Heat Resistance of Salmonella typhimurium and Listeria monocytogenes in Suspension and in a Biofilm Matrix.

by

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(ABSTRACT)

The heat resistance was determined for Salmonella typhimurium and Listeria monocytogenes Scott A suspended in 2% UHT processed milk and in a biofilm matrix. Pure cultures at an initial concentration of 10^5 / ml were used. Heat resistance was determined by two methods. One method was sealed borosilicate glass TDT tubes that were completely submerged in the heating menstrum. Biofilms were grown on Buna-n rubber o-rings (4.46 mm O.D. x 1.41 mm I. D.) for 36 hours. All other cultures used were in stationary phase of growth. The three treatments tested were: inoculated milk, sterile milk and a biofilm on an o-ring, and inoculated milk with a sterile o-ring. At the three temperatures tested (60, 63, 67°C), there was no significant difference ($p > 0.05$) in D-values between treatments. There was a significant difference ($p < 0.001$) between the D-values for Salmonella and Listeria.

The second method used a laboratory scale HTST pasteurizer to determine the difference in heat resistance of the same organisms suspended in 2% milk vs. sloughed off pieces of biofilm in milk. Pure cultures of the

organisms at an initial inoculum of 10^5 / ml were used. Flow rates of the pasteurizer were adjusted to achieve two different F-values for each organism at a reference temperature of 71.7°C. Neither S. typhimurium nor L. monocytogenes Scott A was recovered from pasteurized samples of either treatment. The heating involved in come up and cool down of the transit lines was considered in determining F-value. Under commercial HTST processing, concentrations of 10^5 / ml of S. typhimurium and L. monocytogenes Scott A would not survive pasteurization. The results also show that if pieces of biofilms (3.8×10^{-4} mm^2- 8.8×10^{-3} mm^2) were sloughed off gaskets in the processing lines they would not survive pasteurization.

The heating characteristics of these two systems were so dissimilar they could not be compared. It should however be noted that in the TDT tubes it was necessary to obtain a slightly higher F-value before no growth was seen as compared with the pasteurizer. In the pasteurizer the laminar flow properties would contribute to a more uniform heating. The TDT tube experiences convection heating which can produce cold spots in the tubes and could explain the need for an increased F-value.

DEDICATION

This thesis is dedicated to my parents Barbara and Lowell Moxley.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

SECTION 1

SECTION 2

SECTION 3

INTRODUCTION

Outbreaks of food borne illness due to contaminated pasteurized milk are rare but when they do occur thousands of people can be affected and milk sales are depressed. Pasteurization is relied on to produce milk free of illness causing pathogens. Previous outbreaks involving contaminated pasteurized milk have been assumed to be the result of post-processing contamination. It is possible the source of contamination stems from bacteria in biofilms being released into the milk pipelines. Proper, consistent, cleaning has been shown to effectively remove biofilms. A breakdown in cleaning could enable bacteria in biofilms to survive when otherwise they would not. Studies have shown that bacteria in biofilms have a higher resistance to cleaning and sanitizing agents than bacteria in suspension. Also organisms in biofilms may be more heat resistant than those in suspension. Survival of bacteria in milk after pasteurization may be due to increased heat resistance of a bacterial pathogen as part of a biofilm instead of post-process contamination.

SECTION 1: LITERATURE REVIEW

A. BIOFILMS

1. Occurrence in nature

The study of bacterial attachment is not new. The buried and submerged slide techniques used for the direct observance of soil and aquatic microorganisms by microscope are based on the ability of microorganisms to attach to surfaces. These slide techniques were described by Zobell (1943) in a report frequently referenced as the origin of the study of bacterial attachment. The terms biofilm and biofouling refer broadly to biological deposits on any surface. Biofilms consist of microbes and their extracellular products, usually polysaccharides. They protect microbes from hostile environments and serve as a trap for nutrient acquisition. The only requirements for formation of a biofilm are an acceptable surface and moisture. A deficiency of nutrients may actually increase the biofilm formation capacity of microbes (Dawson et al., 1981). Dead ends, corners, cracks, crevices, gaskets, and joints are the most vulnerable points for biofilm accumulation and formation (Sandholm and Wirtanen, 1992).

Biofilms are a normal occurrence in nature. They are commonly known as plaque on teeth (Russell, 1993). Biofilms can cause numerous

problems in industry. Thin biofilms on wet surfaces dramatically increase fluid frictional resistance to flow (Characklis, 1973). Biofilms affect flow in at least three ways: reducing the cross-sectional area available for flow, increasing the roughness of the surface, and increasing the drag (Sandholm and Wirtanen, 1992). The biggest contributing factor to decreased flow is the increased roughness. Constant fluid velocity can be maintained in many pumped systems where biofilms have formed; however, pumping costs increase. Also, biofilms developing on heat transfer surfaces (tubes) can impede the flow of heat across the interface. According to Characklis (1981), biofilms influence convective and conductive heat transfer.

After a period of time biofouling occurs and may influence the corrosion process in several ways (Characklis and Cooksey, 1983). The biofilm can serve as a molecular sieve that alters ion mobility near the metal surface. The acid produced within the anaerobic microenvironments of biofilms can accelerate corrosion.

There are several industrial systems in which biofilm formation may occur. Sufficient nutrients are present in drinking water systems for planktonic cells to form biofilms. Legionella pneumophila is a dangerous biofilm forming organism and can become a major problem in houses with hot water systems (Wadowski et al., 1982). The choice of surface material becomes important when designing and building equipment and processing

lines for industrial use. Surfaces that are smooth and in good condition are easier to clean and less likely to harbor biofilms. Accumulation of particulates and cells will occur where the cleaning is inadequate. Bends, pockets, and extended joints are prone to accumulation. Especially favorable places for biofilm growth are gaskets, because dirt and different kinds of nutrients accumulate preferentially on gasket material (Mosteller, 1993). Since the biofilm affects heat transfer, sterilization time is increased accordingly. Biofilms also protect microbes against sanitizers and disinfectants (Frank and Koffi, 1990).

2. Attachment

In virtually every habitat studied to date, bacteria grow preferentially on available surfaces and not in the aqueous phases above or around them. Several theories have been proposed to explain the forces involved in the transport of bacteria to surfaces. Marshall (1985) summarized the mechanisms that transport bacteria from a liquid to a contiguous surface as sedimentation, chemotaxis, cell surface hydrophobicity, fluid dynamic forces and Brownian motion. Marshall et al. (1971) described microbial attachment to solid surfaces as a two step process: reversible and irreversible. In the first reversible stage the bacterium is weakly held to the surface by electrostatic attraction and Van der Waals Forces. Cells are easily removed

in this stage. The second irreversible stage is time-dependent and involves the physical attachment of the cell to the surface by complex polysaccharide material produced by the cell. This extracellular material (ECM) has been described as a felt-like polysaccharide glycocalyx by Costerton (1978).

In an extensive review of the role of bacterial attachment in the establishment of microorganisms in food processing plants, Notermans et al. (1991) described three distinct steps. In the first stage, the bacteria attach to the surface. In the second stage, consolidation, the microorganisms produce extracellular material (ECM) that cements the cells to the surface. The ECM contains thread-like fibers, frequently referred to as fimbriae, that extend from the cell wall to the contact surface (Sasahara and Zottola, 1993; Herald and Zottola, 1988; Lewis et al., 1987). During the third stage, colonization, the bacteria colonize the surface, growing and spreading. Many changes occur at the interface between the bacterial microcolony and the surface. The complex polysaccharides present in the glycocalyx may bind metal ions, altering the chemical nature of the biofilm. Metabolic by-products such as organic acids can be trapped in the matrix and result in localized corrosion.

Bacterial biofilm formation can bebeneficial for the bacteria. Biofilms capture inorganic and organic molecules from the bulk liquid making these available as nutrients for the growth of organisms within the biofilm. The

glycocalyx holds cells close together, ensuring an exchange of metabolites to sustain growth. Some microbial habitats operate under low nutrient conditions. Marshall (1985) and Dawson et al. (1981) showed the enhanced rates of adhesion by starved cells. This is significant since most biofouling occurs in low nutrient conditions.

4. Attachment to food contact surfaces

All plant materials and animal surfaces carry a variety of microorganisms at the point of harvest or slaughter. These microorganisms can directly or indirectly contaminate food or food contact surfaces during processing. Direct contamination occurs by contact with the raw material. Indirect contamination can occur by contact with factory surfaces, personnel, environmental sources, rodents, or pests. A particular microorganism becomes indigenous to a processing system if it can survive and multiple under routine cleaning and disinfection. Improper factory layout or equipment design contributes to the proliferation of microorganisms. Areas that are not cleaned effectively such as dead ends, cracks, bends or turns, and gaskets provide sites for accumulation of organisms (Sandholm and Wirtanen, 1992).

Several types of surfaces in a food processing plant have been shown to be suitable to microbial attachment: glass (Fletcher, 1988), stainless steel

(Lewis et al., 1987; Herald and Zottola, 1988), meat (Notermans and Kampelmacher, 1974), rubber (Speers et al., 1985) and polystyrene (Harber et al., 1983). Scanning electron micrographs of four different surfaces contaminated with Escherichia coli show the different surface characteristics (Notermans et al., 1991). The Teflon and tile surfaces are smooth and the organism appears to be attached. Pore sizes are 10pm and 10μm, respectively. The stainless steel surface has channels and crevices of a width equivalent to that of the E. coli. The aluminum surface has more large channels and a sponge-like appearance. In the same study, Notermans examined by SEM the concrete floor of a poultry slaughterhouse, a welded metal joint, and a piece of rubber. The concrete and the welded joint have many uneven surfaces and globules, and the whole surface of the rubber is pitted. These different types of surfaces demonstrate the ease with which bacteria can attach and proliferate.

