Effect of heat and/or chemical shock on ability of

Listeria monocytogenes to survive exposure to sanitizers and attach

to stainless steel and meat surfaces

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfilment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Microbiology, Immunology, and Preventive Medicine
Major: Microbiology

Iowa State University
Ames, Iowa

1994

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ACKNOWLEDGEMENTS

First, I would like to thank God because through God all of this was possible. God has truly been good to me and has blessed me to successfully complete my degree requirements.

I would also like to express my sincere thanks to my family who has supported me throughout these challenging years, especially my mother. My mother, Ms. Eddye Jean Pickett had instilled in me hope, desire and belief that I can achieve even the highest of goals. My sincere thanks and never ending appreciation go to my dear mother for all that she has done.

My thanks go out to my committee members, Dr. Deland Myers and Dr. Dennis Olsen for being there when I needed them. I also would just like to thank them for there many suggestions and words of encouragement.

I would like to express my overwhelming and sincere appreciation to my major professor, Dr. Elsa Murano for her guidance, assistance, suggestions and gracious support throughout my research. Like any good professor, she noticed my weaknesses and gave all it took to help me strengthen them. I am so grateful for her having invested in me.

A special thanks go to Dr. George Jackson the Director of Minority Student Affairs for his encouragement and support throughout the years, especially during the rough times. His warm, friendly and helpful advice gave me the strength to carry on and I can never repay

him for showing such a tremendous amount of concern and consideration for me.

My final thank you go to the members of my family that I so dearly love and appreciate for just being who they are: my sister, Allacy; my brother, John; my cousin, Willie Mae; my Grandmother. Mrs. Dorothy; my Grandparents, Mr. and Mrs. Frank Jones; my nieces and nephews, Johnvis, Dedric, Erica, Johnvondavis, Johnetta, Jeanyarvis, Dantrel and Ashley; and my cousins, Monika, Miesha, Ida, Clantina and Adrian.

INTRODUCTION

Within the past decade *Listeria monocytogenes* has received much attention from regulatory agencies, the public and the food industry as one of the causes of foodborne illness. Although the organism is responsible for few outbreaks, the high mortality rate accompanied by infection, its wide distribution in nature, and its ability to grow at refrigeration temperatures have propelled *L. monocytogenes* to the spotlight among foodborne pathogens.

The organism has been isolated from raw and cooked products, as well as from food contact surfaces in processing plants. Attachment of the cells to surfaces such as stainless steel, plastics, and rubber gaskets occurs by production of polysaccharides that actually protect the organism from treatments with chemical sanitizers.

L. monocytogenes is able to respond to various stresses by synthesizing a series of proteins termed stress proteins. It has been shown that exposure of this organism to a mild heat stress, or heat shock, also increases the ability of the cells to survive a subsequent lethal heat treatment. Several organisms, including *L. monocytogenes* respond to other stresses such as cold, starvation, and chemicals in a similar manner as they do to a heat stress (heat shock). It has been postulated that exposure to one of these stresses may enable the organism to become tolerant to lethal treatments other than heat, and that it may effect such changes in the metabolism of cells as to induce the production of macromolecules such as polysaccharides.

For this study, we hypothesized that exposure of *L. monocytogenes* to sublethal levels of heat (heat shock) or sanitizers (chemical shock) would affect survival of cells to a subsequent exposure to lethal levels of the sanitizer, and that either or both stresses will affect the ability of cells to attach to various surfaces. The research presented as part of this thesis was an attempt at ascertaining the validity of this hypothesis.

Because *Listeria monocytogenes* is tolerant to the adverse conditions, food processing and sanitizing treatments done at plants may not be adequate treatments for eliminating the bacteria. Several studies have indicated that exposing *L. monocytogenes* to harsh and stressful conditions such as processing treatments and exposure to chemical sanitizers can result in survival of the organism to these treatments.

The purpose of this study was to determine the effect of heat and chemical shock on the ability of *L. monocytogenes* to survive the treatment of lethal concentrations of various chemical sanitizers. The effect of heat shock and chemical shock on the ability of *L. monocytogenes* to attach to meat and food contact surfaces was also investigated.

LITERATURE REVIEW

Listeria   monocytogenes

History

The first detailed description of the Gram-positive bacillus now known as *Listeria monocytogenes was* published in 1926. Murray et. al (1926) described a spontaneous epidemic of infection among laboratory rabbits and guinea-pigs caused by a bacterium which they named *Bacterium monocytogenes* because the infection was characterized by a monocytosis. A year later, a similar bacillus was isolated from gerbils by Pirie (1940) and he named it *Listerella hepatolytica.* The designation *Listerella* was chosen to honor Lord Lister, the well known pioneer bacteriologist (Gray & Killinger, 1966).

In 1940, Pirie suggested the bacterium be named *Listeria monocytogenes* because the generic name *Listerella* had already been used. The first report of listeriosis in humans was by Nyfeldt in 1929, who isolated *L. monocytogenes* from the blood of patients with an infectious mononucleosis-like disease (Gray & Killinger, 1966). Burn (1936) later reported that *L. monocytogenes* may cause infection in humans during the prenatal period and also meningitis in adults. The bacterium has now been isolated from an extremely diverse range of environmental sources, and has been reported to cause disease in a wide range of animals, including fish and insects (Gray and Killinger, 1966).

Characteristics

L. monocytogenes is a small, Gram-positive rod, 0.4-0.5-µm in length, with rounded ends (Seeliger and Jones, 1986). It may be occasionally seen in young cultures as short chains, lying parallel or in a "V" shape. Older cultures (3-5 days old) present more filamentous forms and may be gram variable. *Listeria* species are facultative anaerobic, nonsporeforming, non acid fast and do not form capsules. *Listeriae* are catalase positive and oxidase negative, hydrolyze esculin, and ferment glucose without production of gas. They are methyl red and Voges-Proskauer positive, do not produce indole or hydrogen sulfide, and do not hydrolyze urea. The bacterium is motile by a few peritrichous flagella when cultured at 20-25°C. The optimum temperature for growth is between 30-37°C; however, the organism can grow over a wide temperature range from 1 to 45°C.

Listeria cells grow well in complex media at a wide pH range from 5.6-9.8 (Seeliger and Finger, 1976), and in the presence of high concentrations of sodium chloride up to (10-12%) (Bille and Doyle, 1991). On solid medium such as nutrient agar, *Listeria* colonies are translucent, dew-drop-like and bluish gray by normal illumination; however, they show a characteristic blue-green sheen when exposed to 45° incident transmitted light (Seeliger and Jones, 1986). On blood agar at 37°C for 48 h, colonies may be dew-drop-like, and translucent, becoming grayish-white to opaque with age. Hemolysis may be observed on blood agar, but the zone of hemolysis may be so weak that

it may not be detected without removing the colony from the agar surface (Lovett, 1989).

Pathogenesis

Listeria monocytogenes can cause a wide range of symptoms in individuals infected with the bacterium. The minimum number of pathogenic cells causing listeriosis in humans varies depending on the serotype of the pathogen and host susceptibility to listeriosis (Marth, 1988; Bille and Doyle, 1991; Rocourt, 1994). Individuals at high risk for listeriosis are pregnant women, newborns, and immunocompromised patients (alcoholics, cancer victims, transplant recipients, people on hemodialysis, AIDS patients, etc...) (Gantz, 1975;Bortolussi et al., 1985; Fleming et al., 1985; Rocourt, 1994).

Mortality rates world wide range from 13 to 43%, with the highest mortality being among neonates with listeriosis (36%) (Farber and Peterkin, 1991). In 1987 listeriosis was the twelveth most frequent infection here in the United States, and is the fourth most costly in terms of medical intervention and modifications of the food processing network (WHO, 1993).

Meningitis is the most common manifestation in humans. The symptoms include severe headache, dizziness, drowsiness, stupor, stiff neck, and coma, Patients in untreated cases die within 1 to 3 days, and even when treated with drugs (tetracycline), high mortality rates are common.

Encephalitis is another manifestation occurring in humans infected with listeriosis. The flu-like symptoms which continue for 10 days are

headache, fever, and vomiting. This condition is not treatable and the patients die in another 2 to 3 days.

Prenatal infection of *L. monocytogenes* frequently causes fetal damage or infant death. Abortions occur in pregnant women, who have had listeriosis during the early pregnancy period. Usually in prenatal infections, the mother is not seriously ill, and up to 20% of women may actually be symptomatic carriers (Gray and Killinger, 1966).

Foodborne Outbreaks

There have been three well investigated listeriosis outbreaks involving foods during the 1980's. One outbreak reported in 1981 in the Maritime Provinces of Canada resulted in 41 infected victims and in 18 deaths (Schlech, et. al., 1983). The vehicle of infection was coleslaw which had been prepared by a regional manufacturer, and distribution was confined to the Maritime Provinces. A review of the sources of raw vegetable for the plant identified a farmer who raised cabbage grown in fields fertilized with manure of sheep kept by the farmer. Two of the farmer's sheep had died previously from listeriosis. Following the cabbage harvest, the crop was stored in a large cold-storage shed. *L. monocytogenes* is able to grow at temperatures so low that the period of cold storage acted essentially as a period of selective enrichment for the bacterium.

In 1983, a listeriosis outbreak in Massachusetts resulted in 49 people being hospitalized for meningitis and sepsis with 14 of those people dying (Fleming et al., 1985). The outbreak was associated with

consumption of contaminated pasteurized 2% and whole milk. The milk associated with the outbreaks came from a group of farms where bovine listeriosis was known to have occurred at the time of the out break. *L. monocytogenes* was isolated from raw milk from these farms, but there was no evidence of improper pasteurization procedures at the plant.

Another major listeriosis outbreak occurred in 1985 in California, involving 48 deaths from 142 cases (Linnan, 1988). Epidemiological investigation traced the origin of the bacteria to a Mexican-style soft cheese manufactured by a plant in southern California. Environmental samples taken from the plant producing the implicated cheese were positive for the bacteria of the epidemic phage type. The pasteurizer was found to be in good operational order, but *L. monocytogenes* was isolated from test samples of the cheese.

Heat Shock Response

The exposure of cells from a wide variety of species to sublethal temperatures can result in the enhanced synthesis of several proteins, which have been referred to as heat shock proteins (hsps) (Craig, 1985; Lindquist, 1986). The synthesis of these proteins is strongly stimulated by an environmental stress, in particular, as a result from a change in temperature to a few degrees centigrade above the normal physiological temperature (Schlesinger, 1986). The proteins are induced by a wide variety of other stresses, seem to have very general protective functions, and may well play a role in normal growth development

(Lindquist, 1986). This phenomenon has been called the heat shock response even though recovery from anoxia, ethanol, inhibitors of oxidative phosphorylation, and a number of other chemicals have been shown to induce the synthesis of the same proteins. Therefore, the response should, perhaps, more appropriately be referred to as stress response (Craig, 1985). This response is universal. It has been observed in every species examined, from eubacteria to archaebacteria, from mice to soybean (Lindquist, 1986).

The stress response was first seen to occur in *Drosophila melanogaster*, in 1962, when Ritossa observed that upon a shift from 20 to 37°C, as well as treatment with dinitrophenol of sodium salicylate, several new puffs appeared in the salivary gland polytene chromosomes (Ritossa, 1962). Over the next several years it became clear that the puffs were the sites of vigorous RNA transcription and that a number of these RNAs were translated into heat shock proteins (Craig, 1985). Analysis in a number of species has revealed that heat shock or related proteins are commonly present during normal growth and various developmental stages.

Evidence suggests that heat shock proteins may be involved in the development of thermotolerance, although the biochemical and molecular nature of this phenomenon is not well understood (Lindquist, 1988). When bacteria are shifted for a short period of time from lower to higher temperatures within or slightly above their normal growth range, a degree of protection against the lethal effect of a subsequent shift to a higher temperature (or an acquired thermotolerance) is

achieved (Mackey and Derrick, 1986; Neidhardt and vanBogelen, 1987; vanBogelen et al., 1987).

Most of what we know about heat shock in prokaryotes has been gathered from studies of the response in *Esherichia coli* (Neidhardt and vanBogelen, 1987). The heat shock response of *Esherichia coli* was discovered by examining a mutant that was temperature sensitive for growth (would not grow above 43°C) (Cooper and Ruettinger, 1975). Subsequent work led to the characterization of the mutant gene rpoH, as a positive-acting regulatory gene, and its protein product, sigma-32, as a sigma-like transcription factor required for the induction of HSPs (Tobe et al., 1984).

Regarding the applicability of the heat shock response in food microbiology, Tsuchido et al. (1974) exposed *Esherichia coli* K-12 to various temperatures (ranging from 0 to 45°) before challenging the cells at 50°C for 20 minutes, and found that the higher the temperature of the sublethal heat treatments, the greater the number of survivors at 50°C. They also heated *E. coli* to 50°C at different rates and found that the slower the rate of heating, the greater the number of survivors. Mackey and Derrick (1986) increased the heat resistance of *Salmonella typhimurium* by exposing cultures to sublethal temperatures at 42, 45, or 48°C for 30 minutes before exposing the organism to higher temperatures at 50 to 59°C. These investigators (Mackey and Derrick, 1987a) demonstrated the same effect with *Salmonella thompson* when the organism was preheated at 48°C and then heated in food systems such as 10% and 40% reconstituted skim milk, whole eggs and minced

beef. Increased thermotolerance occurred after exposure to 48°C for 30 minutes in all samples, with the increase in survivors ranging from 2 to 10 fold, depending upon the menstruum. Later, Mackey and Derrick 1987b reported that the rate of heating also affects the number of survivors to the heat challenge at 50 to 50°C. The heat resistance of *Salmonella typhimurium* increased by linear heating at rising temperatures before heating at 58°C (Mackey and Derrick, 1987b). A slower heating rate (0.6°C/minute) resulted in greater numbers of survivors than a faster heating rate (10°C/minutes).

