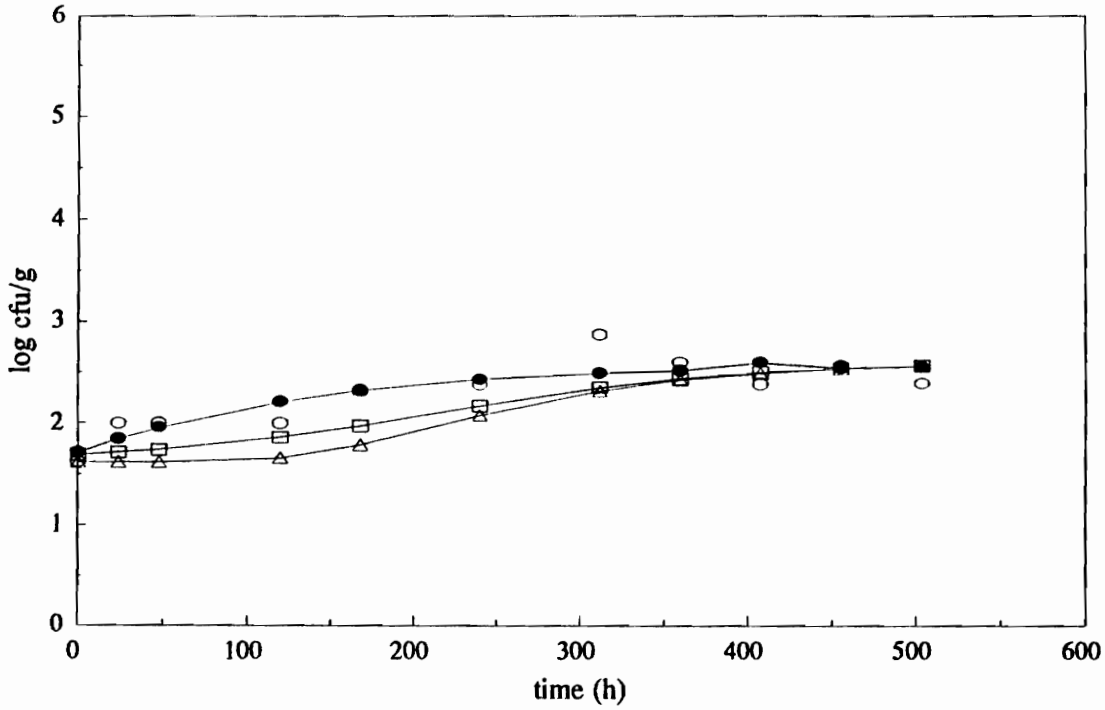

Figure 3. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 2.2°C and initial aerobic plate count less than 15,000 cfu/g.