3. Elimination

The formation of biofilms on surfaces pose serious fouling problems in many systems. Bacteria grow, divide, and produce metabolites within the biofilm. Surface damage and biofouling often result from these processes. The elimination of the biofilm is a very difficult and demanding task. Mechanical cleaning is the most efficient way of removing biofilm, but

equipment structure often makes this difficult. Cleaning-in-place (CIP) systems have not been designed to eliminate biofilms, although CIP can prevent biofilm formation (Stone and Zottola, 1985). The period between cleanings can range from several hours to several days. Longer periods between cleanings allow for buildup of organic material and microorganisms. These researchers reported that with proper treatment temperatures and proper concentrations of detergents and sanitizers attached Pseudomonas fragi were inactivated and biofilms were removed. When intervals between cleaning and sanitizing exceeded 8 hours, the numbers of attached microorganisms in the biofilm increased to a point at which they might resist inactivation by cleaning and sanitizing. LeChevallier et al. (1988B) reported that microorganisms in biofilms were 150-3000 times more resistant to hypochlorous acid (free chlorine, pH 7) and 2-100 times more resistant to monochloramine than unattached cells.

Older biofilms are harder to remove than younger ones due to the nature of irreversible attachment. Pontefract (1991) noted that trends in the food processing industry toward longer processing times with minimum time allotted for cleaning and sanitizing can promote the growth of biofilms. If short cuts were made in cleaning and sanitizing, attached microorganisms could be a potential source for contamination of food.

B. SALMONELLA TYPHIMURIUM

1. Identification and taxonomy

Salmonellae are widespread in the environment and appear in a wide variety of foods and food ingredients, thus posing a great problem to the food industry. The number of human salmonellosis cases per year in the United States is conservatively estimated to be 2-4 million (Foster, 1969; Silliker, 1982). Salmonellae grow at temperatures of 3.5 - 45°C and at Aw values of 0.945 to 0.999. They are short rods (0.7 - 1.5 X 2 - 5 μm) usually motile by peritrichous flagella, facultatively anaerobic, Gram negative, and conform to the definition of the family Enterobacteriaceae. Salmonellae can survive but not grow in media with 15 to 30% sodium chloride (Marth, 1969).

Salmonellae usually reduce nitrate to nitrite, can produce gas from glucose, can produce hydrogen sulfide on triple sugar iron agar, are indole negative, and can utilize citrate as a sole carbon source. If present, salmonellae usually comprise a very small component of the total population. Even one Salmonella cell detected in a sample of food (exceptions are raw chicken and red meat) is considered infective (D'Aoust, 1985B). Therefore, it is necessary to detect very small numbers of the organism. The small number of salmonellae in comparison to the larger number of other organisms, means the presence of salmonellae is unlikely to be demonstrated

by direct plating on selective media. Pre-enrichment of processed foods in a nonselective broth medium facilitates detection of Salmonella. Lactose broth is commonly used for pre-enrichment, because when competing lactose-utilizing bacteria are present in a food sample, a resulting drop in pH generates a bacteriostatic effect on competing microflora (North, 1961). Selective enrichment media contain selective ingredients that allow the proliferation of Salmonella and inhibit the growth of competing non-Salmonella microorganisms. Enrichment broths used for the detection of foodborne salmonellae include selenite broth or selenite broth modified by the addition of cystine or brilliant green dye (Fagerberg and Avens, 1976) and tetrathionate broth (USDA, 1974). Following enrichment, samples of the enrichment broth are streaked onto selective and differential agar plates such as bismuth sulfite (BS), xylose lysine desoxycholate (XLD), or brilliant green (BG) (BAM, 1995). If colonies typical of Salmonella are observed after overnight incubation, the nature of these colonies must be confirmed. Typical reactions on triple sugar iron agar (TSI) and lysine iron agar (LIA) slants are presumed positive for Salmonella (BAM, 1995). Confirmation is accomplished in two ways: (1) examination of additional biochemical characteristics of the isolate, and (2) serologic determination of antigenic nature typical of Salmonella. Salmonella possess O, H, and K antigens (Bailey and Scott, 1966). Usually only the O and H antigens are determined

when typing a culture. The cultures are typed according to the Kauffmann-White scheme (Kauffmann, 1966). The O (somatic) antigens are a constitutive part of the cell wall, and are a complex of lipopolysaccharide (LAPS) and protein. The O antigens are heat stable, resisting boiling for 2 1/2 hr. The H antigens, or flagellar antigens, are found only in motile cultures. They are destroyed at 100°C and by dilute alcohol or acid (Bailey and Scott, 1966).

2. Disease

According to Morgan (1965), there are three main types of salmonellosis. They are: enteric (typhoid) fever, gastroenteritis, and a localized type with foci in one or more organs accompanied by septicemia. Every Salmonella strain is potentially able to produce any of these three clinical types of infection. Typhoid fever is the classic example among the enteric fevers. The incubation period is seven to 14 days. The mortality rate in untreated patients is about 10%, and death generally results from intestinal hemorrhage or perforation.

Gastroenteritis is the most frequent syndrome encountered and is of primary importance to the food microbiologist. It may be caused by any of the numerous Salmonella serotypes, except Salmonella typhi and is usually a self-resolving mild to moderately severe prolonged diarrhea (Marth, 1969).

Severity and duration of the disease vary with the amount of food consumed, the kind of Salmonella, and the resistance of the individual. Intensity varies from slight discomfort and diarrhea in 0.1% of cases to death in two to six days. A definitive diagnosis requires the isolation and identification of the causative agent from the patient's feces. Often the symptoms are so mild that no medical treatment is sought or needed. Medical management usually only consists of supportive care with attention to fluid and electrolyte replacement. Septicemias caused by salmonellae are characterized by a high remittent fever. Organisms may localize in any tissue of the body and may produce local abscesses (Morgan, 1965).

3. Distribution in Nature

The transmission of the disease is usually from animal to human by the ingestion of food of animal origin (D'Aoust, 1989). Various foods have been vehicles of transmission. The major sources of human salmonellosis are eggs, poultry, and to a lesser extent other farm animals, which may frequently be intestinal carriers of the organism. Generally no symptoms of disease are observed in animals, so they usually pass veterinary slaughterhouse inspection without restrictions (Oosterom, 1991). During slaughter intestinal material, often containing Salmonella, contaminates the surface of carcasses, which may lead to contamination of meat and meat

products (Haddock, 1970). Animals most often implicated are poultry and pigs and to a lesser degree, cattle and sheep. Milk may be contaminated with fecal material during collection (Marth, 1969). Eggs may be contaminated externally with fecal material or by transovarial transmission (Thornton, 1991). Human infection occurs when animal products are improperly handled during final preparation. Salmonellae may spread in the environment due to excretion by human patients, wild and domesticated animal carriers, as well as disposal of slaughter sludge and manure (Lillard, 1989).

4. Food borne Outbreaks

Consumer mishandling of poultry, meat and dairy products is the most frequent cause of outbreaks (Silliker, 1982). Salmonellae lead the list of common food borne pathogens in Europe and North America (D'Aoust, 1989). Several large outbreaks of Salmonella typhimurium have occurred internationally. An outbreak in Sweden in 1953 resulted from massive cross-contamination of carcass meat within a single slaughtering plant (Lundbeck et al., 1955). Community consumption of unchlorinated water in Riverside, California, resulted in one of the largest outbreaks of human salmonellosis in the United States (Anon., 1971). A nondairy imitation ice cream dessert supplied by a single manufacturer and served at several

banquets in the Jewish community resulted in an estimated 1790 cases of illness (Armstrong et al., 1970). The product was prepared with unpasteurized egg yolks separated from eggs with hairline cracks. Sale of raw milk by a local producer-retailer in Scotland triggered a widespread outbreak in a rural community. In 1984, a large outbreak was recognized in Canada from Cheddar cheese manufactured from pasteurized or heat-treated (nonpasteurized) cheese milk (D'Aoust et al., 1985A). The largest outbreak of S. typhimurium associated with improperly pasteurized milk occurred in 1984 (Lecos, 1986). The milk was produced in a modern dairy plant with a daily output of 1,500,000 pounds of milk. Although the definitive cause of the outbreak was never found, investigations suggest that a cross-contamination between raw and pasteurized milk lines may have been at fault.

5. Salmonella Biofilms

Reversible or irreversible attachment of Salmonella spp. to surfaces is of primary concern to the poultry industry because Salmonella is indigenous to poultry. The presence of bacteria on poultry carcasses effects shelf-life and safety of the product.

Jones et. al (1981) showed that a wild type strain of S. typhimurium would attach reversibly and irreversibly to HeLa cells if an average of six

flagella were present. When less than one flagellum was present, bacteria failed to become attached. Non-motile strains were shown to attach to HeLa cells when impacted on to the HeLa cells by centrifugation. The researchers found that decreasing the ionic strength also decreased the numbers of bacteria irreversibly attached to the HeLa cells. Other researchers (Notermans and Kampelmacher, 1974) showed that the attachment of flagellated bacteria is greatly dependent on temperature. The optimal temperature for attachment in this study was 20°C.