The previously mentioned studies examined heat resistance in gram negative bacteria, but a study conducted by Quintavalla et. al. (1988) examined heat resistance in a gram-positive organism, *Streptococcus faecium*. They determined that the rate of heating of the cells to 65°C greatly influenced their D-value. The D_{65} value found for cells heated instantaneously was 5.4 minutes. Cells heated at 0.48°C/minute showed a D_{65} of 27.8 minutes, while cells heated at 0.13°C/minute had a D_{65} of 42.9 minutes, 5 and 8 times greater, respectively, than the instantaneously heated cells. Results from their study also indicated that lower initial cell concentrations (10^4 and 10^2 cells/ml) showed even greater D-values when heated at 0.13°C/minute, compared with both the higher cell concentration and the instantaneously heated cells (D_{65}=134.2 minutes for 10^2 cells/ml, D_{65}=1225.7 minutes for 10^4 cells/ml).

As a result of outbreaks of human listeriosis associated with pasteurized milk, a re-examination of the heat resistance of *L*.

monocytogenes was stimulated. Research involving heat resistance in this organism was carried out by several investigators. For example, Fedio and Jackson (1989) established that *L. monocytogenes* acquires increased thermotolerance in 2% milk during ultra high temperature (UHT) pasteurization. They conducted the study by heat shocking *L. monocytogenes* in trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) or 2% UHT treated milk at 48°C for 1 h. Heat-shocked cells survived a challenge of 60°C better than did non heat-shocked cells. Farber and Brown (1990) found that *L. monocytogenes* was capable of acquiring increased heat resistance in a meat product. They examined sausage meat in which *L. monocytogenes* had been heat-shocked for 2 h at 48°C. The meat inoculated with heat-shocked cells was then stored at 4°C for 24 h before challenging the cells at 64 °C for up to 8 minutes. After storage at the refrigeration temperature for 24 h, heat shocked *L. monocytogenes* retained its increased thermotolerance. Knabel et al. (1990) found that *L. monocytogenes* cells grown at 43°C were more thermotolerant than cells grown at lower temperature or cells that had been heat-shocked at 43°C (for 5, 30, or 60 minutes). They also found that increasing the length of heat shock increased the thermotolerance of *L. monocytogenes* cells with a heat shock treatment of 30 and 60 minutes at 43°C resulted in more survivors than a 5 minute heat shock. Knabel et al. concluded that growth of *L. monocytogenes* at 43°C for 18 h and enumeration by the use of strict anaerobic techniques resulted in $D_{66.8}$ values that were approximately 6-fold greater than those of cells grown at 37°C and

enumerated aerobically. Linton et al. (1990) examined thermotolerance in log phase *L. monocytogenes* cells. The cells were exposed to 40, 44, or 48°C for up to 20 minutes, followed by a heat challenge at 55°C for 50 minutes. The authors found that, for log phase cells, the optimum condition for heat shocking was 48°C for 20 minutes. The results from this investigation using log phase cells confirmed similar work by Fedio and Jackson (1989) and Knabel et al. (1990) who used stationary cells.

Smith and Marmer (1991) examined the effect of growth temperature on heat tolerance of *L. monocytogenes*. The lower the temperature of growth, the less thermotolerant the cells were. Protein synthesis appeared to be involved in thermotolerance, although the role of the synthesized proteins was not determined. The temperatures used in the experiment were all within the normal rang of growth for the organism (10, 19, 28 and 37°C).

Heat-shock proteins are generally defined as those whose synthesis is induced at high temperatures (Lindquist, 1986). *L. monocytogenes* has been shown to synthesize 12 to 14 heat shock proteins ranging in size from 20 to 120 kilodaltons following incubation of the organism at 48°C (Sokolovic and Goebel, 1989). One of the proteins produced under heat-shock conditions was found to be listeriolysin, an essential virulence factor in *L. monocytogenes* (Sokolovic and Goebel, 1989). Listeriolysin was found in the supernatant of the heat-shocked cells, whereas the other heat shock proteins remained associated with the cell (Sokolovic and Goebel, 1989).

Sanitizing Agents Used in the Food Industry

Sanitizers are chemical compounds which are used to reduce the number of microorganisms on and within surfaces. Surfaces must be cleaned to remove grease, films, soil and debris, and rinsed before sanitizing solutions are applied. Goldenberg and Relf (1967) described sanitizers or disinfectants suitable for food use as follow: (a) must be efficient for conditions of use; (b) must be safe for use by those applying it; (c) must not influence the flavor or odor of food process by equipment sanitized by its use; (d) should leave no toxic residue; and (e) should be easy to use.

Sanitizer activity or effectiveness is affected by exposure time, pH, temperature, concentration, water hardness, and surface cleanliness (Bakka, 1991). Many sanitizers are available and the selection and use will correspond to the needs of a given food production facility.

Several common sanitizers are discussed here. They include the basic types of sanitizers which are approved for use in the food industry. These are acid anionic sanitizers, chlorine-based sanitizers, iodophors and quaternary ammonium compounds (quats). Also discussed are organic acids such as citric, lactic, and propionic which are used in the food industry for their antimicrobial activity in addition to other properties.

Acid anionic Sanitizers

Acid anionic sanitizers are anionic surfactants used as antimicrobial agents especially in automated cleaning systems, which combine sanitizing with a final rinse (Giese, 1991). The sanitizers are combinations of an acid, usually phosphoric with an anionic detergent, such as dodecyl benzene sulfonate, long-chain alcohol sulfates, sulfonated olefins or sulfated ethers (Troller, 1993). Some studies have shown that the combination is synergistic rather than additive (Dychdala and Lopes, 1991). Acid anionic surfactants are usually present as alkali or amine salts of long-chain fatty acids or alkane sulfonates (e.g. $R\text{-}COO^-Na^+$, $R\text{-}SO_3^-Na^+$; $R = C_{10}\text{-}C_{12}$ alkyl) (Paulus, 1993). Acid anionic sanitizers generally have antimicrobial activity against vegetative cells of both gram-negative and gram positive species; however bacterial and fungal spores are resistant (Troller, 1993). The bactericidal action of acid anionic sanitizers has been reported to be rapid (within 30 seconds) on a number of bacteria that are of particular importance to the dairy industry (Dychdala, 1977). The microorganism are destroyed when the cell membranes and cell permeability are disrupted by the action of the sanitizer (Snyder, 1992). Viruses also are inactivated by these compounds (Troller, 1993).

The maximum antimicrobial effectiveness of this product is at pH below 3.0, thus alkaline waters decrease the effectiveness of the sanitizers (Snyder, 1992). Acid anionic sanitizers are well suited for cleaning stainless steel surfaces and can prevent mineral deposits from accumulating (Giese, 1991)

Chlorine-based   Sanitizers

Chlorine-based sanitizers include hypochlorites, chlorine dioxide, gaseous chlorine, chloramines, and organic chlorinated compounds like sodium or potassium dichloroisocyanurate. The chemistry of chlorine can basically be described as follows:

$$Cl_2 + H_2O \longleftrightarrow HOCl + HCl$$
$$NaOCl + H_2O \longleftrightarrow HOCl + NaOH$$

Free chlorine is defined as hypochlorite ion (OCl^-), hypochlorous acid ($HOCl$) and elemental chlorine (Cl_2) (Hall et al., 1981).

$$HOCl \longleftrightarrow H^+ + OCl^-$$

In solution, hypochlorous acid is formed, the compound believed responsible for microbial destruction. Many mechanisms of action are proposed (Mariott, 1985 and Troller, 1993), the predominant one being enzyme destruction through oxidation of sulfhydryl groups. The formation of hypochlorous acid is temperature and pH dependent: as pH increases, activity decreases. However, at alkaline pH, proteins are cleaved or peptized to chloramines. All microbes are destroyed by hypochlorous acid, and the effectiveness against spores is greater than using iodine (Mariott, 1985). Some typical uses are treatment of drinking water, waste water treatment programs, and sanitizing equipment and surfaces. Chlorine-based sanitizers normally are employed at concentrations that provide 100 or 200 ppm available chlorine when used as surface sanitizers (Troller, 1993).

Iodophors

Iodophors, created when iodine is complexed with carriers such as polyvinylpyroliodone or a surface active agent such as alkylphenoxypolyglycerol ether, is one of the most popular forms of iodine compounds used today (Troller 1993). In aqueous solution iodine could be present in acid form as I_2 or in alkaline form as IO^-, IO_3^-, I^-, or I_3^-. This iodine and surface active compound is active against both gram-positive and gram-negative bacteria and more active against viruses than other sanitizers (Snyder, 1992). The iodophors are used for cleaning and disinfecting equipment and surfaces, in water treatment and as a skin antiseptic. The mechanism of action has not been studied in detail. As with chlorine compounds, when the pH decreases, activity increases. When used in acid solutions, iodophors act as surface active agents, sanitizers and prevents scale build-up. Iodophors are more costly than chlorine-based sanitizers; however, they normally are used at much lower concentrations (12.5-25 ppm) than the latter (Troller, 1993). Iodophors uses are more advantageous than chlorine-based sanitizers because they are less irritating to the skin, less corrosive to metals, and are not affected by organic acids as much (Snyder, 1992). The disadvantages of using iodophors are their narrow effective pH range of 4.5-5.5 and their ability to vaporize above 50°C (Marriott, 1985).

Quaternary Ammonium Compounds

Quaternary ammonium compounds (quats) have wetting and penetrating properties which are good for cleaning porous material, but quats are primarily used as sanitizers. Long chain quaternary ammonium compounds are cationic surface active agents and their general formula is $R_4N^+X^-$, where X is usually chloride or bromide (Paulus, 1993). Quats are synthesized when tertiary amines are reacted with alkylhalides. Because quats are strongly cationic in solution, they are incompatible with anionic detergents (Troller, 1993).

Quats are more effective against gram-positive bacteria and not as effective against gram-negative bacteria. Spores are not killed, but growth is inhibited. The method of action is not well understood, but involves cell leakage and enzyme inhibition (Mariott, 1985). Troller (1983) stated that quats coat the cell which is reasonable, considering their surface active property. Because quats form a film on surfaces, residual activity remain after sanitization, but this may be a disadvantage when quats need to be rinsed from food contact surfaces. Quats are more effective sanitizers in the presence of soil than chlorine and iodine sanitizers.

Organic Acids

Organic acids and their derivatives that are typically used as antimicrobial agents include acetic acid, benzoic acid, propionic acid, sorbic acid, lactic acid and succinic acid. The effectiveness of organic acids as antimicrobial agents was explained in relation to three factors by Ingram et al. (1956). These factors include: (1) the effect of pH; (2)

the effect of acid dissociation and (3) the specific effect of the antimicrobial agent. Differences in pH affect the antimicrobial properties of acids; for example, an unbuffered system consisting of 0.1 M HCl at pH 1.0 can kill most microorganisms, including yeasts and molds, while a 0.1 M acetic acid solution at pH 3 has little effect on microorganisms. The pK_a value (pH at which 50% of an acid is dissociated) of most antimicrobial agents fall in the pH range 3.0-5.0. As the pH is lowered, the concentration of the undissociated acid increases, leading to more effective antimicrobial activity. The undissociated acid may passively diffuse through the cell membrane (Cramer and Prestegard, 1977). Inside the cell, the undissociated acid splits into anions and protons in response to the internal pH of the cell which is close to neutrality. This leads to intracellular acidification which may result in loss of cell viability or in cell destruction. If the pH of the medium is above the pk_a of the weak acid, the acid dissociates outside the cell, and is unable to enter the cytoplasm, rendering the chemical mostly ineffective. In contrast to weak acids, strong acids like HCl inhibit bacterial growth by complete dissociation, instantly reducing the pH of the surrounding medium (Levine and Fellers, 1940).

Most industrial methods to reduce bacterial numbers on the surface of animal carcasses include sprays. Cold, hot and chlorinated water sprayed onto meat significantly reduces the number of microorganisms present (Gill and Newton, 1980; Geer, 1981; Morrison and Fleet, 1985; Statham et al., 1985). Organic acids have also been used effectively to reduce microbial populations on animal carcasses

(Biemuller et al., 1973; Firstenberg-Eden, 1981; Smulders and Woolthuis, 1985). Dickson (1991) used 0.5, 1.0 and 2.0% acetic acid in different spray cycles and reported reductions in the populations of *Salmonella typhimurium*, *Listeria monocytogenes*, and *Esherichia coli* 0157:H7 on treated carcasses. The use of short chain organic acids as decontaminants has emerged as a possible preventive measure. Although many acidic compounds may reduce bacterial counts in meats, they have a negative effect on color and flavor (Firstenberg-Eden, 1981).

Attachment of *L. monocytogenes* to Food Contact Surfaces

L. monocytogenes has received considerable attention in the food industry because it is ubiquitous in nature and has a high resistance to adverse conditions as compared to other pathogens. The bacterium is often isolated from air, dust, drains, floors, walls, ceilings, conveyor belts, external surfaces of milk cartons and machines in food processing plants (COXA et al., 1989). Its high resistance allows it to survive, grow and reproduce in the harsh environment of food processing plants such as the surface of stainless steel equipment. Herald and Zottola (1988) determined that *L. monocytogenes* was capable of attaching to stainless steel at various pH values and temperatures. Their findings suggested that *L. monocytogenes* attached to stainless steel at 10°C, 21°C, and 35°C. However, attachment and development of fibrillar material was most common at 21°C. The pH of the medium was important and when cells

were grown at pH 8.0 the attachment matrix was more evident at 21°C than at 35°C. When grown in a shaking environment, the attachment of *L. monocytogenes* to stainless steel was reduced. The investigators also reported a direct relationship between numbers of attached cells and incubation time. Spurlock and Zottola (1991) demonstrated attachment of *Listeria* to free-standing cast iron floor drains containing reconstituted skim milk or tryptic soy broth with yeast extract and survival after a month. Populations of *L. monocytogenes* from drains containing reconstituted skim milk were an average of (10^6-10^7 CFU/ml) 2-log cycles lower than populations recovered from drains containing trypticase soy broth with yeast extract (a nutrient rich medium for the growth of the test organism) when compared to dilute solution of dried nonfat milk. The work of Krysinski et al. (1992) showed adherence of *L. monocytogenes* to polyester and polyurethane conveyor belts. They found that the type of surface (stainless steel, polyester, polyester/polyurethane) had little effect on the rate of cell attachment but affected the efficacy of various sanitizers and cleaners. This work also confirmed adherence of *L. monocytogenes* to stainless steel. The micrographs in these studies always showed sparse cell attachment and the lack of large microcolony development rather than confluent accumulation.