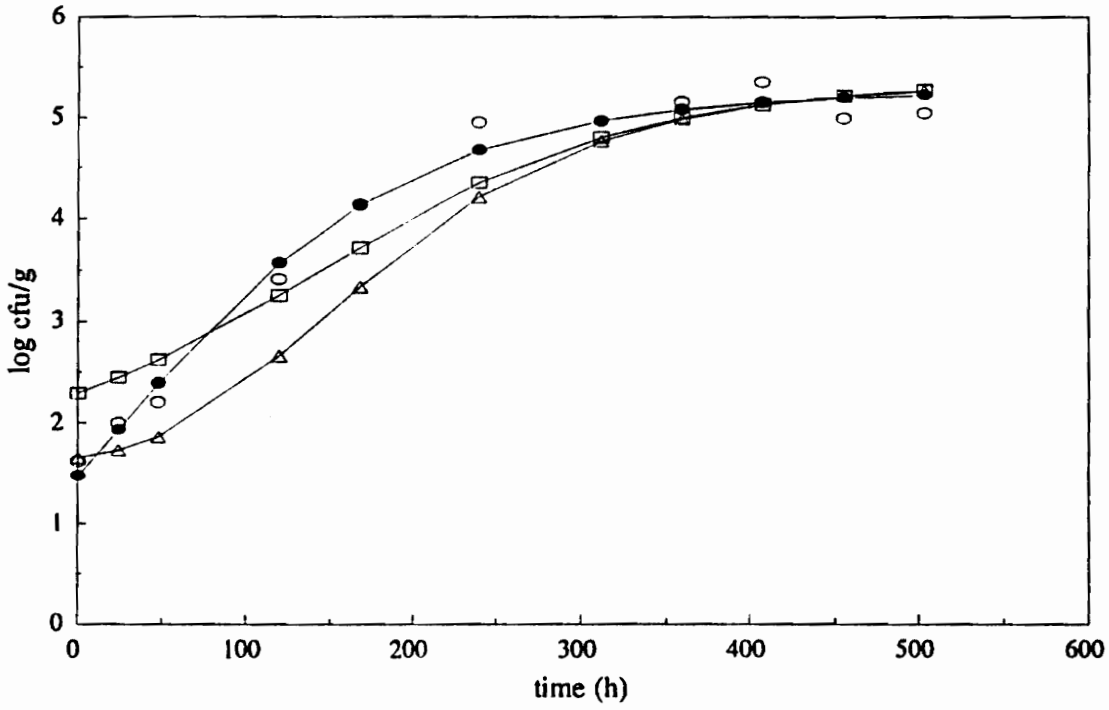

Figure 4. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-▵-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 5°C and initial aerobic plate count less than 15,000 cfu/g.

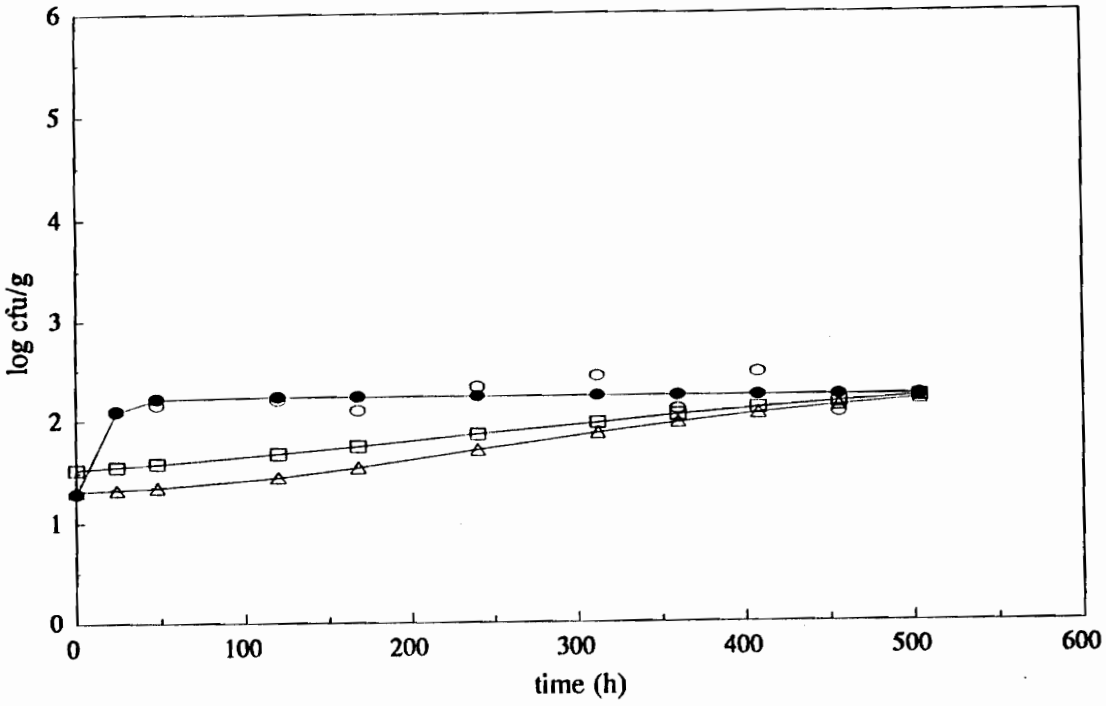

Figure 5. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 1.1°C and initial aerobic plate count between 15,000 and 60,000 cfu/g.