Chlorine has been shown to effectively reduce the number of Salmonella on broiler breast skin by 2.44 to 3.93 logs when combined with sonification (Lillard, 1993). Thomas and McMeekin (1981) showed that saline prevented firm attachment of bacteria to the fascia or perimysium of chicken muscle. Lillard (1988) showed however, that a significantly higher number of salmonellae attached to skin when saline was used. This discovery is important because muscle fascia is not normally exposed to external contaminants during the processing of broilers. Lillard in the same study also showed that ionic strength had little effect on the attachment of salmonellae to poultry skin or muscle. This research shows that electrostatic attraction between tissue and bacterial cells is not the main mechanism of attachment.

Ronner and Wong (1993) showed that S. typhimurium would grow

well and develop biofilms on stainless steel. They also found that S. typhimurium adhered better to Buna-n than Listeria monocytogenes. When adhered to Buna-n Salmonella was also found to be more resistant to sanitizers. Helke et al. (1993) found that pretreating cells with skim milk significantly decreased attachment to both stainless steel and Buna-n.

C. LISTERIA MONOCYTOGENES

1. Identification and taxonomy

Listeria monocytogenes is the causative agent of the disease listeriosis. Prior to 1981, Listeria was recognized mainly as an animal pathogen. It was suspected that humans acquired the disease through contact with infected animals. Consumption of foods contaminated with Listeria is now known to cause sporadic illness as well as foodborne disease epidemics. L. monocytogenes was first described by Murray et al. in 1926. They named the organism Bacterium monocytogenes after the mononucleosis-like illness it produced. L. monocytogenes is a relatively small (1.0 - 2.0um x 0.5um) rod shaped bacterium (Lovett, 1989). It is facultatively anaerobic, gram-positive, and non-spore forming. L. monocytogenes multiplies from 1° - 45°C with optimum temperatures being between 30° - 37°C. It thrives under anaerobic to microaerophilic conditions and prefers a 10% carbon

dioxide environment. The pH levels under which the organism will grow are pH 5.0 - 9.6 with an optimum pH of 7.0 (Brackett, 1988). It will however grow outside these parameters. L. monocytogenes will grow in media containing 10% NaCl and exhibits survival in 26% salt at 10°C (Hudson, 1992).

Identification of L. monocytogenes is based on colony morphology, gram reaction, tumbling motility, catalase reaction, and β-hemolysis. The production of β-hemolysis on blood agar plates is closely linked to pathogenicity (Rocourt and Seeliger, 1987).

The most common methods for isolation and identification of L. monocytogenes were developed by the Food and Drug Administration and the U.S. Department of Agriculture (BAM, 1995). The FDA procedure is used when culturing from milk and dairy processed foods. Listeria enrichment broth (LEB) is used as the primary enrichment and contains trypticase soy broth supplemented with yeast extract for optimum growth of Listeria. Typical colonies are isolated on Oxford Agar (OXA) which selects against Gram-negative microorganisms. A typical non-selective medium used to grow Listeria is Trypticase Soy + 0.6% Yeast Extract (TSYE). Listeria produces a blue-green sheen by obliquely transmitted light on TSYE. Confirmation and identification tests may include API™-Listeria strips and Listeria O-antigen tests.

2. Disease

Human listeriosis cases are sporadic and the source and route are usually unknown. L. monocytogenes is frequently shed in stools of healthy humans, who otherwise show no signs of the illness (Gellin and Broome, 1989). Humans who carry the organism do not necessarily contract listeriosis. Several large foodborne outbreaks suggests the contaminated food may be the primary source of the organism. In adults the disease manifests as a flu-like illness and may include the following complications: septicemia, meningitis, endocarditis, conjunctivitis, and pharyngitis. The majority of cases of listeriosis occur in immuno-suppressed individuals. These individuals include pregnant women, neonates, organ-transplant recipients, or those receiving immunosuppressive therapy. Persons suffering from such chronic disorders as alcoholism, malignancy, diabetes, heart disease, or acquired immunodeficiency syndrome (AIDS) also have shown to be at risk (Donnelly, 1994). In a recent study of listeriosis in the United States, it was estimated that a minimum of 90 out of every 100,000 AIDS patients had listeriosis infections (Gellin et al., 1991). This was 150 times the cases in the general population of the same age group.

The highest incidence of listerial infection is usually seen in neonates (McLauchlin, 1990). L. monocytogenes is transmitted early on in pregnancy transplacentally from infected amniotic fluid and late in pregnancy via vaginal

discharge (Seeliger and Finger, 1976). The major symptom observed in early transmission is septicemia. Mortality rate is 15-50% of cases and death of the fetus usually occurs by spontaneous abortion or stillbirth. Late transmission usually occurs during birth by contact of the fetus with vaginal discharge. Mortality rate is 0-5% of cases and is usually a result of meningitis. Unlike infection by early transmission, late onset listeriosis is usually not accompanied by maternal symptoms (McLauchlin, 1990). Pregnancy predisposes women to listeriosis but not to carriage of the organism (Lamont and Postlethwaite, 1986). While it is well known that transmission occurs from mother to child through the transplacental barrier, environment to animal and animal to human transmission is not well understood.

3. Distribution in Nature

L. monocytogenes is distributed very widely in nature. This organism can be isolated readily from soil, water, sewage, green plant material, decaying vegetation, and numerous species of birds and mammals, including humans (Gray and Killinger, 1966). A close relationship between onset of listeriosis in cattle and feeding of contaminated silage has long been recognized (Gray, 1960; Kalac, 1982). Previous investigations have identified sheep as a major reservoir of Listeria in nature. In one study in

Spain, 88% of tested sheep were identified as carriers of some member of the genus Listeria (Rodriguez et al., 1984). Infected animals displaying symptoms of listeric infection may excrete L. monocytogenes in milk, blood, and feces. High excretion rates of L. monocytogenes in milk from asymptomatic cows and goats has frequently been reported (Loken et al., 1982).

L. monocytogenes has often been found in raw milk (Lovett et al., 1987). It has also been widely isolated from cheese. Levels as high as 10^7 CFU/g have been found in naturally contaminated cheese. Soft cheese contamination is localized exclusively on the surface of the rind. There is a wide pH gradient during ripening of the cheese and presence of L. monocytogenes parallels increase in pH values of the cheese (Ryser and Marth, 1987).

L. monocytogenes has been found in many varieties of meats and has been widely attributed to surface contamination. L. monocytogenes growth on a variety of processed meat and poultry products correlates well with product pH (Glass and Doyle, 1989). L. monocytogenes grew well on products of pH near or above 6.0 such as ham, bologna, and bratwurst. Most contamination is observed on the surface of meats. Johnson et al. (1990) found L. monocytogenes in the interior muscle cores of 5 of 110 total samples of beef, pork, and lamb roasts. These organisms were

probably present at the time of slaughter.

4. Food borne Outbreaks

In 1979, listeriosis was diagnosed in at least 23 hospitalized patients in the Boston, Massachusetts area (Ho et al., 1986), although it was not reported until years later. The vehicle of infection in this outbreak was most likely hospital food. Patients who had consumed lettuce, carrots, and radishes were more likely to contract the illness. Isolates from 20 of 23 cases were identified as serotype 4b. Symptoms of afflicted patients include bacteremia and meningitis.

In 1981, an outbreak of listeriosis occurred in the maritime provinces of Canada (Schlech et al., 1983). The vehicle of transmission was tentativly identified as commercially prepared coleslaw. The cabbage used in the manufacture of the coleslaw was harvested from fields known to be fertilized by both composted and noncomposted manure from sheep, two of which had a form of ovine listeriosis. The cabbage was held under refrigerated conditions for several months prior to manufacture of the coleslaw. The overall mortality rate for this outbreak was 41%, two adults and 17 infants. All patient isolates were identified as serotype 4b. Also, L. monocytogenes isolates from unopened packages of coleslaw also were identified as serotype 4b.

A third outbreak occurred in Massachusetts in 1983. Pasteurized whole and 2% milk were implicated, although both products underwent pasteurization procedures with times and temperatures in excess of legal requirements (Fleming et al., 1985). Fetuses or infants were involved in seven of the cases. Of the 42 adults affected, all had pre-existing illnesses causing immunosuppression or were taking immunosuppressive drugs. No Listeria was recovered from the incriminated milk.

In 1985 an outbreak implicated Jalisco brand Mexican-style cheese as the vehicle of transmission in Orange County, California (James et al., 1985). Of the cases 65.5% were reported in pregnant women or their offspring and 49 cases were reported in nonpregnant adults, resulting in a total case-fatality rate of 33%. In this instance the cheese was most likely manufactured from a combination of raw and pasteurized milk. Again L. monocytogenes serotype 4b was implicated, and the cheese plant that manufactured the incriminated cheese was found to be contaminated with Listeria.

An Italian report in 1994 described a fatal neonatal listeriosis after maternal infection that was acquired with ingestion of fresh home-made cheese (Negri et al., 1994). The newborn died on the third day of life because of a sepsis due to L. monocytogenes.

A 1995 Swiss outbreak associated with the consumption of a soft

cheese resulted in 57 cases of listeriosis in adults (Bula et al., 1995). Twenty-one percent of the cases were of bacteremia, 40% were of meningitis, and 39% were of meningoencephalitis. The overall mortality associated with the outbreak was 32%.