The ability of microorganisms to become more resistant to sanitizers and other antimicrobial agents once they become attached to a surface has been documented in aquatic environments (LeChevallier et al., 1988) and medical prosthetic devices (Anwar et al., 1990). Frank

and Koffi (1990) examined the effect of sanitizers on adherent microcolonies of *L. monocytogenes*. They found that attachment of cells to surfaces and subsequent biofilm formation provided cells with resistance against sanitizers. Lee and Frank (1991) reported that allowing *L. monocytogenes* to grow on stainless steel surfaces for a matter of days (8 days in which microscopic examination revealed the presence of microcolonies) significantly increased its resistance to hypochlorite sanitizer. Mafu et al., (1990) reported that the concentration of each sanitizer needed to be higher at 4°C than at 25°C to destroy *L. monocytogenes* attached to stainless steel, glass, and rubber.

Attached microorganisms, sometimes referred to as biofilms, represent a potential problem to the food industry especially when pathogenic bacteria attach to food processing equipment. Biofilm consists of extracellular products; mainly polysaccharides, and of surface-colonizing microbes (Wirtanen and Mattila-Sandholm, 1993). It is produced by the microbe to protect the cells from hostile environments and to trap nutrients (Brown et al., 1988; Costerton et al., 1985). Unless the biofilm is removed, the attached organisms could contaminate a processing system even though the system was flushed with a sanitizer prior to production (Fleming and Gessey, 1991).

L. monocytogenes is of particular interest since this pathogen is widespread in the environment (Tiwari and Alenrath, 1990), grows under refrigerated conditions and is a frequent resident in certain food processing establishments (Gellin and Broome, 1989). *L. monocytogenes*

has been reported to attach to a variety of surfaces (Mafu et al., 1990) and in the case of stainless steel, the adherent organisms demonstrated significant resistance to conventional chemical sanitizers and heat (Frank and Koffi, 1990).

Bacterial Attachment to Meats

Bacterial attachment is presumed to be the first step in the contamination of solid surfaces. It is generally accepted that bacterial attachment occurs as a two stage process: reversible and irreversible attachment (Marshall et al., 1971). The first stage in which attachment is reversible the bacteria still show Brownian motion and are easily removed (e.g. merely by rinsing). In the second phase, called irreversible, much stronger forces are required to remove the bacteria (e.g. scraping, scrubbing). Marshall et al. (1971) defined irreversible attachment as a time dependent firm adhesion, where bacteria did not show Brownian motion any longer, and could not be removed by washing. The authors also suggested that polymer bridging was responsible for the strong hold of bacteria onto the surface, the polymers, being regarded as bacterial response to nutrients accumulated on the surface.

Bacterial attachment to meat is a complex phenomenon which, at present is not fully understood. Several studies have attempted to find the mechanism and factors that may influence bacterial attachment. Studies have shown bacterial attachment to meat surfaces is influenced

by cell surface charge (Fletcher and Loeb, 1979; Dickson and Koohmaraie, 1989), hydrophobicity (Van Loosdrecht et al., 1989a,b), and by the presence of particular cell surface structures such as flagella, fimbriae, and extracellular polysaccharides (Fletcher and Floodgate, 1973).

The nature of the surface structures involved in attachment (e.g. cell wall and surface meat) can also play an important role in the attachment (Beachey, 1981). Other factors such as temperature, pH, culturing method, etc., may also be involved (Firstenberg-Eden, 1981).

The study by Dickson and Koohmaraie (1989) described the involvement of cell surface charge in bacterial attachment. They reported that the major contributing factor to attachment to lean beef tissue was the net negative charge on the bacterial cell. They found that there was a linear correlation between the relative negative charge on the bacterial cell surface and initial attachment to lean beef muscle.

In regards to hydrophobicity, Dickson and Koohmaraie (1989) also reported that an increase in attachment to fat tissue surfaces correlated with an increase in both negative charge and hydrophobicity. Van Loosdrect et al. (1987b) indicated that bacterial cell surface hydrophobicity is important in bacterial attachment. They demonstrated that attachment increased as both negative charge (electrophorectic mobility) and hydrophobicity (contact angle) increased.

Several reports indicate that the flagellated bacteria attach more readily than nonflagellated bacteria to poultry and red meat surfaces

(Butler et al., 1979; Farber and Idziak, 1984; Notermans and Kampelmacher, 1974). There is disagreement over the role and importance of flagella in the attachment process. The early work of Notermans and Kampelmacher (1974) emphasized the importance of flagella and their activity in the attachment of bacteria to skin of broiler chicken. These authors found that flagellated bacteria consistently attached to poultry skin while non-flagellated bacteria rarely attached. However, McMeekin and Thomas (1978) were unable to confirm these results and Lillard (1985) indicated that nonflagellated bacteria attached as readily as flagellated bacteria to poultry skin. Research by Lillard (1986) using electron microscopy indicated that microbes approach the surface of chicken skin and muscle in a thin water layer following immersion in aqueous suspensions of from various post-slaughter cleaning regimens. The author concluded that attachment of bacteria to poultry skin is a very complex phenomenon which may involve other mechanisms besides fimbriae, flagella, or water uptake.

In natural habitats, bacteria will attach firmly to surfaces, and the immobilized bacteria grow, forming microcolonies and produce exopolymers which often extend from the cells. Firstenberg-Eden et al. (1978) showed that bacterial attachment to both chicken and beef can be expressed in terms of and "S" value, which was intended to differentiate between bacterial cells which were "strongly" attached to the tissue surface and those which were "loosely" attached (trapped in a moisture layer on the surface). Firstenberg-Eden (1981) reviewed the early literature on the attachment of bacterial cells to meat and

concluded that during the initial stage, attachment was regulated by physical forces and the secondary stage was regulated by bacterial production of extracellular polysaccharides.

Costerton et al. (1978) claims that bacteria stick by means of a mass of tangled fibers of polysaccharides or branching sugar molecules that extend from the bacterial surface and form the "glycocalyx" that surrounds the cell or the colony. The "glycocalyx" positions the bacteria to the surface, channels various nutrients and conserves digestive enzymes released by the bacteria.

Some bacteria are able to attach to meat surfaces better than others (Firstenberg-Eden et al., 1978). The kinetics of attachment depend on the meat surfaces as well as the individual bacterial species. Dickson (1988), working with beef muscle and fat tissue washed with various compounds, reported that there were significantly more bacteria removed or destroyed from fat tissue than from lean tissue. He hypothesized that this difference could be attributed to the bacteria being more protected from the toxic effect of the compounds on muscle tissue than on fat and it may also be partially attributed to enhanced physical removal of the bacteria by saponification of the fat. Chung et al. (1989) reported no competitive interactions between several strains of spoilage and pathogenic bacteria during attachment to lean or fat tissue. The different bacteria neither enhanced or interfered with the attachment of the other bacteria.

The effect of temperature and pH on the process of bacterial attachment has also been studied. Notermans and Kampelmacher

(1974) reported attachment to be optimal at 21°C and pH 8.0, while Butler et. al. (1979) found pH and temperature to have very little effect on the extent of bacterial attachment. Notermans and Kampelmacher (1974) found that attaching rate increased with an increasing number of bacteria in the attachment medium. McMeekin and Thomas (1978) however, found that the time of immersion in the bacterial suspension had little effect on the retention of bacteria.

Bacterial attachment may also be affected by the nutrients available. When nutrients are limited, slime and fibers are produced, possibly affording protection and concentration of nutrients (Firstenberg-Eden, 1981). While studying the mechanism of secondary attachment to cow teat, Firstenberg-Eden (1981) observed production of extracellular polymers for all test bacteria examined. Costerton et al. (1978) stated that "in nature, bacteria are subject to many sources of stress, against which fibers of polysaccharides may offer protection".

L. monocytogenes has been found in meat, milk, and vegetable products in which each have been implicated in human listeriosis outbreaks (Schlech et al., 1983; Fleming et al., 1985; Schwartz et al.,1989). The association of *L. monocytogenes* with several large foodborne outbreaks raised immediate concerns in the food industry. Consequently, this led to a series of studies that examined the heat resistance of *L. monocytogenes* which in some investigations found the bacteria to have increased thermotolerance (Mackey and Derrick, 1986; Knabel et al., 1990; Linton et al., 1990).

Since *L. monocytogenes* is ubiquitous in nature, the potential for surface contamination is high. As indicated before, attachment is the first step in bacterial contamination (Firstenberg-Eden, 1981). Studies have indicated that bacteria production of extracellular material can assist in attachment to surfaces, and can also provide protection against heat and sanitizers (Herald and Zottola, 1988). The resistance of *L. monocytogenes* to heat can allow it to survive, grow and reproduce in harsh environment of food processing plants. The objective of this study was to investigate whether heat/and or chemical shock can have an effect on the survival of *L. monocytogenes* to industrial sanitizers, and whether it also can affect the attachment of this organism to meat and stainless steel surfaces.

MATERIALS AND METHODS

Growth of *Listeria monocytogenes* Culture and Preparation of Sanitizing Solutions

Culture

Listeria monocytogenes strain Scott A was obtained from the Iowa State University Department of Food Science and Human Nutrition (Ames, IA). The stock culture was maintained through monthly transfers on slants of trypticase soy agar (TSA; BBL Microbiological Systems, Cockeyville, MD) supplemented with 0.6% yeast extract (Difco Laboratories, Detroit, MI) (TSAYE) and stored at 4°C. Before use, the culture was grown in trypticase soy broth (TSB; BBL) supplemented with 0.6% yeast extract (Difco) (TSBYE) overnight (at least 12 h) at 37°C.

Sanitizers

L. monocytogenes was exposed to four commonly used plant sanitizers which included: an acid anionic sanitizer containing 15% dodecylbenzene sulfonic acid and 50% phosphoric acid as the active ingredients (CD-640, Chemidyne Corporation, Macedonia, OH), a chlorine-based containing 10% sodium hypochlorite (10-Chlor, Birko Corporation, Denver, CO), an iodophor containing Alpha-(P-nonylphenyl)-Omega-hydroxypoly (oxyethylene)-iodine complex (providing 1.75% titratable iodine) (Birkodyne, Birko Corp.) and a quaternary ammonium compound (quat) containing n-Alkyl (68% C_{12}, 32% C_{14}) dimethyl ethylbenzyl ammonium chlorides and n-Alkyl (60%

C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) (Bi-quat, Birko Corp.). Organic acids included certified grade citric, lactic, and propionic acids (Fisher Scientific Co., St. Louis, MO).

Preparation of sanitizing solutions

All dilutions of sanitizing agents were prepared in 99-ml quantities in sterile 250-ml dilution bottles on the day of the test. Chlorine, iodophor, quat and organic acids were diluted in sterile 0.1% peptone (Difco, Detroit, MI), while acid anionic sanitizer was diluted in sterile deionized distilled water. After the solutions were prepared each was filter-sterilized using a pre-sterilized filter apparatus (Costar Corporation, Cambridge, MA) containing a membrane pore size of 0.45 μm.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was obtained by inoculating 10^3-10^4 *L. monocytogenes*/ml into sterile 13 x 100-mm tubes containing serial two-fold dilutions of the sanitizing solutions, and incubating at 37°C for 48 h. Each test was done in triplicate. Growth was measured by turbidity at 650 nm after 24 and 48 h using a spectrophotometer (Spectronic 20, Bausch & Lomb, Rochester, NY). The average of the lowest concentration of each sanitizer that showed an optical density (O.D.) value greater than or equal to 0.1 was designated as the MIC. The number of bacteria present in the cell suspension was determined by O.D., using a standard curve relating bacterial numbers (obtained by standard plate count) with optical densities.

Effect of Heat Shock on Survival of *L. monocytogenes* to MICs of Sanitizers

Heat Shock of *L. monocytogenes*

A 0.1-ml inoculum of an overnight (12 h) culture of *L. monocytogenes* was inoculated into 5 ml of TSBYE. The culture was incubated for 6 h and one ml was transferred into 5-ml of TSBYE that was pre-heated at 48°C in a water bath (model 730, Fisher Scientific, Pittsburgh, PA). The inoculated tube remained in the water bath for 15 min. This temperature was chosen since Linton et al., 1990 reported that optimal heat shock condition for increased thermal resistance in log phase *L. monocytogenes* cultures was 48°C for 20 minutes. They also reported that more consistent results were achieved at 48°C for 10 minutes, therefore 15 minutes was chosen in order fall in the middle of the two times. The internal temperature of the tube was monitored by inserting an iron-constantan thermocouple (Omega Engineering, Inc., Stamford, CT) into a pre-heated uninoculated tube of TSBYE and logging the temperature with a datalogger model LI-1000 (LI-cor, Lincoln, NE). Non heat-shocked or control cells were prepared by transferring one-ml of the 6 h culture into 5-ml of TSBYE and incubating at room temperature (25°C).