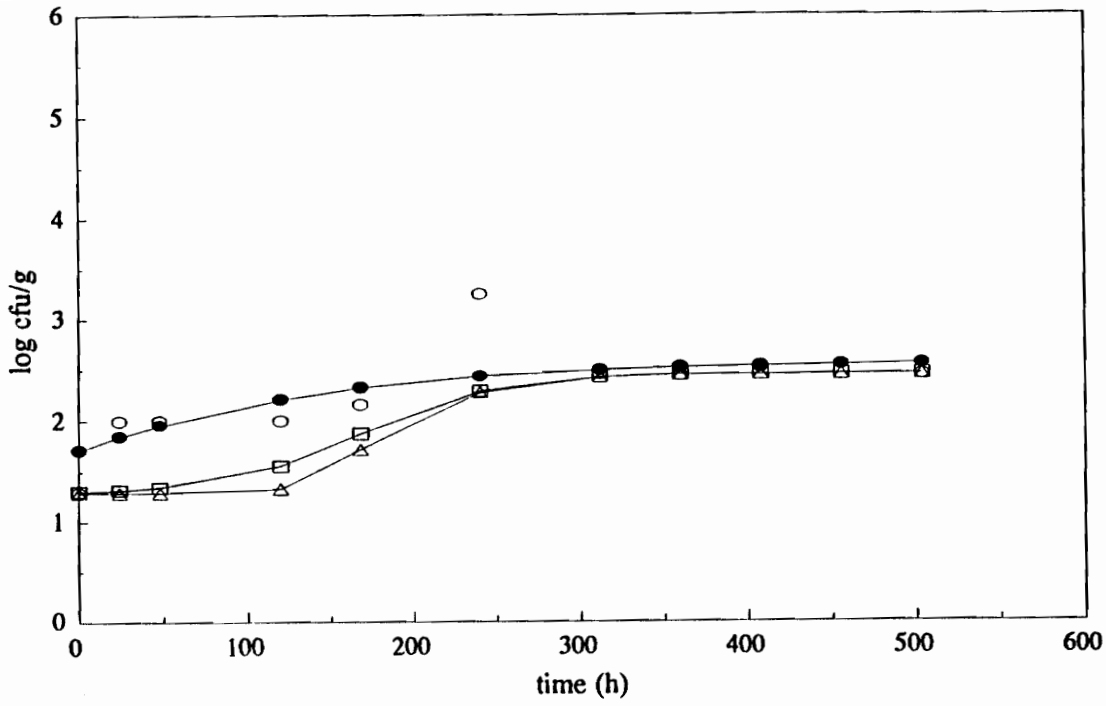

Figure 6. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 2.2°C and initial aerobic plate count between 15,000 and 60,000 cfu/g.