5. Listeria Biofilms

Listeria monocytogenes has been shown to attach to a variety of surfaces (Mafu et al., 1990; Ronner and Wong, 1993; Krysinski et al., 1992). Once attached to a particular surface, L. monocytogenes can develop resistance to surface sanitizers and heat (Frank and Koffi, 1990; Ren and Frank, 1993). Krysinski et al. (1992) found that the type of surface had little effect on the rate of cell attachment but affected the efficacy of various sanitizers and cleaners. It has been demonstrated that very low nutrient levels produced more resistant biofilms (LeChevallier et al. 1988A). In another study (Ronner and Wong, 1993) however, L. monocytogenes cells in biofilms grown in low nutrient medium were no more resistant to sanitizers than those grown in higher nutrient conditions. Cells and the extracellular matrix of the biofilms grown in higher nutrient conditions seemed to persist better through the rigors of fixation and critical point drying for SEM. Ronner and Wong (1993) also showed that Buna-n rubber had a bacteriostatic effect on L. monocytogenes that was most pronounced under low nutrient

conditions. Czechowski (1990) reported that bacterial attachment to Buna-n gaskets in dairy processing lines increased with length of time in the line and the physical deterioration of the gasket surfaces. Numerous researchers have reported that L. monocytogenes can attach to stainless steel surfaces (Herald and Zottola, 1988; Mafu et al., 1993; Krysinski et al., 1992; Ronner and Wong, 1993; Mustapha and Liewen, 1989). Ronner and Wong (1993) showed that biofilms of L. monocytogenes were more resistant to sanitizers when grown on buna-n than on stainless steel. Once attached the cells of L. monocytogenes are more resistant to chemical sanitizers (Krysinski et al., 1992; Frank and Koffi, 1990; Mustapha and Liewen, 1989). Spurlock and Zottola (1991) showed that L. monocytogenes Scott A was found to survive in cast iron drains regardless of pH or growth media for 28 days. Mosteller (1993A) showed that a temperature of 63°C for 10 - 12 minutes was not sufficient to kill Yersinia enterocolitica and L. monocytogenes in biofilms. The calculated D-values for Y. enterocolitica and L. monocytogenes indicate that the heat alone should have reduced the population by several logs.

C. HEAT TREATMENT

1. Pasteurization

The milk industry has used the process of pasteurization for many years. Pasteurization is a low order heat treatment given foods that will not

support growth of the more heat-resistant organisms or that are refrigerated or frozen to prevent significant growth of the more heat-resistant organisms (Stumbo, 1973). Pasteurization of milk is specifically designed to destroy pathogenic organisms that may be associated with the food and could have public health significance. Pasteurization is based on log reduction and counts can never reach zero. High initial counts can decrease the effectiveness of the process and shorten the shelf life. Pasteurized products need to be further preserved. Milk may be kept stored in a home refrigerator for a week or longer without developing significant off flavors (PMO, 1993).

Milk pasteurization destroys lipase and other natural milk enzymes. Times and temperatures for proper heat treatment are based on the destruction of Coxiella burnetii. Processors must provide the heat that will ensure the remotest particle of food in a batch or within a container sufficient heat treatment. In milk the aim is to apply a heat treatment sufficient to destroy C. burnetii (Potter, 1986). The two most prevalent methods for milk pasteurization are: the batch method of heating every particle of milk to not less than 63°C and holding at this temperature for not less than 30 min, and the high temperature-short time (HTST) method of heating every particle of milk to not less than 72°C (161°F) and holding for not less than 15 sec (PMO, 1993). Pasteurized milk must be quickly cooled following treatment to prevent multiplication of surviving bacteria. Raw milk

contains the enzyme alkaline phosphatase that has heat destruction characteristics that closely resemble the time-temperature exposures of proper pasteurization. It's presence found beyond a certain level in pasteurized milk is evidence of inadequate processing.

2. Microbial Death

From a practical standpoint a bacterium is dead when it has lost it's ability to reproduce. When bacteria are subjected to moist heat, death expressed in terms of reduction of individuals is orderly (Stumbo, 1973). The number of viable cells reduces exponentially with time of exposure to a lethal temperature. Bacteria are killed by heat at a rate very nearly proportional to the number present in the system being heated. This is a logarithmic order of death. When survivors are logarithmically plotted against times of exposure, a straight line will be obtained. Under constant thermal conditions the same percentage of the bacterial population will be destroyed in a given time interval, regardless of the size of the surviving population. The D-value can be obtained from this data. A D-value is the time in minutes required at any temperature to destroy 90% of the spores or vegetative cells of a given organism. Organisms generally have a different resistance to different temperatures. The z-value is the number of Fahrenheit degrees required for the TD curve to traverse one log cycle

(Stumbo, 1973).

One of the factors causing deviation from the logarithmic order is clumped cells (Stumbo, 1973). If cells initially occur in clumps of two or more cells, at the beginning of the heating process each colony will originate from two or more cells. There will be no change in colony count until at least some clumps have been reduced to one viable cell per clump. The graph will originally start out as a curve and when all clumps have been reduced to no more than one viable cell per clump, the curve will become a straight line (Stumbo, 1973). Thermal destruction curves (TD) demonstrate the relative resistance of bacteria to different lethal temperatures. The curve is plotted as the logarithm of D against exposure temperature.

To compare heat processes it is necessary to have a unit of lethality. Processes involving different time-temperature relationships are equivalent if they have the same F-value. The F-value is the equivalent in minutes at a given temperature of all the heat considered with respect to its capacity to kill spores or vegetative cells of a particular microorganism. Equivalent pasteurization holding times (assuming instantaneous heating and cooling of product) to achieve a 15 log cycle reduction in the number of viable cells of non-spore-forming organisms is important to pasteurization (Stumbo, 1973).

3. Factors affecting wet heat resistance

Many factors influence the resistance of bacteria to heat. These factors can be generally classified as Inherent or Environmental (Pflug and Holcomb, 1983). Inherent factors can include cell type, concentration, and phase of growth. Different strains of the same species have different heat resistances. Gram positive cells have a tendency to be more heat resistant than Gram negative cells. A higher population of bacteria requires more time to destroy than does a less populated culture. Pasteurization effectiveness is affected greatly by high initial counts. Shelf-life is also shortened when initial counts are high before pasteurization. The protective mechanism of large microbial populations may also be a result of the production of substances like polysaccharides by the cells. This is basic to the theory of increased resistance of biofilms (Costerton and Lappin-Scott, 1989). In 1910, Schultz and Ritz found the heat resistance of Escherichia coli to be greatest in the early lag, late logarithmic, and early maximum stationary phases.

Environmental factors affecting the growth of bacteria are numerous. Generally speaking nonsporulating bacteria have their highest resistance at or near their optimum growth temperature (Stumbo, 1973). The pH of the growth medium will determine which of the survivors of a heat treatment will grow. Heat resistance of microbial cells increases with decreasing moisture or humidity (Jay, 1992). Addition of soluble salts may decrease

the water activity and increase the resistance of bacterial cells. Low concentrations of NaCl (up to 4%) increase heat resistance of many organisms, whereas higher concentrations decrease resistance (Stumbo, 1973).

4. Thermal resistance methodology

There are many common methods used in measuring thermal resistance of bacteria. The thermal death time tube method (TDT) will be the only one discussed. In this method, inoculated material is distributed in small diameter tubes which are sealed near the mouth (Bigelow and Esty, 1920). The volume in the tubes is usually between 1 - 4 ml. The tubes are heated in a thermostatically controlled bath of mineral oil or water. At specific intervals replicate tubes are removed and plunged into water at or below 70°F. Then the tubes can be incubated directly if the medium in which the bacteria were suspended for heating is favorable for growth of the organism. This method can give reasonable accuracy at $\leq$ 240°F (Stumbo, 1973).

Ideally the glass TDT method should be performed using capillary tubes (Stern and Proctor, 1954). With this method very rapid heating and cooling is possible. Some researchers (Donnelly et al., 1987; Bradshaw et al., 1985) have used borosilicate glass tubes with a somewhat larger

diameter. One of the drawbacks to this method is the appreciable heating and cooling lag. To correct for this it is possible to use tubes made of aluminum. Odlaug and Pflug (1977) found the lag correction factor of aluminum tubes (1/4" OD x 0.058" wall) to be less than 0.3 minutes.

One of the methods used to describe the thermal destruction of microorganisms is the end point method. Multiple replicate tubes are monitored for positive or negative growth. Thermal death time is defined as between the longest heating time when a positive tube was obtained and the shortest heating time when all tubes were negative (Pflug and Holcomb, 1983).

4. Heat Shock/Injury

Heat injury can be characterized by a transformation of selective permeability mechanisms and the biosynthetic capabilities of the stress cells. For example sublethal heating has been shown to cause the loss of cellular membrane integrity, the degradation of ribosomal ribonucleic acid, and some metabolic damage (Tomlins and Ordal, 1971; Gomez and Sinskey, 1973; Pierson and Ordal, 1971A; Pierson et al., 1971B).