Minimum Inhibitory Concentration (MIC) Assay

An appropriate inoculum from either the heat-shocked or control cell suspension was inoculated into TSBYE to yield 100-ml volumes of 10^3-10^4 cells/ml. Two-ml aliquots of either cell suspension was added to tubes containing serial two-fold dilutions of each of the sanitizing

solutions, and incubated at 37°C for 48 h. The number of bacteria was determined as previously described.

Effect of Heat Shock on Ability of *L. monocytogenes* to Attach to Meat Surfaces

Attachment to Meat

Pork chops were purchased from a local grocery store and cut into 25 gram (g) portions. The meat was dipped for 20 min in a suspension of either heat-shocked or control cells diluted in 0.1% peptone buffer to 10^8 cells/ml. This population was chosen in order to obtain an attachment of at least 10^3-10^4 cells/g to the meat. The samples were then placed on a sterile rack to allow the excess liquid to drain. To remove loosely attached cells, the meat was rinsed three times in sterile peptone buffer for five min each time. After rinsing, the meat samples were placed in sterile petri plates and stored at room temperature (25°C) for 0, 3, 9, and 18 h. The cells attached to the meat were enumerated. The experiment was done in triplicate.

Enumeration of *L. monocytogenes*

Twenty-five g of meat were diluted in Stomacher bags (Stomacher Model '400' closure bags, Seward Medical, London, U.K.) containing 225 ml of 0.1% peptone buffer, using a Stomacher blender (Tekmar Co., Cincinnati, OH), serially diluting in peptone, and plating onto a non selective (TSAYE) or selective medium Oxoid Agar (OX) (Oxoid, Basingstoke, England,). Plates were incubated at 37°C for 48 h. To determine the effect of heat-shock on attachment of *L. monocytogenes*

in fresh pork, the number of colony forming units/g of heat-shocked cells was compared to that of control cells. A qualitative analysis was done using scanning electron microscopy (SEM) to evaluate the presence of extracellular material (glycocalyx) on the meat after storage for 0, 3, 9, and 18 h of storage at 25°C.

Scanning Electron Microscopy

Duplicate samples of the meat stored up to 18 h were processed for SEM by fixing the samples in 4 % glutaraldehyde/3 % paraformaldehyde in 0.05 molar (M) cacodylate buffer, pH 7.2, overnight at 4°C. Then the samples were washed in the same buffer three times, 10 min. Later fixed in 1 % osmium tetraoxide (OsO_4) followed by a 10 min wash in the same buffer three times. The samples were dehydrated in an ethanol series to 100 % (50, 70, 75, 80, 85, 90, 95, 100, 100, 100 %).

The fixed specimens were infiltrated to 100 % propylene oxide as an intermediate fluid from 100 % ethanol. The specimens were prepared for scanning electron microscopy (SEM) as follows: critical point dried from 100 % ethanol in CO_2, mounted on brass discs and sputter coated in Polaron E5100 sputter coater with platinum/target (60:40). Observations were made with a JEOL JSM-35 scanning electron microscope at 20kV.

Effect of Heat Shock on Ability of *L. monocytogenes* to Attach to Stainless Steel Surfaces

Attachment to Stainless Steel

Heat-shocked and control cells were prepared in 0.1% peptone buffer at a concentration of 10^8 cells/ml. A 100-ml volume of either cell suspension was inoculated into sterile 250-ml Erlenmeyer flasks containing a 1 in^2 piece of stainless steel. Three flasks each were inoculated with 100-ml of either heat-shocked or control cells and incubated at 25°C in a controlled environment shaker incubator (Model 129, Fisher Scientific, Pittsburgh, PA) at 70 rpm. The three flasks were incubated for either 18, 24 or 48 h before the cell suspension (planktonic cells) and cells attached to stainless steel (adherent cells) were enumerated. The experiment was repeated three times.

Enumeration of *L. monocytogenes*

Planktonic cells were enumerated by serially diluting one-ml of the cell suspension in 0.1% peptone buffer. The diluted cell suspension was plated onto the non selective medium, TSAYE and the selective medium, OX. For enumeration of adhered cells the stainless steel pieces were removed from each culture flask with a pair of sterile forceps. Each stainless steel piece was rinsed in separate beakers containing 0.1% peptone buffer. The stainless steel was rinsed three times for five min in order to remove loosely attached cells. The stainless steel was then removed from the peptone buffer rinse and placed on sterile filter paper and tilted to drain excess liquid. A sterile template containing a 1 cm^2 opening was placed on the stainless steel and this area was

swabbed using a sterile cotton swab. The cotton tip was broken aseptically into 9 ml of 0.1% peptone buffer and vortexed vigorously. After vortexing, the cell suspension was enumerated by serially diluting and plating onto non selective (TSAYE) and selective (OX) medium. The effect of heat shock on the ability of *L. monocytogenes* to attach to stainless steel was determined by comparing the number of CFU/cm^2 of heat-shocked and control cells. Qualitative analysis of the stainless steel was done using SEM, as previously described for meat.

Effect of Chemical Shock on Ability of *L. monocytogenes* to Survive Exposure to Sanitizers

Chemical Shock of *L. monocytogenes*

Two-ml aliquots of *L. monocytogenes* cells cultured in TSBYE at a concentration of 10^3-10^4 cells/ml were exposed to sublethal concentrations (Table 1) of each of the sanitizing solutions. Each sample was prepared in a 30-ml centrifuge tube and immediately vortexed (Fisher Scientific, Pittsburgh, PA) and allowed to expose for various time intervals (0, 10, 20, 40, and 60 min). At each exposure time the sample was centrifuged at 8,000 x g (Beckman Instrument, Inc., Palo Alto, CA) for 15 min at 4°C. The supernatant was decanted and the pellet was resuspended in TSBYE.

Sanitizer Treatments

The chemically shocked cell suspension was transferred to tubes containing serial two-fold dilutions of the sanitizing solutions of one of the following three chemical treatments: exposure to MIC (Treatment 1),

exposure to lethal concentration (Treatment 2), or exposure to both (Treatment 3). Survivors were measured by turbidity after 24 and 48 h of incubation at 37°C, as described in the MIC assay procedure.

Effect of pH

The pH of the acid anionic sanitizer and the citric acid solution was determined by inserting an electronic pH probe (model 910, Fisher Scientific, Pittsburgh, PA) into the medium. The pH of the acid anionic sanitizer solution and the citric acid solution was adjusted (using 1 N HCl or 2 N NaOH) to 2.8, 5.0 and 7.0. *L. monocytogenes* (10^3 cells/ml) was inoculated into tubes containing serial two-fold dilutions of the acid anionic sanitizer and into tubes of the citric acid at each of the adjusted pHs. The inoculated tubes were incubated at 37°C and turbidity was measured at 24 and 48 h. The number of bacteria present was determined as described in the MIC assay procedure.

Effect of Chemical Shock on Ability of *L. monocytogenes* to Attach to Stainless Steel Surfaces

L. monocytogenes cells (10^3-10^4/ml) were chemically-shocked as previously described. Serial two-fold dilutions of the lethal concentration (750 ppm) of acid anionic sanitizer solution was added to the chemically-shocked cell suspension. Control cells were serially diluted two-fold with either 0.1% peptone for the positive control, or 750 ppm acid anionic sanitizer for the negative control. After each sample was shaken vigorously, each was transferred to separate sterile 250-ml flasks containing a 1 in^2 piece of stainless steel. The flasks were

done in duplicate. Each flasks was incubated at 25°C in a controlled environment shaker incubator (Fisher Scientific) at 70 rpm. In 24 h the cell suspension (planktonic cells) and cells attached to stainless steel (adherent cells) were enumerated as previously described. The experiment was repeated three times.

RESULTS

Effect of Heat Shock on Survival of *L. monocytogenes* to MICs of Sanitizers

The minimum inhibitory concentrations (MIC) determined for the various sanitizers against *L. monocytogenes* are shown in Table 1. Heat shocking *L. monocytogenes* at 48°C for 15 min did not result in the ability of the cells to survive exposure to the MICs of each sanitizer (Table 2).

Effect of Heat Shock on Ability of *L. monocytogenes* to Attach to Meat Surfaces

In determining the effect of heat shock on attachment of *L. monocytogenes* to meat, there was no significant difference in the number of heat-shocked cells attached to meat compared with controls cells (Fig. 1). The number of CFU/g of both heat-shocked and control *L. monocytogenes* cells attached to meat immediately after dipping (at time 0) into inocula of 10^8 cells/ml was 10^4 CFU/g. After 3 h of storage, the number increased to 10^5 CFU/g and after 9 h to 10^7 CFU/g. At the maximum storage period (18 h) the number of cells attached increased to 10^{10} CFU/g.

Qualitatively, there was an apparent difference between heat-shocked and control cells that were attached to the meat after 18 h of storage. The SEM micrograph (Fig. 2) shows that meat containing heat-shocked cells stored for this time period were coated with small

unknown particulate, whereas the controls were not. However, the heat-shocked cells attached to meat stored for shorter periods (0, 3, or 9 h) did not appear any different from the control cells attached to meat stored for the same periods.

Effect of Heat Shock on Ability of *L. monocytogenes* to Attach to Stainless Steel Surfaces

The effect of heat shock on the ability of *L. monocytogenes* cells to attach to stainless steel was examined. Figure 3 shows the number of planktonic heat-shocked and control cells after 18, 24 and 48 h of incubation at 25°C. The initial inoculum of 10^8 cells/ml in the culture flask had increased to approximately 10^{11} cells/ml after 18 h of incubation. After 24 h, this cell population increased by 1 $\log_{10}$ (Fig. 3). After 48 h of incubation the number of cells remained the same. Regardless of the time of incubation (18, 24, or 48 h), the number of cells attached to the stainless steel blocks and the number of cells remaining in the culture supernatant was the same for both heat-shocked and control cells (Fig. 3 and 4). The qualitative analysis of heat-shocked cells and control cells attached to the stainless steel are shown in the SEM micrograph in Fig. 5, with no significant difference in the appearance of the cells.

Effect of Chemical Shock on Ability of *L. monocytogenes* to Survive Exposure to Sanitizers

The effect of chemical shock on the ability of *L. monocytogenes* to survive exposure to lethal levels of sanitizers is shown in Fig. 6-12. Cells exposed to a chemical shock (sublethal levels) with most of the sanitizers (chlorine, iodophor, quat, citric acid, lactic acid, and propionic acid) did not survive exposure to treatment with MIC or lethal levels of these sanitizers (Fig. 7-12). However, cells exposed to a chemical shock (sublethal levels) of the acid anionic sanitizer survived exposure to the MIC (500 ppm) of this sanitizer (Fig. 6). In addition, exposure of the cells to the chemical shock for 40 minutes followed by the MIC (500 ppm) for 40 minutes resulted in survival to lethal levels of this sanitizer (750 ppm) (Fig. 6). There were no survivors when cells were chemically shocked and then exposed directly to lethal levels (750 ppm).

Since chemically treated *L. monocytogenes* seemed to have a tolerance to lethal concentrations of the acid anionic sanitizer but not to any of the other organic acids, we investigated the differences between these compounds. Acid anionic sanitizer, with a pk_a of 2.1 is dissociated at the pH of the medium (2.6-3.0). However, at this pH, citric acid (with a pk_a of 3.1) is equally dissociated and undissociated. We speculated that increasing the pH of the medium would result in dissociation of the citric acid, and that this would cause treatment of the cells with citric acid (now dissociated) to be the same as treatment with acid anionic. Survival of *L. monocytogenes* was observed upon exposure of cells to

dissociated citric acid (Fig. 13), suggesting that dissociation plays a role in survival of chemically-shocked cells to lethal concentrations.

Effect of Chemical Shock on Ability of *L. monocytogenes* to Attach to Stainless Steel Surfaces

The effect of sublethal chemical shock on the ability of *L. monocytogenes* to attach to stainless is shown in Figure 14. The initial inoculum (at time 0) in culture flasks containing non chemically-shocked cells incubated in TSBYE; non chemically-shocked cells incubated in lethal levels; and chemically-shocked cells incubated in lethal levels was approximately 10^4 CFU/ml (Figure 14). After 24 h of incubation, the non chemically-shocked cells in TSBYE increased to 10^6 CFU/ml (Fig. 14) while non chemically-shocked cells in lethal levels and chemically-shocked cells in lethal levels increased to approximately 10^5 CFU/ml (Fig. 15). The number of non chemically-shocked cells in TSBYE attached to stainless steel incubated for 24 h was 10^6 CFU/cm^2 (Fig. 16). The non chemically-shocked cells in lethal levels and chemically-shocked cells in lethal levels attached were approximately 10^3 CFU/cm^2.