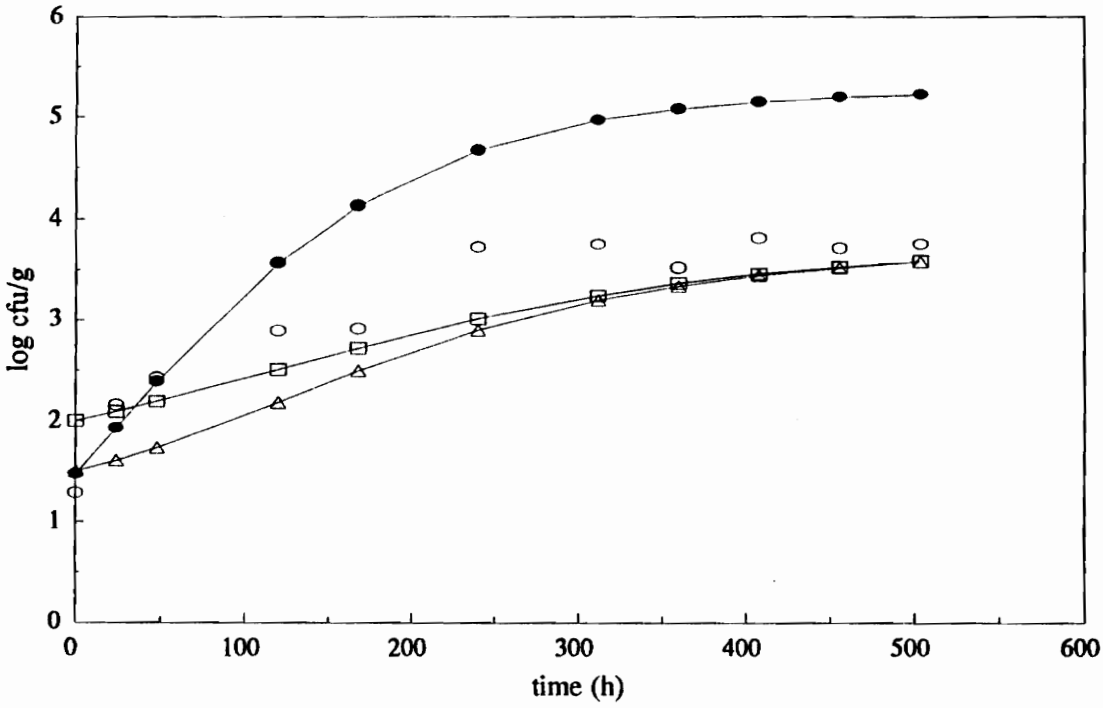

Figure 7. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 5°C and initial aerobic plate count between 15,000 and 60,000 cfu/g.

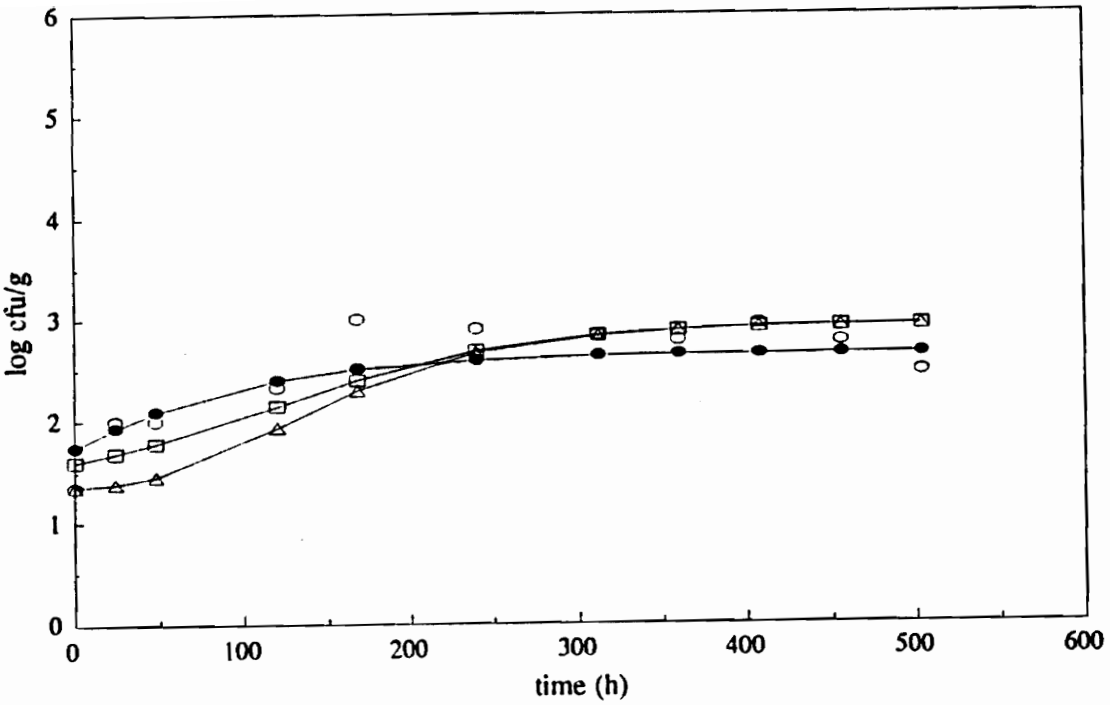

Figure 8. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 1.1°C and initial aerobic plate count between 60,000 and 100,000 cfu/g.