The treated population can be plated on selective and non-selective agars to determine the amount of injured cells. The difference in growth between the two populations is considered the injured population. Injured

cells develop a sensitivity to many of the chemicals in the medium and fail to be detected. A population of bacteria after a sublethal treatment contains three physiologically different types of cells: the injured, that are viable and are capable of multiplying in a nonselective medium, but not in a selective medium; and the dead, that are incapable of multiplication even in a nonselective and nutritionally rich medium (Ray, 1989). When injured cells are placed in an appropriate environment for recovery they can repair and grow. The following characteristics are desirable in a repair environment: the environment should be nonselective, it should provide the nutrients necessary for the repair process, and it should be optimum pH, temperature, and time for the repair (Ray, 1989).

5. Heat Resistance of Salmonella

The sensitivity of Salmonella sp. to moist heat is widely accepted. Milk pasteurization treatment is reportedly sufficient to kill even exceedingly large numbers of Salmonella cells (Goepfert et al., 1970). D'Aoust et al. (1987) studied thermal inactivation of Salmonella species in fluid milk. They found that heating at 63°C produced a 4 $\log_{10}$ or greater reduction in the number of viable Salmonella, and that heating at 60°C caused a minimum 2 $\log_{10}$ decrease. Other D- and Z-values can be found in Table (1). Goepfert et al. (1970) found that the heat resistance of 8 strains of Salmonella

increased as the water activity (a_w) of the heating menstruum was reduced. The heat resistance was not dependent on the a_w of the environment alone, but on the substance that was causing the reduced a_w. So it is not possible to determine the heat resistance of salmonellae in one menstruum and to apply the data to other media. Rubin (1985) reported a direct correlation between the increase in casein concentration and length of survival of Salmonella in yogurt whey. This suggests that casein exerts a protective effect on S. typhimurium in acid dairy products.

Sublethal heat injury of S. typhimurium has been associated with degradation of ribosomal RNA and DNA (Tomlins and Ordal, 1971; Gomez and Sinskey, 1973), reduced enzyme activities (Tomlins and Ordal, 1971), changes in transport kinetics (Pierson and Ordal, 1971A), and lipid content (Pierson et al., 1971B).

Table 1. D- and Z-values for Salmonella spp.

STRAIN	INITIAL COUNT	TEMP °C	D-VALUE (min)	Z-VALUE °C	MEDIUM	REFERENCE
enteridis		64.4	<0.2		egg yolk	Palumbo, 1995
typhimurium	10^5	71.7	0.24	5.3°C	milk	Bradshaw, 1987A
typhimurium		60	0.24	6.2	whole egg	Jaeckle, 1987
typhimurium		60	0.2		TSB	Wolfson, 1994
seftenberg 775W		60 63 67.5	0.122 0.067 0.044		raw milk	D'Aoust, 1987
muenster		60 63	0.063 0.040		raw milk	D'Aoust, 1987

6. Heat Resistance of Listeria

There are conflicting results concerning the ability of Listeria to survive the temperatures used in milk pasteurization (Table 2). Bearns and Girard (1958) and Garayzabel et al. (1986) found that pasteurization at 61.7°C for 35 min and 78°C for 15 sec respectively would not kill viable cells of Listeria. Doyle (1988) reported survival of L. monocytogenes in milk after pasteurization at 72.2°C for 16.4 sec. Others have claimed the organism does not survive the lowest legal high-temperature short-time (HTST) pasteurization temperature (Donnelly and Briggs, 1986, Donnelly et al., 1987). Donnelly et al. (1987) compares the works of Bearns and Girard (1958) and Bradshaw et al. (1985) and concludes the differing results are due to variations in technique and that Listeria will not survive commercial pasteurization. Bradshaw et al. (1985) found that 15 $\log_{10}$ of L. monocytogenes per milliliter of raw milk would be killed if heated at 71.7°C for 15 sec., which is the minimum heat treatment milk must be given for HTST pasteurization. Moore (1988) concluded that pasteurization is a safe process that reduces the number of L. monocytogenes in raw milk to levels that do not pose any risk to human health. If the level of contamination is greater than 4000 bacteria / g, the pasteurization temperature may be raised to 77.2°C. Listeria contamination is most likely a function of post process contamination (USDA, 1987; Charlton et al., 1990). Possible sources of L.

monocytogenes in a dairy plant include floors in coolers, freezers, processing rooms, floor mats and drains (Klausner and Donnelly, 1991).

Other researchers have speculated that differing results are due to whether the Listeria is freely suspended in milk or engulfed in bovine leukocytes or macrophages. Again on this topic, results are conflicting. Doyle et al. (1987) found that intracellular L. monocytogenes Scott A survived HTST pasteurization and extracellular L. monocytogenes did not. Bunning et al. (1988) concluded that the intracellular position did not significantly increase the thermal resistance and that L. monocytogenes could survive pasteurization regardless. Farber et al. (1988) recovered intracellular L. monocytogenes when heating at 60 - 66°C but not at 69°C and above for 16.2 sec.

Another suggestion for the possible thermotolerance of Listeria involves its response to heat shock. Log phase cells of L. monocytogenes Scott A were shown to be significantly more heat resistant when heat shocked at sublethal temperatures (Linton et al., 1990). The study showed that the heat resistance of L. monocytogenes could be increased 2-fold after heating at 50 to 65°C. Fedio and Jackson (1989), Knabel et al. (1990), and Farber et al. (1992) all found that when L. monocytogenes was exposed to sublethal temperature before the final lethal temperature, the cells acquired an enhanced thermotolerance. This implied that L. monocytogenes in

refrigerated foods acquires enhanced thermotolerance if temperature abused. Repair of heat injured Listeria in milk at 4 and 10°C was complete in 16 and 4 days respectively (Meyer and Donnelly, 1992). These results suggest that commercially temperature abused milk could allow the recovery and growth of Listeria. Stephens and Cole (1994) showed that an increased thermotolerance was observed when the rate of heating was less than or equal to 0.7°C per minute. They postulated that the increased thermotolerance during slow rates of heating was analogous to the induction of the heat-shock response.

Table 2. D- and Z-values for Listeria monocytogenes

STRAIN	INITIAL COUNT	TEMP °C	D-VALUE	Z-VALUE	MEDIUM	REFERENCE
	10^6	62.8 71.7	53.8 s 4.1 s	5.6°C 8°C	whole milk	Bunning, 1988
	10^4	74.4 71.7	0.7 s 0.9 s	6.3°C 6.3°C	whole milk	Bradshaw, 1985
Scott A		71.7	0.9 - 2.7 s	5.8 - 7.1°C	milk	Bradshaw, 1987
		66	0.2 min	7.2°C	liquid egg	Foegeding, 1990
		63 65	42 s 20 s	6.1°C 6.1°C	milk	Mackey, 1989
		62.7	0.1 min		milk	Beckers, 1987
		64.4	0.44 min		egg yolk	Palumbo, 1995

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SECTION 2: Heat Resistance of *Salmonella typhimurium* and *Listeria monocytogenes* in Suspension and Attached to a Buna-n Rubber Gasket.

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ABSTRACT

The thermal death time tube (borosilicate glass tubes) method was used to determine the heat resistance of *Listeria monocytogenes* Scott A and *Salmonella typhimurium* suspended in 2% UHT processed milk and adhering to Buna-n o-rings in biofilms. Cultures were grown to the early stationary phase and diluted to 10^5 / ml. Biofilms were grown on Buna-n rubber o-rings (4.46 mm O.D. x 1.41 mm I.D.) for 36 hours giving an initial concentration of $\sim 10^5$. The three treatments were: Control - inoculated milk, Treatment 1 - sterile milk plus an o-ring with a biofilm on it, and Treatment 2 - inoculated milk with a sterile o-ring. Time spent at each temperature was corrected for lag time of heating and cooling through thermocouple measurements. There was no survival recorded at an $F^{6.7}_{71.7} = 0.52$ min. for *L. monocytogenes* and at an $F^{5.0}_{71.7} = 0.41$ min. for *S. typhimurium*.

For the same microorganism at the three temperatures tested (60, 63, 67°C), there was no significant difference in D-values between treatments ($p > 0.05$). There was a significant difference ($p < 0.001$) between the D-values for *Salmonella* and *Listeria*.

INTRODUCTION

Various studies have been performed to determine the heat resistance of *Salmonella typhimurium* and *Listeria monocytogenes* in suspension in a defined phase of growth. Milk pasteurization treatment is reportedly sufficient to kill even exceedingly large numbers of *Salmonella* cells (9). D'Aoust (5) studied thermal inactivation of *Salmonella* species in fluid milk. He found that heating *Salmonella* at 63°C produced a 4 $\log_{10}$ or greater reduction in the number of viable salmonellae, and that heating at 60°C caused a minimum 2 $\log_{10}$ decrease. There are conflicting results concerning the ability of *Listeria* to survive the temperatures used in milk pasteurization. Bearns and Girard (1) and Garayzabel et al. (8) found that heating at 61.7°C for 35 min and 78°C for 15 sec respectively would not kill viable cells of *Listeria*. Donnelly et al. (6) compared the works of Bearns and Girard (1) and Bradshaw et al. (2) and concluded that the differing results were due to variations in technique and that *Listeria* will not survive commercial pasteurization.

Studies on the heat resistance of *L. monocytogenes* in a biofilm matrix have shown that there is an increase in heat resistance over cells suspended in a heating medium. Once attached to a particular surface, *L. monocytogenes* can develop resistance to surface sanitizers and heat (7, 14). Ronner and Wong (15) showed that biofilms of *L. monocytogenes* were

more resistant to sanitizers when grown on Buna-n rubber than on stainless steel. Mosteller (12) showed that a temperature of 63°C for 10-12 minutes was not sufficient to kill *L. monocytogenes* in biofilms.