Table 1. The minimum inhibitory concentrations (MICs), sublethal and lethal concentrations of various sanitizers against *L. monocytogenes* at 37°C for 48 h

Sanitizer	MIC[a]		Sublethal[b]		Lethal[c]	
Acid anionic	500	ppm	350	ppm	750	ppm
Chlorine-based	15	ppb	10	ppb	20	ppb
Iodophor	8	ppb	4	ppb	10	ppb
Quat	5	ppm	2.5	ppm	10	ppm
Citric	0.4%		0.2%		0.5%	
Lactic	0.4%		0.2%		0.5%	
Propionic	0.3%		0.1%		0.4%	

[a]The minimum concentration which inhibited the growth of *L. monocytogenes* for 48 h at 37°C

[b]Defined as the concentration of sanitizer corresponding to one dilution lower than the minimum inhibitory concentration

[c]Defined as the concentration of sanitizer corresponding to one dilution greater than the minimum inhibitory concentration

Table 2. Effect of heat shock on ability of *L. monocytogenes* (10^3-10^4 cells/ml) to survive a 48 h exposure to MIC of various sanitizers at 37°C

Sanitizer	MIC		O.D. at 650 nm	
			Heat-shocked	Control
Acid anionic	500	ppm	0.00	0.00
Chlorine-based	15	ppb	0.00	0.00
Iodophor	8	ppb	0.00	0.00
Quat	5	ppm	0.00	0.00
Citric	0.4%		0.00	0.00
Lactic	0.4%		0.00	0.00
Propionic	0.3%		0.00	0.00
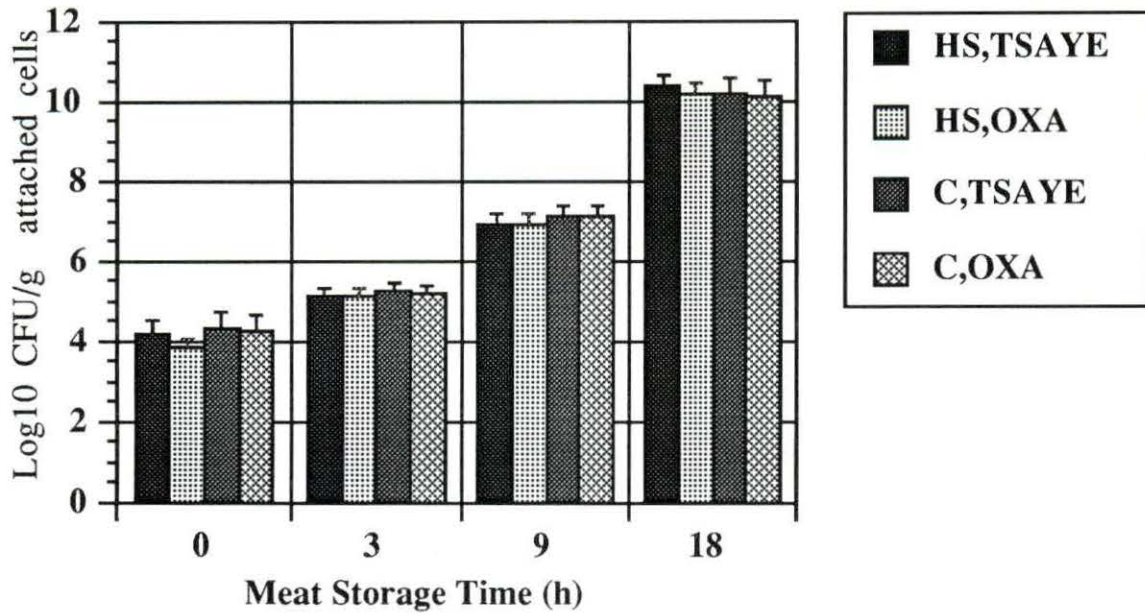
Fig 1. Enumeration (log$_{10}$ CFU/g) of heat-shocked (HS) and control (C) *L. monocytogenes* cells attached to meat dipped in inocula of 10^8 cells/ml for 20 min and stored at 25°C for 0, 3, 9, and 18 h. (TSAYE=trypticase soy agar supplemented with yeast extract, OXA=Oxford agar)

Fig 2.   SEM micrographs (4800x) of (A) heat-shocked and (B) control *L. monocytogenes* cells attached to surface of meat stored at 25°C for 18 h. (Bar=2μm)
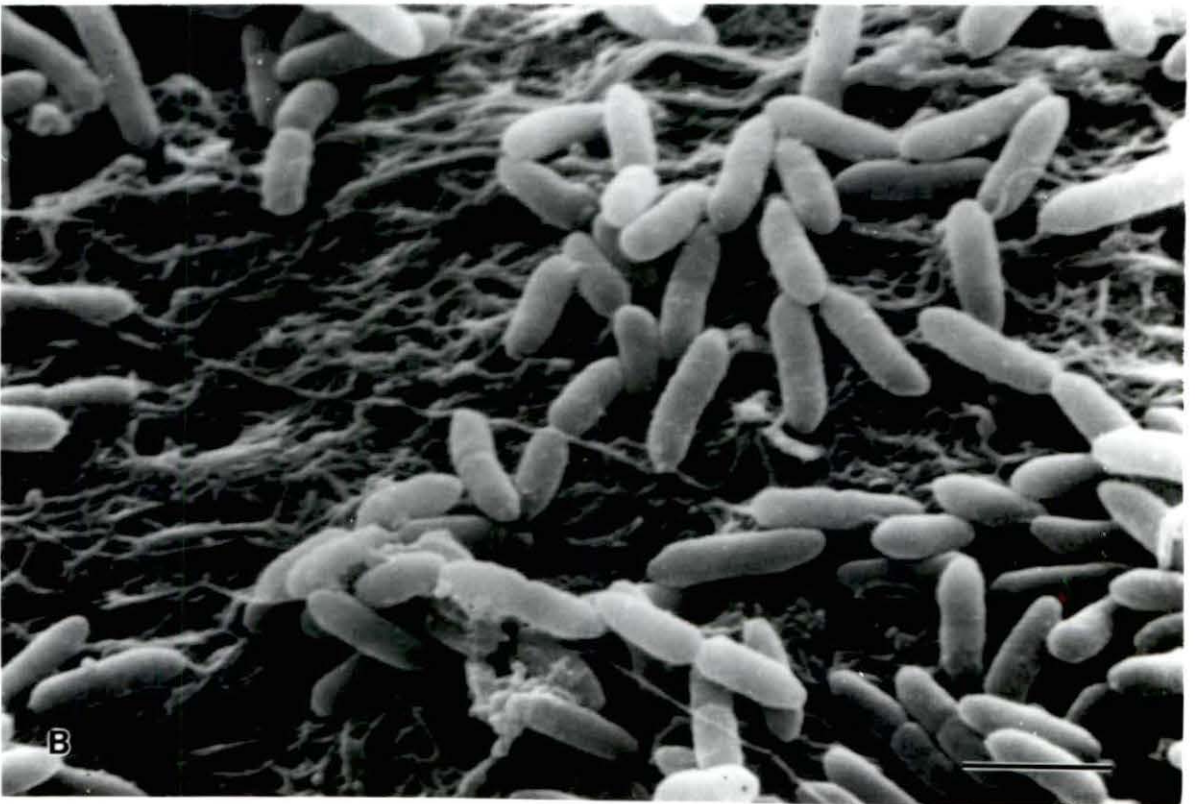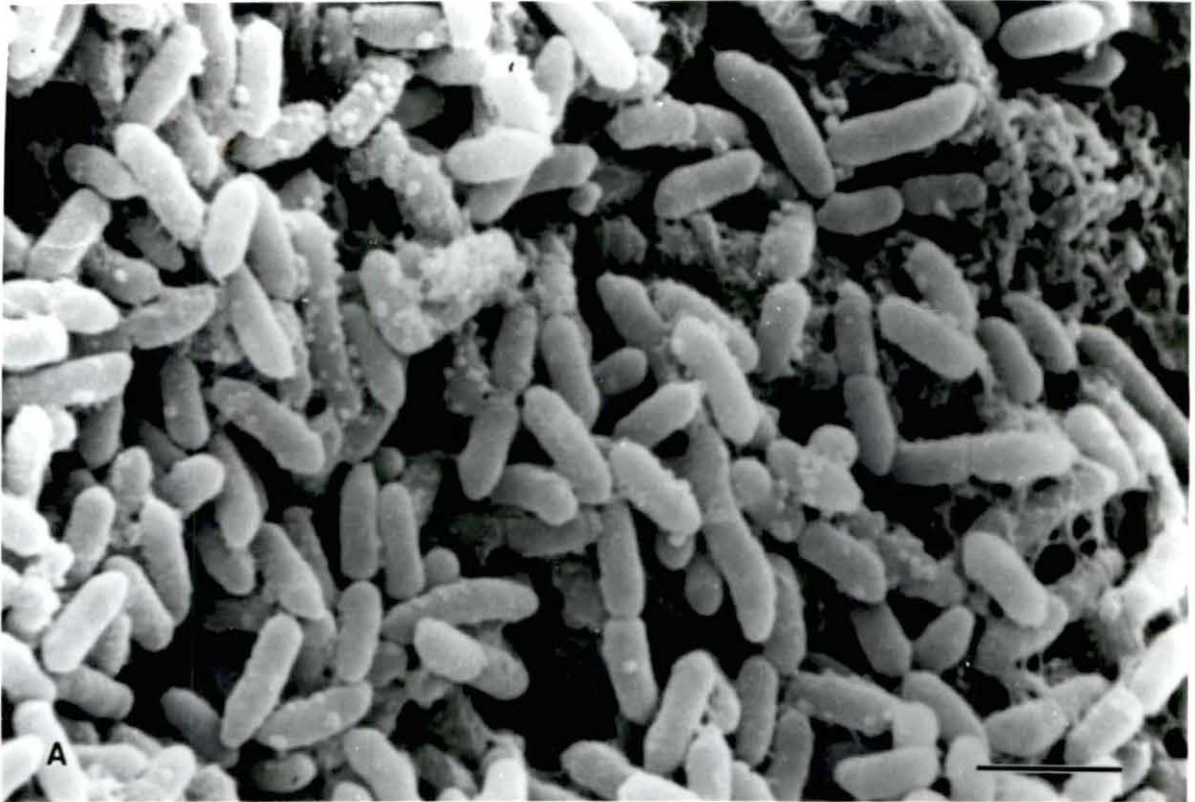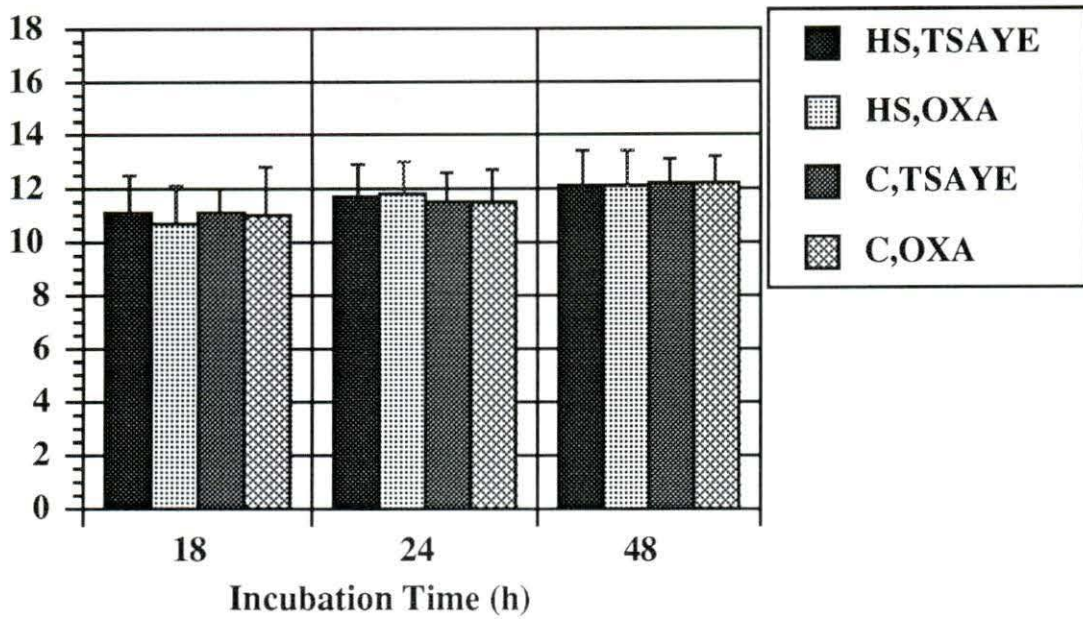
Fig 3. Enumeration ($\log_{10}$ CFU/cm^2) of heat-shocked (HS) and control (C) *L. monocytogenes* planktonic cells in culture flasks after 18, 24, and 48 h of incubation at 25°C. (TSAYE=trypticase soy agar supplemented with yeast extract, OXA=Oxford agar)
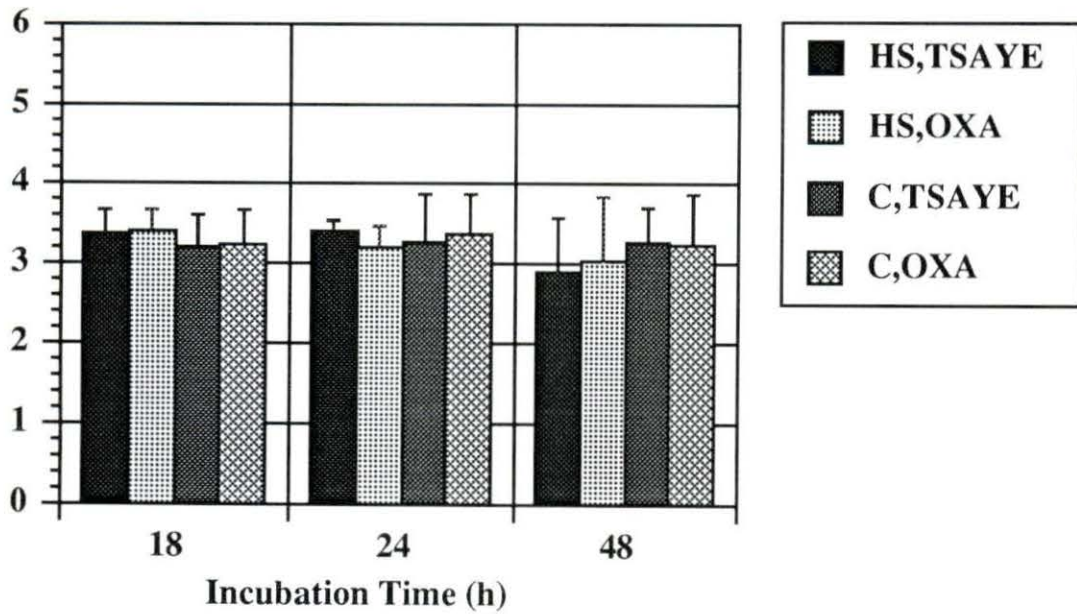
Fig 4. Enumeration ($\log_{10}$ CFU/cm^2) of heat-shocked (HS) and control (C) *L. monocytogenes* cells attached to stainless steel incubated in culture flask for 18, 24, and 48 h at 25°C. (TSAYE=trypticase soy agar supplemented with yeast extract, OXA=Oxford agar)