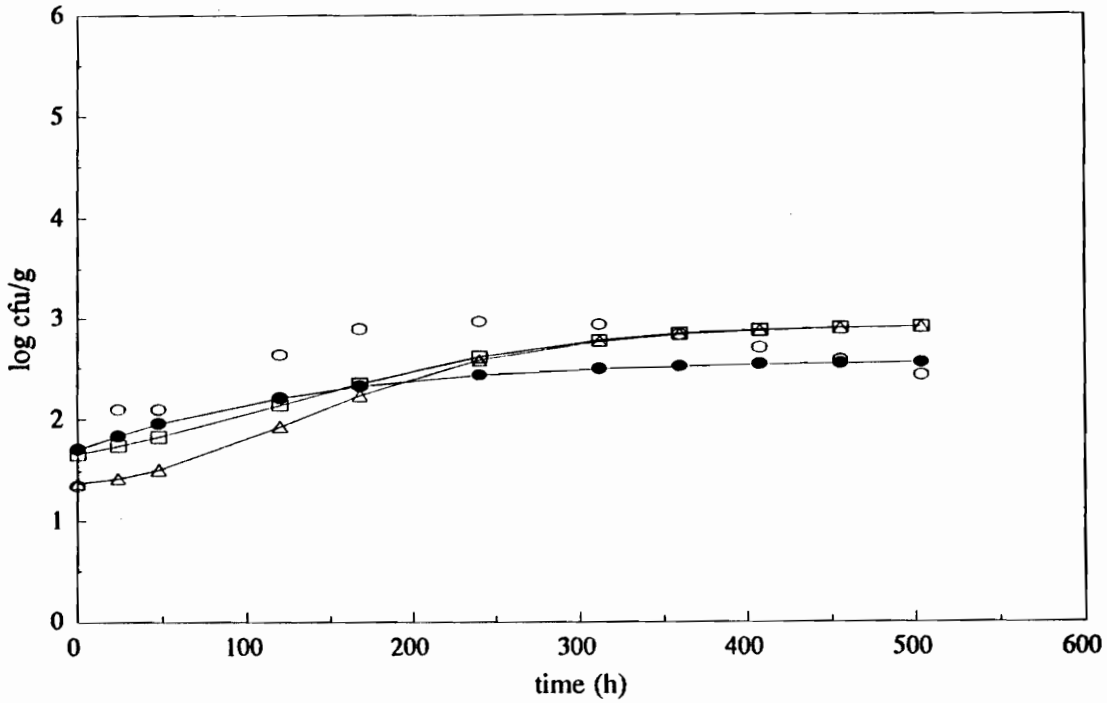

Figure 9. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 2.2°C and initial aerobic plate count between 60,000 and 100,000 cfu/g.