In one of the studies done on attachment of *Salmonella* to surfaces (15), *S. typhimurium* was shown to form good biofilms on Buna-n and stainless steel. In the same study, *S. typhimurium* was also found to be more resistant to sanitizers while attached to Buna-n. Researchers (10) have shown that attachment of *S. typhimurium* to Buna-n was significantly decreased when the surface was pretreated with skim milk.

Traditionally, the method for studying the resistance of bacteria to heat has been the thermal death time (TDT) tube method (16). This method has not been used to determine the resistance of cells in a biofilm matrix. One of the prerequisites of a TDT study is that the cells be in singlet form, because clumped cells can result in a deviation from the logarithmic order of death (16).

The purpose of this study was to determine the heat resistance of organisms contained in a biofilm matrix using a laboratory scale pasteurizer, and to compare that heat resistance with those same organisms in suspension.

MATERIALS AND METHODS

Cultures

Listeria monocytogenes Scott A was obtained from the VPI&SU Food Science and Technology culture collection and transferred daily on Tryptic Soy Agar + 0.6% Yeast Extract (TSAYE) slants. *Salmonella typhimurium* (ATCC# 19585) was also obtained from the VPI&SU Food Science and Technology collection and transferred daily on Plate Count Agar (PCA) slants. Cultures were maintained on slants at 35°C. Monthly transfers were made of stock cultures.

Studies on both organisms were done in parallel. An inoculum was transferred from a slant to Tryptic Soy Broth (TSB) for *Salmonella* and to Tryptic Soy Broth + 0.6% yeast extract (TSBYE) for *Listeria* eight hours before the experiment was begun. All cultures were maintained at 35°C. All media was obtained from Difco Laboratories (Detroit, MI) unless otherwise noted.

Biofilms

Biofilms were grown on Buna-n rubber o-rings (4.46mm O.D. x 1.41mm I.D.) (McMaster Carr, New Brunswick, NJ) with a surface area of 68 mm^2. UHT processed 2% milk purchased commercially was used as the growth medium. The UHT milk was given no further heat treatment. One-

hundred fifty ml of milk and 100 sterile o-rings and 10 ml of culture were added to a 250 ml bottle. The bottle was placed on a Precision Scientific 360 Orbital Shaker Bath (Chicago, IL) set at 150 rpm and 21°C and incubated for 36 hours to form the biofilms (13).

Removal of non-adherent cells

After biofilms had formed on the o-rings, non-adherent cells were rinsed off. Preliminary experiments were done to determine how many rinsings were sufficient to remove non-adherent cells. The o-rings with biofilms grown on them were added to a sterile 50 ml portion of potassium phosphate buffer (pH 7.2), mixed gently by swirling, and then poured over a sterile plastic filter to separate o-rings from buffer. This step was repeated with successive 50 ml portions of potassium phosphate buffer. Each rinsing was plated on either TSAYE for *Listeria* or PCA for *Salmonella* to determine the number of rinsings that were sufficient to obtain <200 cfu/ml at the 10^0 dilution. Twelve rinsings were adequate for removal of non-adherent cells.

Preliminary numbers

Ten o-rings (10 o-rings = 0.2 g) were set aside and blended in a Waring (New Hartford, Connecticut) blender cup (37 ml capacity) with sterile potassium phosphate buffer to determine the initial cell numbers in the

biofilms. The blender cup and the phosphate buffer were kept at 4°C until use to minimize the amount of heat added during blending. Microscopic (Olympus BH2-UMA, Lake Success, NY) evaluation at different blending times was performed to ensure the presence of singlet cells without clumps. The blending time for *Listeria* was 1.5 minutes and the blending time for *Salmonella* was 2.0 minutes. After blending the organisms were enumerated on appropriate agar (PCA or TSAYE) via spread plating. Number of cells contained in the biofilms was approximately 10^5 cfu/o-ring for each experiment.

Growth Curve

A growth curve was determined for each organism using absorbance (ABS) readings at 650 nm (Bausch & Lomb Spectronic 70 spectrophotometer). A 24 hour broth culture was used to inoculate a tube of sterile broth. ABS readings were then taken every hour and those readings were graphed vs time to determine the stationary phase of growth. The stationary phase was reached for both organisms after eight hours of growth. Simultaneously, the broth culture was enumerated every hour to determine the concentration at each phase of growth. The concentration after 8 hours was 10^8 cfu/ml for both organisms. For each experiment the broth culture was diluted with sterile peptone to achieve an initial inoculum

level of 10^5 cfu/ml. Care was taken to ensure that the initial inoculum counts and the initial biofilm counts were equal.

Water bath

The heating unit used for this experiment was a Blue M (Blue Island, IL) constant temperature agitating water bath with a 56 liter capacity. Omega Engineering (Stamford, CT) copper - constantan thermocouples placed at 4 locations in the bath ensured the temperature was equivalent throughout the bath. Temperature readings were obtained using a Campbell Scientific 21X Micrologger (Logan, UT). The temperature was maintained at ±0.5°C of set temperature. Water level was maintained to ensure complete submergence of the tubes.

Experimental Design

Sterile borosilicate glass (6.5 cm L x 5.5 mm I.D. x 7.0 mm O.D.) was used as the thermal death time (TDT) tube vessel. The glass tube was sealed with an acetylene torch at one end, cooled, filled, and then sealed at the other end. This method allowed for quick closure of the tubes, minimal heat transfer from the torch within the milk medium, and minimal head space. Each tube contained 2 ml of milk.

Experiments for both organisms were done in parallel. There were

three treatments per organism per time and ten tubes per treatment. All treatments for both organisms at a given time were placed in the same rack. The three treatments were designated as: control, treatment 1, and treatment 2. Control tubes consisted of sterile milk that had been inoculated with 2×10^4 - 2×10^5 cfu/ml of organism. Treatment 1 tubes consisted of sterile milk inoculated with 2×10^4 - 2×10^5 cfu/ml of organism plus a sterile o-ring. Treatment 2 tubes consisted of sterile milk plus an o-ring with a biofilm grown on it at a concentration of 2×10^4 - 2×10^5 cfu/o-ring. Each rack of tubes contained six rows, three for *Listeria* and three for *Salmonella* arranged in a random order. Survival data was determined at three temperatures. The heating times and temperatures tested were as follows: 67°C (0, 5, 10, 15, 20, 25 min), 63°C (0, 6, 12, 18, 24, 30 min), and 60°C (0, 6, 12, 18, 24, 30 min).

One o-ring was placed in each tube using sterile tweezers. Tubes were filled with milk using a sterile Micromatic (Popper and Sons, New Hyde Park, NY) repeatable glass pipet and then sealed. All six racks were simultaneously placed in the water bath and then individually removed at the appropriate times. Before and after heat treatment the tubes were kept at 4°C.

After the tubes had received heat treatment and were cooled to 4°C they were incubated at 35°C for 48 hours. Milk was considered a sufficient

recovery medium so subsequent subculturing was not performed. After 48 hours, the tubes were opened and the entire contents, including the o-ring, were added to an enrichment broth. Selective enrichment broths used were *Listeria* Enrichment Broth (LEB) for *Listeria* and Selenite Cysteine (SC) broth for *Salmonella*. Enrichment broths were incubated for 24 hours at 35°C and then each tube was streaked onto a selective agar. Xylose Lysine Desoxycholate (XLD) was used as the selective agar for *Salmonella* and Oxford Agar (OXA) was used as the selective agar for *Listeria*. Presence or absence of the organism in the tube was indicated by typical black colonies on either XLD or OXA. Results were recorded as survival vs no survival. Selected colonies were confirmed by *Listeria* API (bioMerieux, l'Etoile, France) or *Salmonella* O Antiserum Poly A (Difco, Detroit, MI).

F-value and Z-value determination

Heating and cooling curves were determined using a Campbell Scientific 21X Micrologger (Logan, UT) and an AM32 Input Multiplexer (Logan, UT). Omega Engineering (Stamford, CT) copper - constantan thermocouple wire was used. Thermocouples were placed in three locations within the heating tube to correctly assess the cold spot in the glass tubes: geometric center, top 1/3 of the tube, and bottom 1/3 of the tube. A wire mesh was placed on the inside of the glass tube to ensure that the

thermocouple wire was centrally located and freely suspended. The thermocouple wire was threaded through this mesh. The tube was then filled with milk and sealed with silicone sealant.

A total of 20 sample tubes with thermocouple couple wires were used. Two thermocouples were also placed directly in the water bath. All racks were loaded with tubes. Each rack had at least three thermocouple tubes in it. Racks were removed at appropriate times. Before and after heating, the racks were kept at 4°C. This procedure was performed for each of the temperatures and times used in the experiment. Campbell Scientific PC208 Software (Logan, UT) was used to retrieve the data from the datalogger. As described below an F-value was calculated with a program that utilizes the General Method of calculation (16).

A Z-value was determined for the control tubes from the inverse slope of the log (D-value) vs. temperature graph.

Formulas and Statistics

Survival was recorded as growth or no growth following the 'end point' technique of Stumbo (16). Formula 1 (16) was used to estimate the most probable number (mpn) of survivors per ml. That value was then used in Formula 2 (16) to calculate a D-value at a given temperature.