Fig 5.   SEM micrographs (4800x) of (A) heat-shocked and (B) control *L. monocytogenes* cells attached to stainless steel cultured in trypticase soy broth supplemented with yeast extract incubated at 25°C for 24 h. (Bar=2μm)
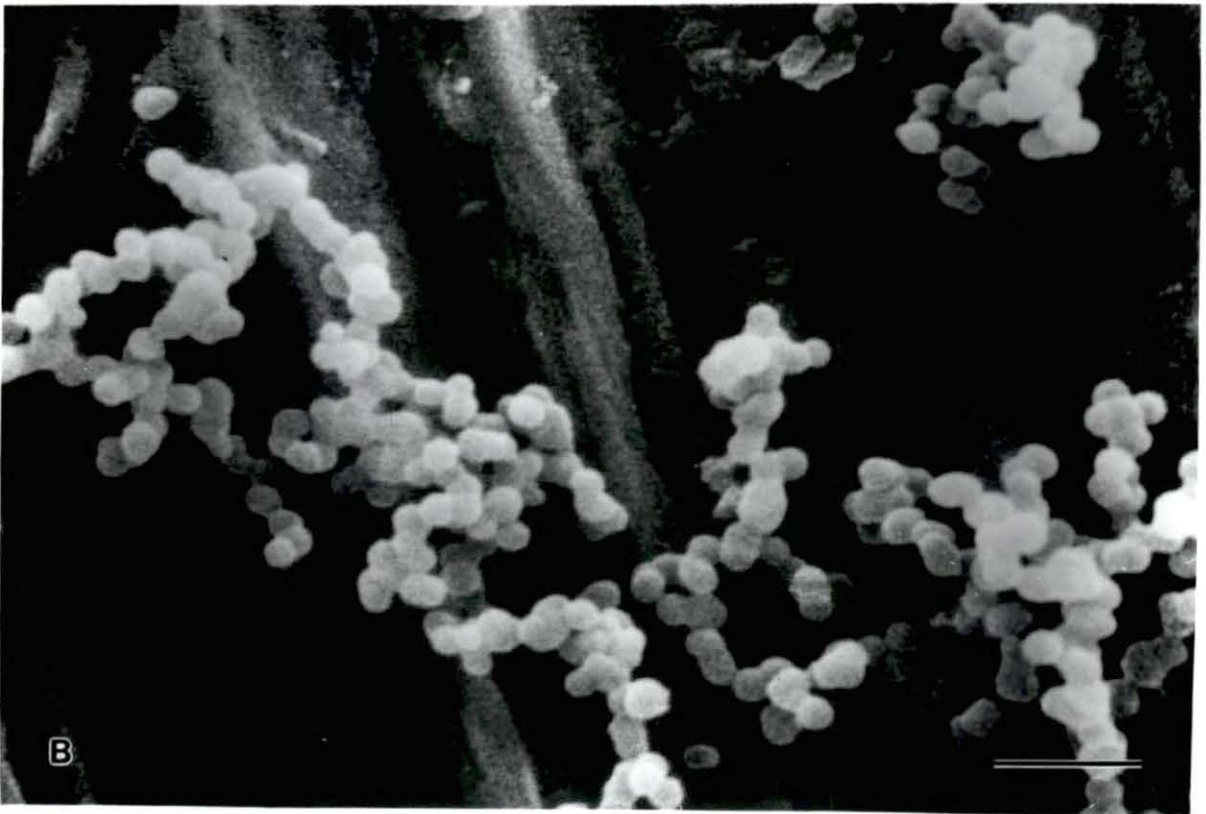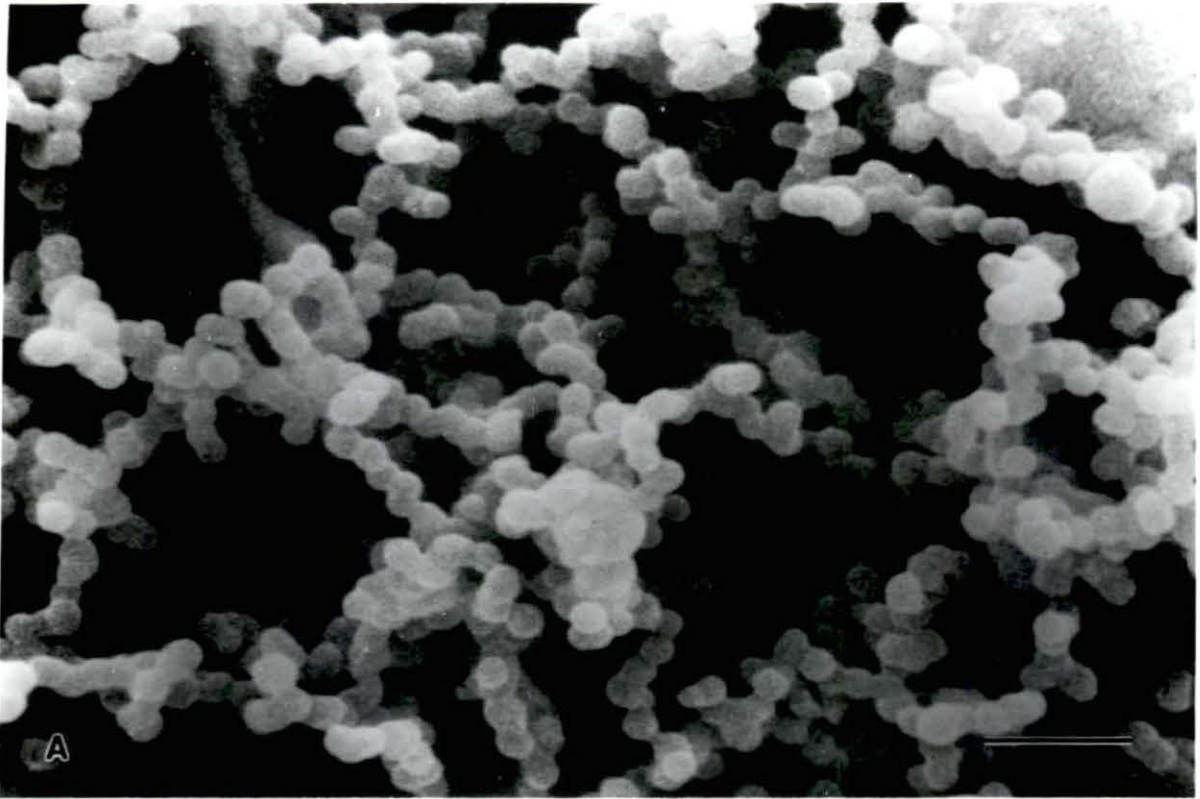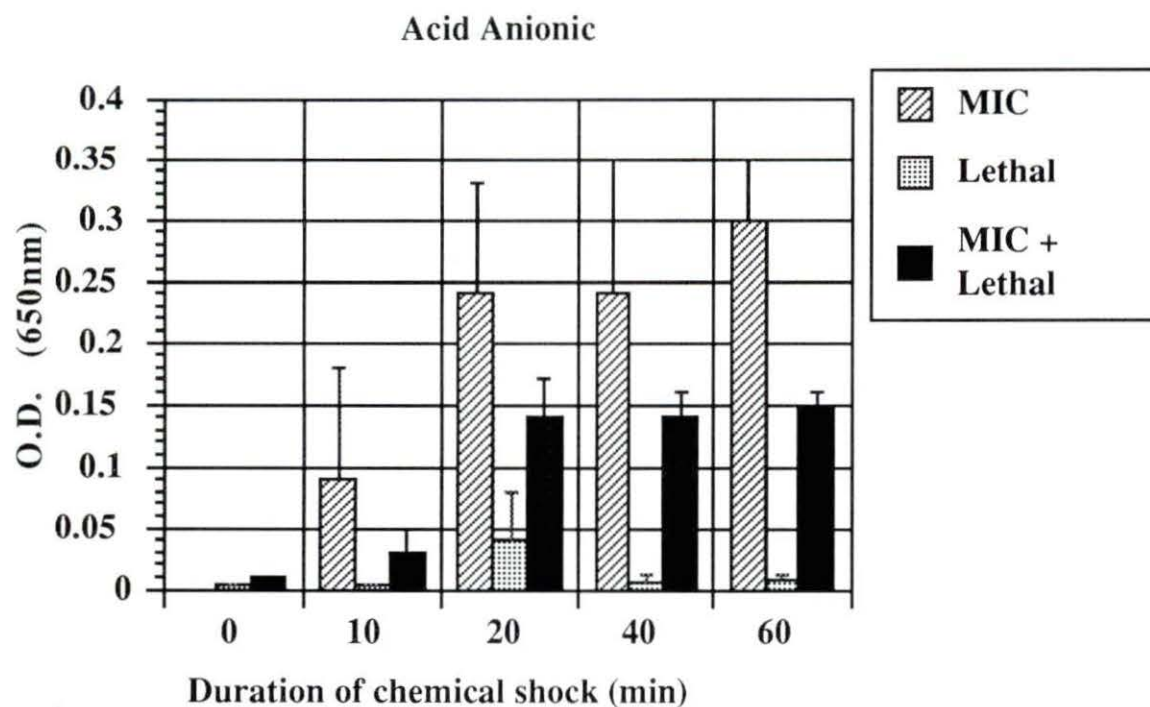
Acid Anionic