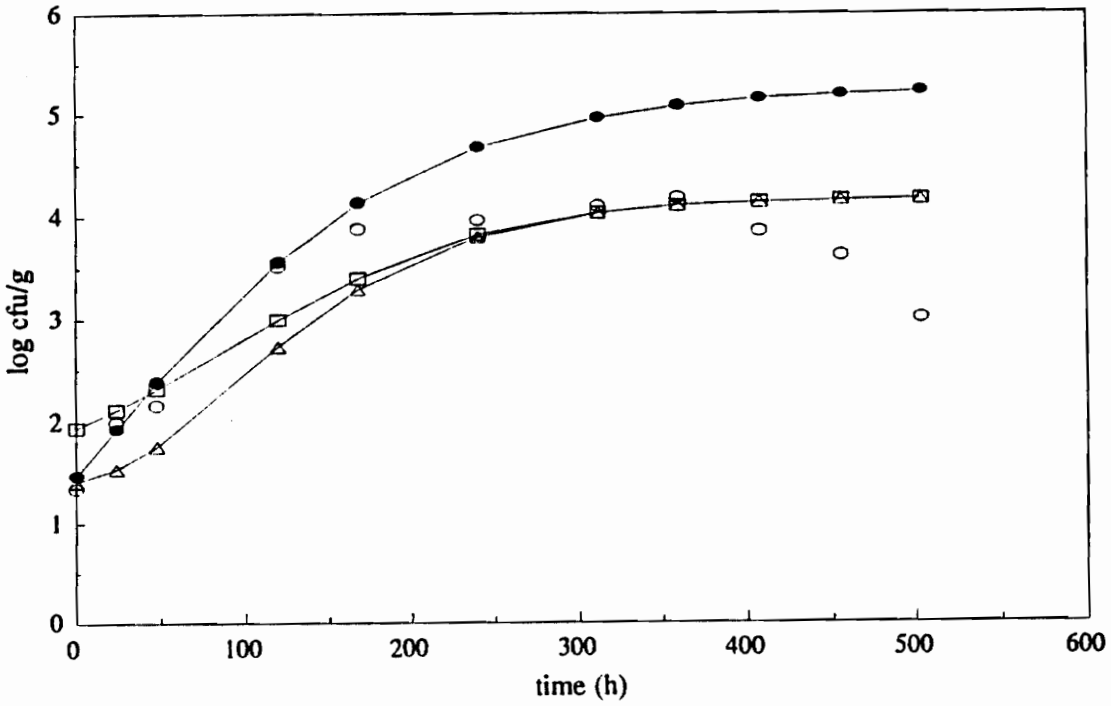

Figure 10. Correlation of estimates for the growth of *Listeria monocytogenes* (-○-) in fresh blue crab meat. Estimates obtained from a logistic function (-△-), Gompertz function (-□-), and a modified Gompertz equation (-•-). Storage temperature 5°C and initial aerobic plate count between 60,000 and 100,000 cfu/g.

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SUMMARY

In this study, the incidence of *Listeria monocytogenes* in freshly cooked and picked blue crab (*Callinectes sapidus*) meat was determined at approximately 8%. The levels at which the organism was found in this product were less than 100/g, averaging 50 cfu/g, with only one exception which was 1,100/g. These low levels agree with those previously reported by Ryu et al. (1992), who isolated *L. monocytogenes* in 6.1% of samples of fish and fish products, including ready-to-eat foods in Japan. The investigator also estimated the levels of *L. monocytogenes* to be less than 50 cfu/g. The ability of *L. monocytogenes* to grow at refrigeration temperatures was confirmed (Wilkins et al., 1972; Junttila et al., 1988), and generation times of 68.7, 31.4, and 21.8 h were observed at 1.1, 2.2, and 5°C in pasteurized crab meat inoculated with *L. monocytogenes* and stored for 21 days. No significant differences were observed in the growth of either *L. monocytogenes* or *L. innocua* inoculated at two levels, in three mixure ratios, simultaneously in pasteurized crab meat and incubated at 4 and 36°C. These results confirm the report by Petran and Swanson (1993) for the growth of these species in non-selective media and food systems. Although the natural microflora in crab meat had an inhibitory effect upon the growth of *L. monocytogenes* at refrigeration temperatures (1.1, 2.2, and 5°C), the suppression occurred after 13 days of storage at which the meat was inedible due to the high microflora (9-10 $\log_{10}$) levels. During the first 10 days of storage,

temperature had a significant effect ($p < 0.05$) on the growth of *L. monocytogenes*. The population of *L. monocytogenes* remained practically constant throughout the 21 days of storage at 1.1 and 2.2°C (between 2-3$\log_{10}$). Similar results were reported for precooked crawfish tail meat stored at 0°C, in which an increase in population of *L. monocytogenes* of less than 1 $\log_{10}$ was observed during 20 days of storage (Dorsa et al., 1993). During storage at 5°C, a higher growth rate in *L. monocytogenes* was observed, reaching a population maximum between 4 and 6 $\log_{10}$ after 10 days of storage. This low temperature *Listeria* growth, reinforces the importance of proper refrigeration for ready-to-eat products, since a population of *L. monocytogenes*, if present, increases exponentially with increasing temperatures. Fresh crab meat should constantly be maintained at 1.1°C or below until consumption, to decrease the potential of infection risk. A commercial microwave oven, with wave frequency of 2,450 MHz, was able to apply sufficient thermal process which resulted in non detection of *L. monocytogenes* after 5 days of storage. The results obtained in this experiment are encouraging in terms of the applicability of a microwave process to reduce or eliminate *L. monocytogenes* in ready-to-eat crab meat. Even though the experimental design was limited, it nevertheless reflects the *L. monocytogenes* incidence levels and commercial packaging and storage conditions for this product. A more detailed and extensive study should be performed for a final process design, however, the present feasibility study indicates potential success. A modified Gompertz function, to include the effect of temperature in the growth of *L.*