Formula 1: $x = 2.303/a * \log(n/q)$
x = mpn survivors/ml
a = volume in ml of each sample
n = total replicates = 10
q = number of negative tubes

Formula 2: $D = t/(\log a - \log b)$
D = D-value at a given temperature
t = time in minutes corrected for lag
a = initial number per 10 tubes
b = mpn survivors per 10 tubes = x*2ml/tube*10tubes

Statistics were performed using SAS (Release 6.07), Statistical Analysis System, Cary, NC. A general linear model procedure was performed for analysis of variance followed by a Tukey's multiple means comparison test.

RESULTS AND DISCUSSION

Both organisms formed good biofilms on Buna-n o-rings. The biofilms formed by *Salmonella* seemed to adhere better to the Buna-n because a longer blending time was required to remove the biofilm cells. This is consistent with previous research (15).

D-values are given in Table 1 for *Listeria monocytogenes,* and in Table 2 for *Salmonella typhimurium*. The time used in the Formula 2 calculation was corrected for come-up and cool down by using the equivalent time spent at the given temperature or the F-value. This time was

appreciable. The total lag factor ranged from 50% of the total time for the shorter heating times and lower temperatures to 8% of the total time for the longer times and higher temperatures tested.

There was no statistical difference ($p > 0.05$) between D-values for the different treatments (Control, Treatment 1, Treatment 2). A significant difference ($p < 0.0001$) between D-values was found between organisms and when temperature and organism were accounted for together.

The results of this study indicated that an organism as part of a biofilm is not significantly more heat resistance than an organism freely suspended. At the temperatures tested there was an appreciable lag factor in the glass tubes for some of the times tested. Linton (11) showed that minimal thermal processes involving long come up times may cause a heat shock response and increase heat resistance of certain bacteria. The lag factor at 60°C was the greatest and lasted up to 50% of the test time. Z-value obtained for *S. typhimurium* was 9.3°C. This values was substantially higher than published Z-values (5.3°C (3)). The published Z-value for *L. monocytogenes* is 8.0°C (4). A Z-value could not be obtained for the experimental *L. monocytogenes* data, because the data was to erratic.

When a biofilm was introduced into this procedure deviations were expected from usual logarithmic death. For *S. typhimurium* the heat

resistance in suspension was very similar to the heat resistance in a biofilm. Although *Salmonella* formed very good biofilms it did not seem to have an increased heat resistance in the biofilm over this temperature range.

Introducing an o-ring into the system added an additional variable. The o-ring may have established a heat gradient that may have produced a cooling effect on the surface of the o-ring where the biofilm was formed. This would be especially evident for lower temperatures and times tested. If a gradient were established in a processing line then milk processed under these conditions could be susceptible to contamination from biofilms. As processing continued the gradient would disappear.

Within each experiment death of both organisms was not orderly. Survival values would fluctuate up and down as the time increased. This fluctuation may have contributed to the higher than published Z-values for *S. typhimurium* and the inability to determine a Z-value for *L. monocytogenes*.

Table 1. Average[a] calculated D-values (min) for *Listeria monocytogenes* Scott A at the given temperatures. z = 6.7°C (2)

Temperature °C	Control: inoculated milk	Treatment 1: inoculated milk and sterile o-ring	Treatment 2: sterile milk and biofilm on o-ring
60	0.64	1.05	0.78
63	1.35	0.73	1.36
67	1.41	1.46	1.71

a: 2 repetitions

Table 2. Average[a] calculated D-values (min) for *Salmonella typhimurium* at the given temperatures. z = 5.0°C (3)

Temperature °C	Control: inoculated milk	Treatment 1: inoculated milk and sterile o-ring	Treatment 2: sterile milk and biofilm on o-ring
60	2.37	2.85	2.11
63	1.71	1.38	1.76
67	1.30	1.53	1.50

a: 2 repetitions

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SECTION 3: Heat Resistance of *Salmonella typhimurium* and *Listeria monocytogenes* in Suspension and in a Biofilm Matrix as Determined by an HTST pasteurizer.

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ABSTRACT

A laboratory scale pasteurizer was used to determine the heat resistance of pure cultures of *Listeria monocytogenes* Scott A and *Salmonella typhimurium* suspended in 2% milk vs. those same organisms in a biofilm matrix. Biofilms were grown on Buna-n rubber o-rings and then sloughed off through blending. Microscopic evaluation determined the size of the sloughed off pieces to be 3.8×10^{-4} mm^2 - 8.8×10^{-3} mm^2. The sloughed off pieces were added to commercially purchased UHT 2% milk and pasteurized at different flow rates. As a control the same organism freely suspended in milk was pasteurized. All the heat in the transit lines that incorporate the come up and cool down was also considered. The different flow rates all incorporated a process in excess of 71.7°C for 16 sec (Z = 6.7°C for *Listeria*, Z = 5.0°C for *Salmonella*).

Following heat treatment samples were allowed to recover in the milk and were enriched before plating for growth on selective agar. *Listeria monocytogenes* Scott A and *Salmonella typhimurium* were not recovered from any sample of freely suspended cells or any sample of sloughed off biofilm cells. Previous research citing the increased heat resistance of *L. monocytogenes* in a biofilm always involved cells attached to a surface. These results would suggest that neither organism would survive the commercial pasteurization process if pieces of the biofilm matrix were to

slough off during processing.

INTRODUCTION

Several types of surfaces in a food processing plant have been shown to be suitable to microbial attachment: glass (9), stainless steel (16, 18), meat (22), rubber (25) and polystyrene (14). The dairy industry relies on cleaning-in-place (CIP) systems to ensure sanitary processing. CIP systems have not been designed to eliminate biofilms, although CIP can prevent biofilm formation (27). Pontefract (23) noted that trends in the food processing industry toward longer processing time with minimum time allotted for cleaning and sanitizing can promote the growth of biofilms. If short cuts were made in cleaning and sanitizing, attached microorganisms could be a potential source for contamination of food. Czechowski (3) reported that bacterial attachment to Buna-n gaskets in dairy processing lines increased with length of time in the line and the physical deterioration of the gasket surface.

Two organisms of concern for the dairy industry are *Salmonella typhimurium* (17) and *Listeria monocytogenes* (8). The organisms can be isolated readily from soil, water, sewage, green plant material, decaying vegetation, and numerous species of birds and mammals, including humans (13). *L. monocytogenes* is distributed widely in nature and has often been found in raw milk (19). Transmission of *S. typhimurium* is usually from animal to human by the ingestion of food of animal origin (5).

Various studies have been performed to determine the heat resistance of *S. typhimurium* and *L. monocytogenes* in suspension in a defined phase of growth. Milk pasteurization treatment is reportedly sufficient to kill even exceedingly large numbers of *Salmonella* cells (12). D'Aoust et al. (4) studied thermal inactivation of *Salmonella* species in fluid milk. They found that heating at 63°C produced a 4 $\log_{10}$ or greater reduction in the number of viable salmonellae, and that heating at 60°C caused a minimum 2 $\log_{10}$ decrease. There are conflicting results concerning the ability of *Listeria* to survive the temperatures used in milk pasteurization. Bearns and Girard (1) and Garayzabel et al. (11) found that pasteurization at 61.7°C for 35 min and 78°C for 15 sec respectively would not kill viable cells of *Listeria*. Donnelly et al. (6) compares the works of Bearns and Girard (1) and Bradshaw et al. (2) and concludes the differing results are due to variations in technique and that *Listeria* will not survive commercial pasteurization.

Studies exploring the heat resistance of *L. monocytogenes* in a biofilm matrix have yielded significantly increased resistance values. Once attached to a particular surface, *L. monocytogenes* can develop resistance to surface sanitizers and heat (10, 24). Ronner and Wong (25) showed that biofilms of *L. monocytogenes* were more resistant to sanitizers when grown on Buna-n than on stainless steel. Mosteller et al. (20) showed that a temperature of 63°C for 10-12 minutes was not sufficient to kill *L. monocytogenes* in

biofilms.

S. typhimurium was shown (25) to form good biofilms on Buna-n and stainless steel. *S. typhimurium* was also more resistant to sanitizers while attached to Buna-n. Other researchers (15) have shown that attachment of *S. typhimurium* to Buna-n was significantly decreased when the surface was pretreated with skim milk.

Traditionally the method for studying resistance to heat has been the thermal death time (TDT) tube method. This method has not been used to determine the resistance of cells in a biofilm matrix. One of the prerequisites of a TDT study is that the cells be in singlet form, because clumped cells cause deviation from the logarithmic order of death (28). A laboratory scale pasteurizer was used to determine the heat resistance of organisms contained in a biofilm matrix.

MATERIALS AND METHODS

Cultures

Listeria monocytogenes Scott A was obtained from the VPI&SU Food Science and Technology culture collection and transferred daily on Tryptic Soy Agar + 0.6% Yeast Extract (TSAYE) slants. *Salmonella typhimurium* (ATCC# 19585) was also obtained from the VPI&SU Food Science and Technology collection and transferred daily on Plate Count Agar (PCA)

slants. Cultures were maintained on slants at 35°C. Monthly transfers were made of stock cultures.

Studies on both organisms were done in parallel. An inoculum was transferred from a slant to Tryptic Soy Broth (TSB) for *Salmonella* and to Tryptic Soy Broth + 0.6% yeast extract (TSBYE) for *Listeria* eight hours before the experiment was begun. All cultures were maintained at 35°C. All media was obtained from Difco Laboratories (Detroit, MI) unless otherwise noted.