Fig 6.   Effect of chemical shock (by exposure of cells to sublethal concentration of acid anionic sanitizer (350 ppm) for various times) on survival *L. monocytogenes* to MIC (500 ppm) ▨, lethal concentration (750 ppm) ▦ or to both MIC and lethal concentration of the same sanitizer after 48 h at 37°C. ■
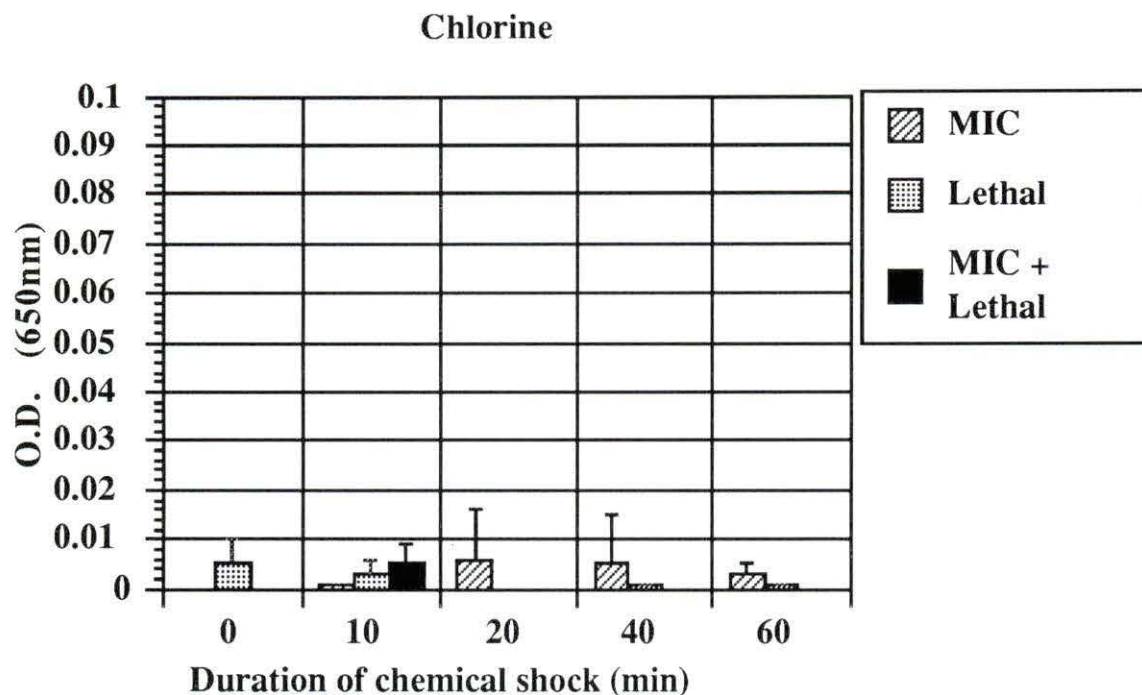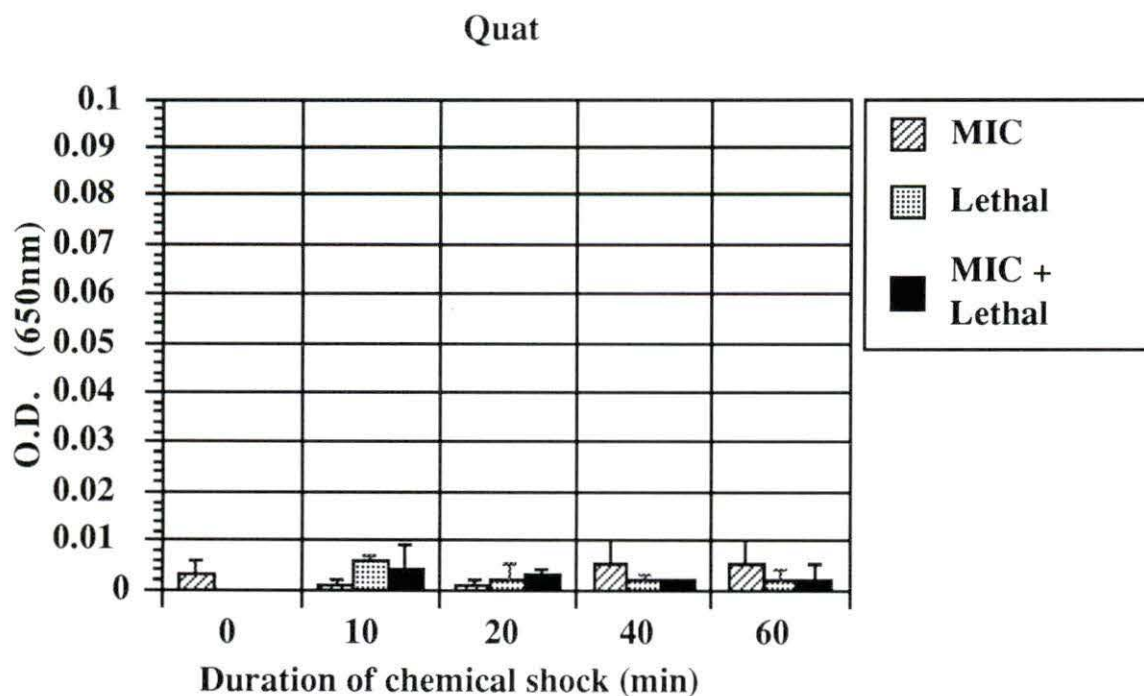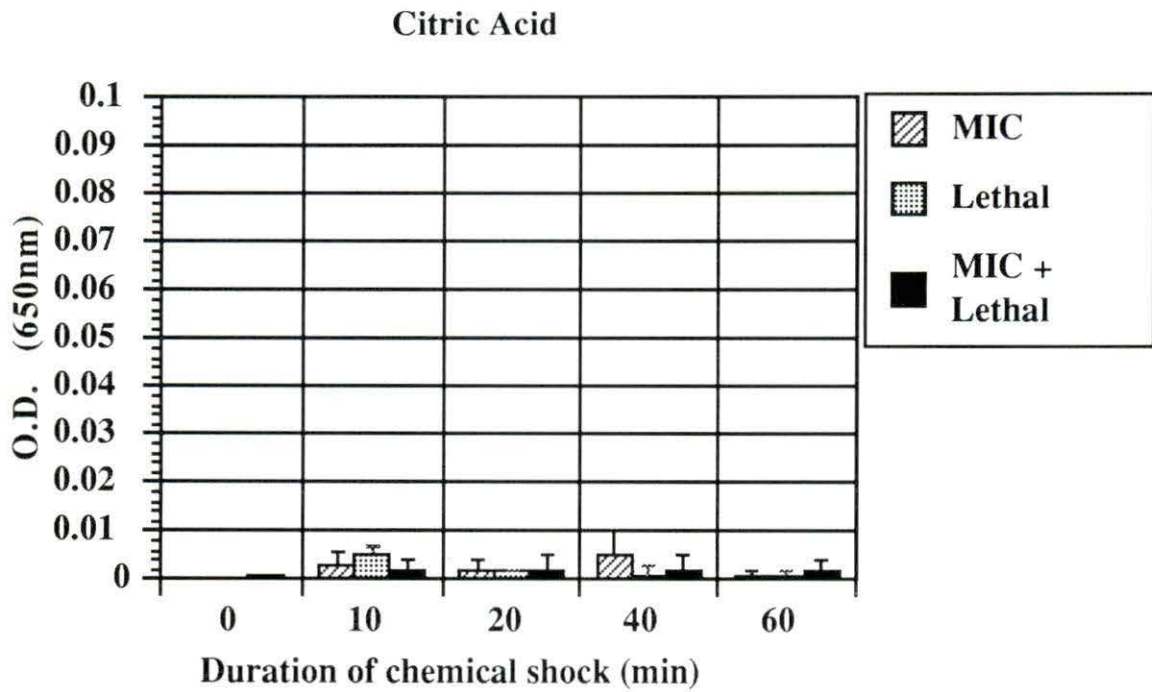
Fig 7.   Effect of chemical shock (by exposure of cells to sublethal concentration of chlorine-based sanitizer (10 ppb) for various times) on survival *L. monocytogenes* to MIC (15 ppb) ▨, lethal concentration (20 ppb) ▦ or to both MIC and lethal concentration of the same sanitizer after 48 h at 37°C. ■
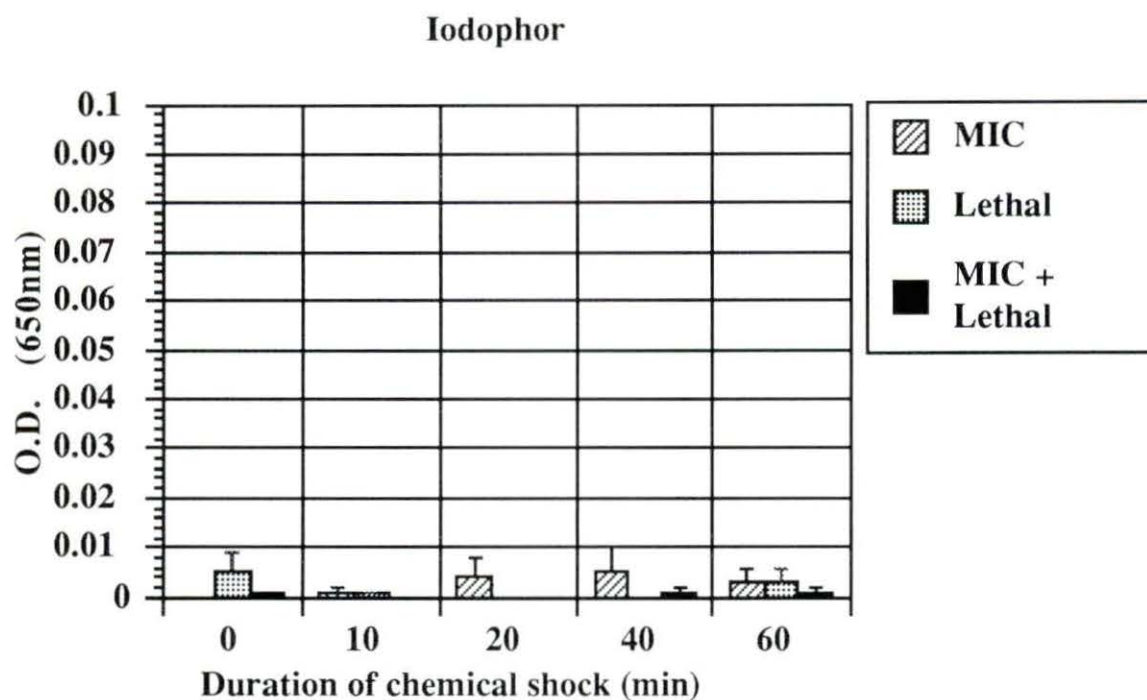
Iodophor

Fig 8. Effect of chemical shock (by exposure of cells to sublethal concentration of iodophor sanitizer (4 ppb) for various times) on survival *L. monocytogenes* to MIC (8 ppb) ▨, lethal concentration (10 ppb) ▦ or to both MIC and lethal concentration of the same sanitizer after 48 h at 37°C. ■

Quat

Fig 9.   Effect of chemical shock (by exposure of cells to sublethal concentration of quaternary ammonium compound (2.5 ppm) for various times) on survival *L. monocytogenes* to MIC (5 ppm) ▨, lethal concentration (10 ppb) ▦ or to both MIC and lethal concentration of the same sanitizer after 48 h at 37°C. ■
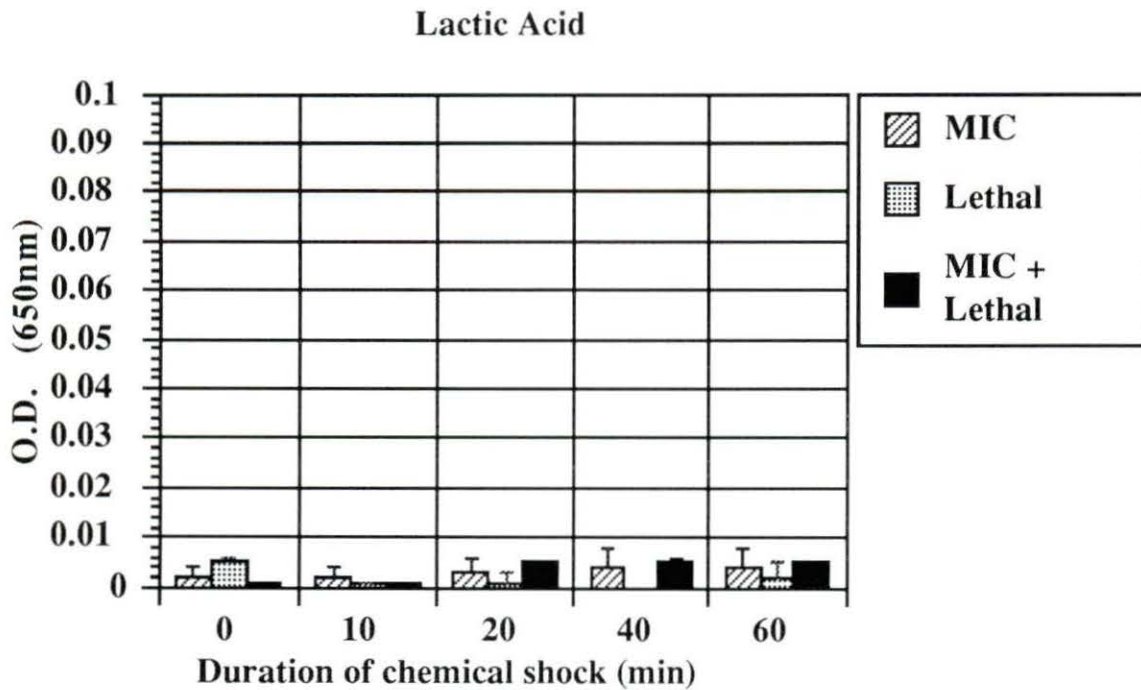
Citric Acid

Fig 10. Effect of chemical shock (by exposure of cells to sublethal concentration of citric acid (0.2%) for various times) on survival *L. monocytogenes* to MIC (0.4%) ▨, lethal concentration (0.5%) ▦ or to both MIC and lethal concentration of the same sanitizer after 48 h at 37°C. ■

Lactic Acid

Fig 11. Effect of chemical shock (by exposure of cells to sublethal concentration of lactic acid (0.2%) for various times) on survival *L. monocytogenes* to MIC (0.4%) ▨, lethal concentration (0.5%) ▦ or to both MIC and lethal concentration of the same sanitizer after 48 h at 37°C. ■
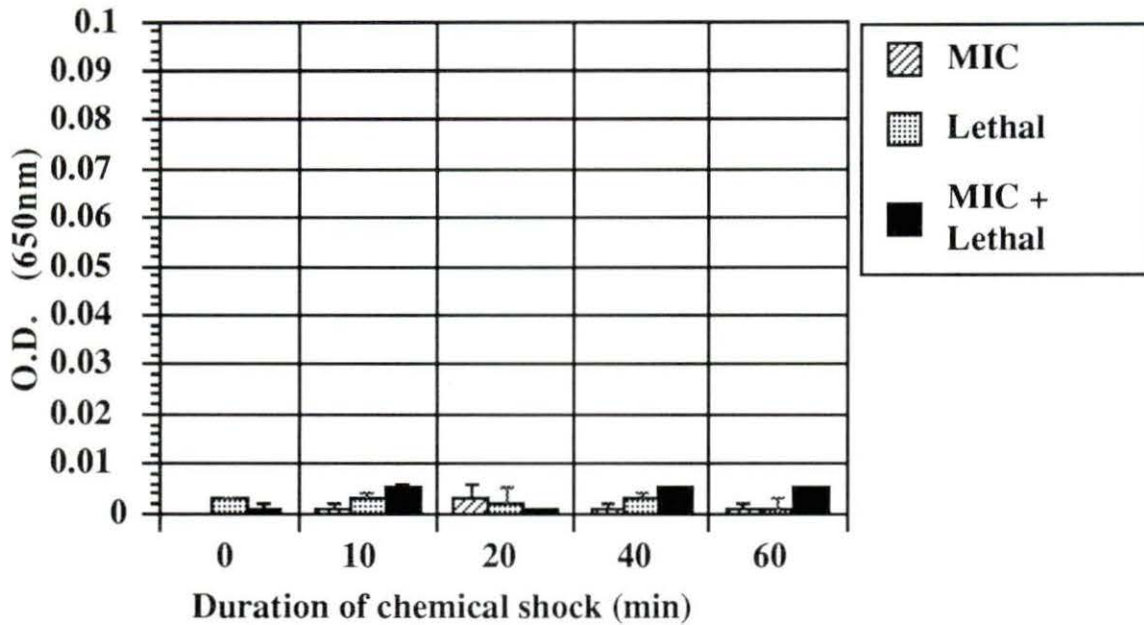
Propionic Acid

Fig 12. Effect of chemical shock (by exposure of cells to sublethal concentration of propionic acid (0.1%) for various times) on survival *L. monocytogenes* to MIC (0.3%) ▨, lethal concentration (0.4%) ▦ or to both MIC and lethal concentration of the same sanitizer after 48 h at 37°C. ■