monocytogenes, was developed to model the growth of this pathogen during refrigerated storage. An extended version of this modified equation was developed to estimate the growth of *L. monocytogenes* in the range between 1.1 and 5°C.

VITAE

PERSONAL:

Born September 10, 1959 in Santiago, Chile.
Mother of two daughters, Daniela and Tatiana Canaval.
Married to Howard D. Rawles

EDUCATION:

Doctor of Philosophy in Food Science and Technology. May 1993 to present. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Master of Science in Food Science and Technology. January 1991 to April 1993. Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Thesis title: Constant current electronarcosis of market weight broilers and turkeys.

Bachelor of Science in Food Engineering. March 1977 to January 1984. Universidad de Chile, Santiago, Chile. Thesis title: Specific velocity of death and test of germicide efficiency, parameters to evaluate disinfectant action.

PUBLICATIONS:

Rawles, D., G. Flick, M. Pierson, A. Diallo, R. Wittman, and R. Croonenberghs. "Growth of *Listeria monocytogenes* in fresh blue crab (*Callinectes sapidus*) meat in the presence of naturally occurring microflora during refrigerated storage". In preparation.

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Diez de Medina, D., J. A. Marcy and M. Hulet. 1994. "Constant Current Stunning of Market Weight Broilers". **J. Poultry Res.** In Press.

Diez de Medina, D., J. A. Marcy and M. Hulet. 1994. "Constant Current Electronarcosis of Market Weight Turkeys". **J. Poultry Res**. In Press.

Diez de Medina, D. 1986. "Specific Velocity of Death and Test of Germicide Efficiency, Parameters to Evaluate Disinfectant Action". **Soc. Chilena de Tecnologia de los Alimentos**, Vol.11/3.

Diez de Medina, D. 1988. "Food Conservation Systems". **Revista Alimentacion y Salud**, Vol.23.

AWARDS:

Second place in the IFT Student Association/Phi Tau Sigma Graduate Research Competition held during the 1994 Annual Meeting of the Institute of Food Technologists.

IFT Seafood Technology Division Student Paper Competition Award, 1994.

PRESENTATIONS AT PROFESSIONAL MEETINGS:

Institute of Food Technologists, Atlanta, Georgia.
June 1994.

- Presentation of paper "Simultaneous Growth of *Listeria monocytogenes* and *Listeria innocua* in Fresh Blue Crab (*Callinectes sapidus*) Meat".
- Graduate Competition

Southeastern Poultry & Egg Association's Exposition, Atlanta, Georgia.
January 1993.

- Presentation of paper "Gender Differences of Broilers in Response to Constant Current Stunning".

PROFESSIONAL AFFILIATION:

Member of the **Honor Society of Phi Kappa Phi**.

Member of the Biological Sciences **Honor Society Phi Sigma, Alpha Psi Chapter**.

Member of the **Honor Society of Agriculture Gamma Sigma Delta**.

Member of the **Institute of Food Technologists**.

Member of the **American Society for Microbiology**.

Member of the **International Association of Milk, Food and Environmental Sanitarians, IAMFES**.

INDUSTRY EXPERIENCE:

Quality Control Manager
Alimentos Naturales Vitafoods, San Fernando, Chile.
August 1989 - December 1990.

Assistant Mgr., Quality Assurance and Production Coordination
Consorcio Agroindustrial Andes, S. A., Santiago, Chile.
February 1987 - July 1989.

Quality Control Manager
Agroindustria Agrofoods, San Fernando, Chile.
September 1985 - January 1987.

Consultant
Dimyl Ltda., Santiago, Chile.
January 1984 - August 1985.