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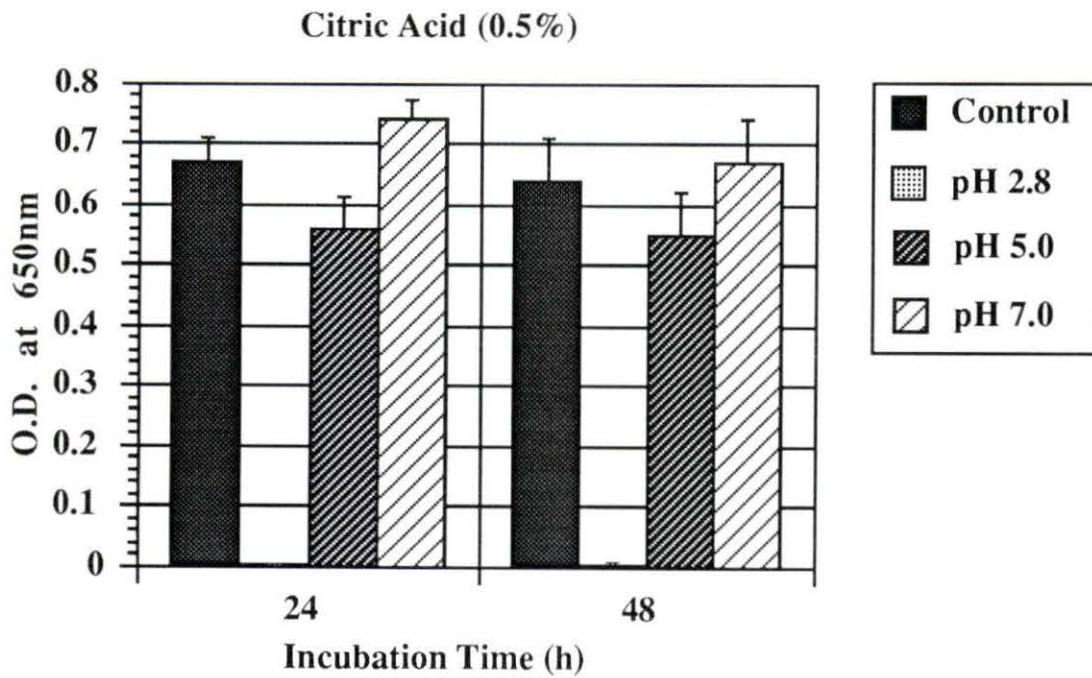
Fig 13.   Effect of pH on survival of *L. monocytogenes* to lethal level (0.5%) of citric acid sanitizer
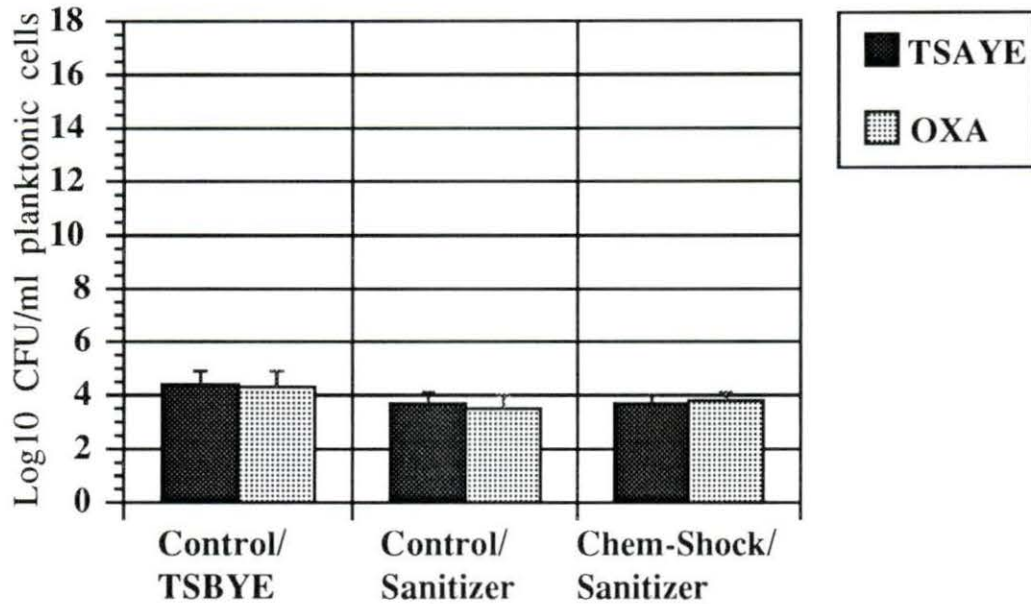
Fig 14. Enumeration (log$_{10}$ CFU/ml) of *L. monocytogenes* control (non chemically-shocked) cells and chemically-shocked cells immediately after exposure to TSBYE or to lethal levels of acid anionic sanitizer. (TSAYE or TSBYE=trypticase soy agar or broth supplemented with yeast extract; OXA=Oxford agar)
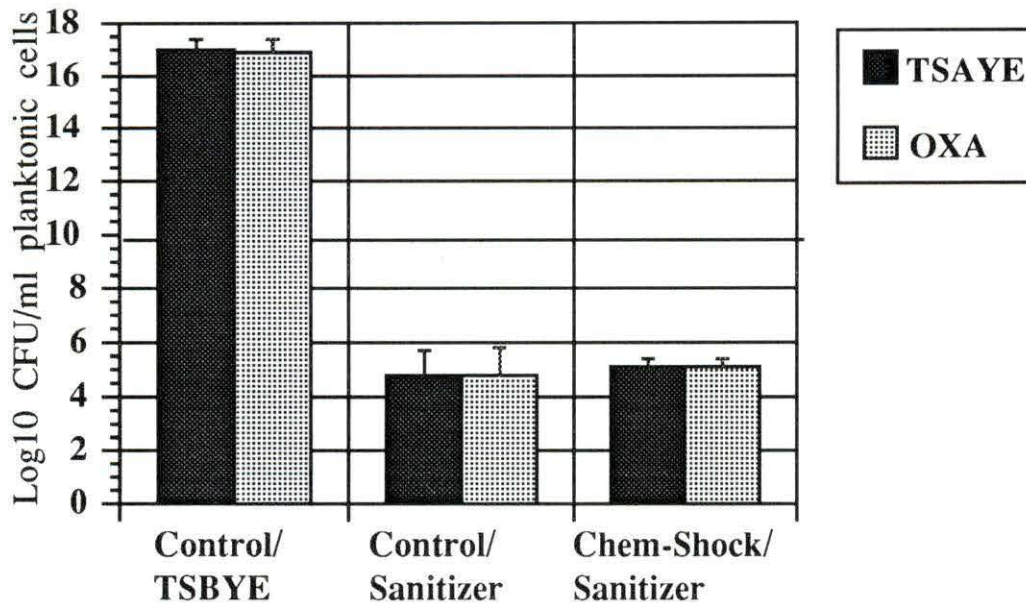
Fig 15. Enumeration ($\log_{10}$ CFU/ml) of *L. monocytogenes* control (non chemically-shocked) cells and chemically-shocked cells after 24 h of exposure to TSBYE or to lethal levels of acid anionic sanitizer. (TSAYE or TSBYE=trypticase soy agar or broth supplemented with yeast extract; OXA=Oxford agar)

Fig 16. Enumeration of attached ($\log_{10}$ CFU/cm^2) *L. monocytogenes* control (non chemically-shocked) cells and chemically-shocked cells to stainless steel after exposure to TSBYE or to lethal levels of acid anionic sanitizer. (TSAYE or TSBYE=trypticase soy agar or broth supplemented with yeast extract; OXA=Oxford Agar)
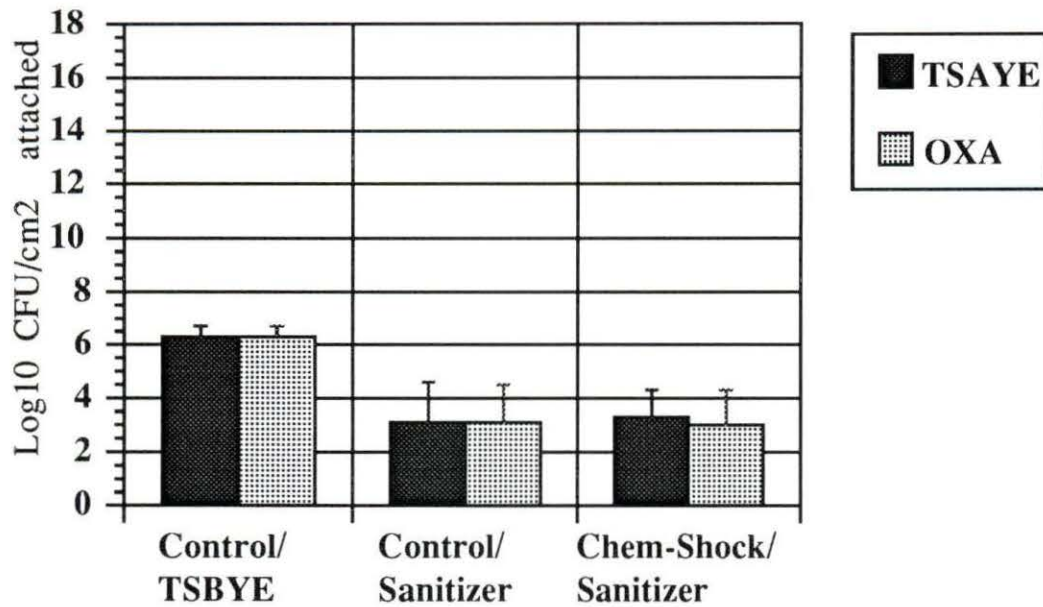
DISCUSSION

Heat shocking *L. monocytogenes* at 48°C for 15 minutes did not enhance cell survival to MIC of the various sanitizers in broth medium (TSBYE) for 48 h. Previous studies have indicated that heat shock enhances the ability of *L. monocytogenes* to survive environmental stress, with elevated temperatures being the type of stress applied in most instances. Linton et al (1990) noticed increased thermotolerance in *L. monocytogenes* that had been heat shocked for 20 minutes. There have been no studies to show the effect of heat stress on the resistance of bacteria to another environmental stress such as chemical shock, thus we cannot compare our results with those of other investigators.

The attachment of *L. monocytogenes* to meat, as noted earlier, is presumed to be the first step in the contamination of solid surfaces. The attachment and subsequent survival and growth of the contaminating bacteria is of importance for the overall safety of the food supply. The environmental conditions can influence the nature of the attachment in regards to the cells being 'loosely' or 'strongly' attached. Environmental conditions can also have an effect on the bacteria production of extracellular polysaccharide layers (glycocalyx). In examining the ability of heat-shocked cells to attach to meat, heat shocking had no effect on the number of cells attached to the meat stored for 3 to 18 h compared to control cells. The number of cells attached were comparable in both heat-shocked and control cells for each storage time. The presence of glycocalyx was speculated in heat-

shocked cells after observing some differences in surface characteristics by SEM. The heat-shocked cells present on meat stored at 18 h appeared different, containing small noticeable particulate on their surface compared to control cells. Since the substance was never analyzed, it is only mere speculation that a difference exists between heat shocked and control cells on meat after 18 h of storage. This substance was not observed in meat stored for shorter times.

The number of heat-shocked cells attached to stainless steel was the same as the number of control cells. The numbers of both heat-shocked and control cells did not increase after extensive h of storage. This may be due to the unavailability of nutrients on the stainless steel blocks. The number of cells in suspension for both heat-shocked and control cells also remained the same after extensive h of incubation, probably due to the cells having reached the stationary phase of growth. There was no apparent difference in heat-shocked and control cells attached to the stainless steel blocks. However, those heat-shocked and control cells attached to stainless steel were morphologically different from heat-shocked and control cells attached to meat. The cells attached to stainless steel were smaller and more coccoid and those attached to meat were larger and more bacillus in shape. This difference in morphology could be due to the type of surface in which the cells have attached. The cells attached to the stainless steel may not have grown as large cells attached to meat since less nutrient is provided on the stainless steel surface.

Exposure of *L. monocytogenes* cells to sublethal levels of most of the sanitizers (chlorine-based, iodophor, quat, citric acid, lactic acid and propionic acid) did not result in the ability of the cells to become resistant to lethal level of these sanitizers. Acid anionic sanitizers seem to provide some resistance to exposure of the cells to the MIC and lethal level of this sanitizer. Given the fact that the same results were accomplished with the citric acid sanitizer after a pH adjustment, it appears that the resistance exhibited by *L. monocytogenes* to the MIC of weak acids is based on the level of dissociation of the acid. It is interesting to note that the cells chemically-shocked with acid anionic sanitizer were not able to survive direct exposure to lethal level, but rather survived when subjected to MIC. It is possible that the cells became acclimated to the undissociated acid by such a stepwise increase in concentration. Mackey and Derrick (1987b) found that a slow exposure to increasing temperatures can result in higher numbers of survivors than a sudden shift.

This phenomenon is believed to trigger the heat-shock response thus it is possible that a similar mechanism resulted in survival of the cells to increasing levels of acid anionic sanitizer.

The effect of exposure of *L. monocytogenes* cells to sublethal concentrations of the various sanitizers did not result in the ability of the cells to attach to surfaces of stainless steel.

CONCLUSIONS

Although some studies have indicated that heat shocking bacteria results in the ability of cells to survive exposure to various lethal treatments, this study did not draw the same conclusion. *L. monocytogenes* cells exposed to a heat shock were not able to survive treatment with chemical sanitizers, nor did they become better able to attach to meat or stainless steel. Chemical shock when applied with certain compounds will somehow result in resistance to some chemical sanitizers. The type of sanitizer used will be a factor with the use of acid anionic sanitizers and dissociated organic acids resulting in increased survival of cells to lethal levels of these chemicals. These results suggest that careful attention should be paid when using acid anionic sanitizers to make sure that the pH and concentration being used will not result in exposure of bacterial pathogens to a chemical shock. The user should be sure that the manufacturer's recommended concentration and contact time is applied.

Attachment of *L. monocytogenes* to meat and other surfaces, however, did not seem to be affected by either heat shock or chemical shock, thus the potential danger in exposure of the pathogen to environmental stress is only that of increased survival. However, given that bacterial cells are difficult to remove once attached to surfaces, the lack of enhancement offered by chemical and heat shock should not give sanitarians a false sense of security.

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