Biofilms

Biofilms were grown on Buna-n rubber o-rings (4.46mm O.D. x 1.41mm I.D.) (McMaster Carr, New Brunswick, NJ) with a surface area of 68 mm^2. UHT processed 2% milk purchased commercially was used as the growth medium. The UHT milk was given no further heat treatment. One-hundred fifty ml of milk and 100 sterile o-rings and 10 ml of culture were added to a 250 ml bottle. The bottle was placed on a Precision Scientific 360 Orbital Shaker Bath (Chicago, IL) set at 150 rpm and 21°C and incubated for 36 hours to form the biofilms (13).

Removal of non-adherent cells

After biofilms had formed on the o-rings, non-adherent cells were

rinsed off. Preliminary experiments were done to determine how many rinsings were sufficient to remove non-adherent cells. The o-rings with biofilms grown on them were added to a sterile 50 ml portion of potassium phosphate buffer (KH_2PO_4, pH 7.2), mixed gently by swirling, and then poured over a sterile plastic filter to separate o-rings from buffer. This step was repeated with successive 50 ml portions of potassium phosphate buffer. Each rinsing was spread plated on either TSAYE for *Listeria* or PCA for *Salmonella* to determine the number of rinsings that were sufficient to obtain <200 cfu/ml at the 10^0 dilution. Twelve rinsings were adequate for removal of non-adherent cells.

Sloughing

Biofilms formed on o-rings were blended for a short amount of time in a sterile stainless steel Waring (New Hartford, CT) blender cup (110 ml capacity) with sterile potassium phosphate buffer to simulate the possible sloughing off of pieces in a processing line. Thirty-six ml of buffer was added to 200 o-rings (200 o-rings = 4g) to achieve an initial dilution of 10^{-1}. The blender cup and the phosphate buffer were kept at 4°C until use to minimize the amount of heat added to the biofilms on blending. Microscopic (Olympus BH2-UMA, Lake Success, NY) evaluation at different blending times was performed to ensure the presence of clumps 3.8×10^{-4} mm^2 -

8.8×10^{-3} mm^2. The *Listeria* blending time was 15 sec., and the *Salmonella* blending time was 25 sec.

Initial numbers

After the first blending, a 300 ml sample of UHT milk was inoculated with 10 ml of the blended buffer solution. A portion of the sample was reserved to determine the initial count by further blending to get singlet cells. The *Listeria* sample was blended for an additional 1.25 min. and the *Salmonella* sample was blended for an additional 1.5 min. The 300 ml sample contained an initial count of approximately 10^5 cfu/ml for all experimental runs.

F-value

In commercial pasteurization, the lethality given the product is determined by only the time the product spends in the hold tube. The heat the product receives in the transit lines on come-up and cool down is not considered. All the heat given a specific particle needs to be considered to determine an F-value. A laboratory scale pasteurizer was used to simulate commercial pasteurization. The unit was a Microthermics 25DH (Raleigh, NC) operated at a flow rates of 400, 1800, and 2200 ml/min with a hold tube length of 212.15 cm. Heating and cooling curves for the process were

determined using a Campbell Scientific 21X Micrologger (Logan, UT). Temperature readings were taken at selected points in the processing line using Omega Engineering (Stamford, CT) copper-constantan thermocouple wire and Type T plugs. Heating and cooling curves were calculated based on the minimum residence times for laminar flow. Campbell Scientific PC208 (Logan, UT) Software was used to retrieve the data from the datalogger. An F-value was calculated with a program that utilizes the General Method of calculation (28).

Pasteurization

The Microthermics unit was used to simulate commercial pasteurization. Samples were pasteurized to two previously determined F-values per organism (*Listeria* $F^{6.7}_{71.7}$ = 1.6 and 0.21 min.; *Salmonella* $F^{5.0}_{71.7}$ = 2.5 and 0.21 min). Table 1 is an example of how these F-values were determined. Six flasks of inoculated culture were prepared at an initial cell concentration of 10^5 cfu/ml. In addition six flasks with sloughed off pieces of biofilms were prepared at an initial concentration of 10^5 cfu/ml. *Listeria* and *Salmonella* were done on different days to prevent cross-contamination. The milk was kept at room temperature (24°C) for not greater than 1 hour until pasteurization to ensure proper function of the machine. The milk was pasteurized and collected in a sterile flask and incubated for 48 hours at

Table 1. Time, temperature history for 25DH pasteurizer at a flow rate of 400 ml/min.

Thermocouple placement	Residence time (sec)a	Cumulative time (sec)	Cumulative time (min)b	Temperature °Cb
product entry	0	0	0	26.1
preheater	8.67	8.67	0.15	76.8
transit preheater to jumper panel	6.29	14.96	0.25	76.3
beverage hold tube	3.75	18.71	0.31	74
transit hold tube to jumper panel	1.89	20.6	0.35	73.8
transit jumper panel to cooler	3.97	24.57	0.41	73
cooler	16.89	41.46	0.69	11.4

a: minimum residence time for laminar flow

b: these two columns are used to calculate an F-value that is obtained by the general method of calculation.

35°C. A representative sample of 20 ml was then placed in 50 ml of enrichment broth, *Listeria* Enrichment Broth (LEB) for *Listeria* or Selenite Cysteine (SC) broth for *Salmonella*. The enrichment broth was incubated for 24 hours at 35°C and then streaked on to a selective media. Xylose Lysine Desoxycholate (XLD) was used as the selective agar for *Salmonella* and Oxford Agar (OXA) was used as the selective agar for *Listeria*. Results were recorded as growth or no growth. Experiments were repeated until 18 samples of each type were obtained at each flow rate.

RESULTS AND DISCUSSION

Milk was pasteurized at two different flow rates that yielded two different $F_{71.7}$ for each organism. At a flow rate of 400 ml/min. (*Salmonella* $F^{5.0}_{71.7}$ = 2.6 min, *Listeria* $F^{6.7}_{71.7}$ = 1.6 min) no survival was recorded for initial concentrations of 10^5 / ml of the inoculum alone or the sloughed biofilm trials for either organism. At a flow rate of 1800 ml/min ($F^{6.7}_{71.7}$ = 0.22 min), no survival was recorded for either treatment of *L. monocytogenes*. At a flow rate of 2200 ml/min ($F^{5.0}_{71.7}$ = 0.22 min), no survival was recorded for either treatment of *S. typhimurium*.

Previous research (6), has concluded that *L. monocytogenes* freely suspended will not survive commercial HTST pasteurization. The results of this study support this finding. No freely suspended *L. monocytogenes* at an

initial concentration of 10^5 / ml was recovered. Researchers have used other methodologies to determine whether *L. monocytogenes* will survive pasteurization. The heating characteristics in a TDT tube experiment (2) are very different from those in a HTST pasteurizer and results can differ based on the different methodologies. Doyle (7) used an HTST pasteurizer to determine heat resistance characteristics and found that when *L. monocytogenes* was encased in polymorphonuclear leukocytes (PMNL) a protective effect on *L. monocytogenes* may allow it to survive pasteurization. This study used unpasteurized milk from cows that had been inoculated over time with *L. monocytogenes*. The effect of PMNL's was not seen after holding unpasteurized milk 4 days at 4°C.

L. monocytogenes has previously been found to be more resistant to heat while attached to a surface (10, 20, 24). During pasteurization, flow through pipelines may cause pieces of biofilms to slough off the surface. This study shows that these sloughed off pieces (3.8×10^{-4} mm^2 - 8.8×10^{-3} mm^2) are not able to survive pasteurization at the flow rates tested.

No previous research was found that addressed the heat resistance of *S. typhimurium* in a biofilm matrix. Numerous researchers have shown that the heat treatment of pasteurization is in excess of that necessary to kill 10^5 cells / ml of freely suspended *S. typhimurium*. The findings of this study support the previous conclusions. *S. typhimurium* has been shown to form

strong biofilms on Buna-n (15, 25). If pieces of these biofilms were to slough off during pasteurization then this study shows that these pieces would not survive pasteurization.

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SECTION 4: APPENDICIES

APPENDIX A: F-value program

The following is the program used to compute the f-value for the glass tube experiment and the pasteurizer runs.

```
$DEBUG
      PROGRAM FVALUE
C-----------------------------------------------------------------
C  Program name: FVALUE
C  Written By  : J. BOLING
C  Date        : 05-06-1986 09:21:38
C  Purpose     : fortran program
C-----------------------------------------------------------------
C  THIS PROGRAM CALCULATES THE FVALUE FOR A THERMAL PROCESS.
C  THE USER IS PROMPTED FOR THE REFERENCE TEMPERATURE, Z
VALUE,
C  THE NUMBER OF THERMOCOUPLES USED IN THE PROCESS (UP TO 8),
AND
C  THE FILE NAME CONTAINING THE TIME-TEMPERATURE DATA.
C
      REAL LO(8),LH(8)
      REAL TIME(600),TEMP(600,8),LE(600,8),F(8)
      CHARACTER FNAME*12
C
      WRITE(*,6005)
6005  FORMAT(' TYPE REFERENCE TEMPERATURE: '\)
      READ(*,*)REF
      WRITE(*,6007)
6007  FORMAT(' TYPE THE Z VALUE: '\)
      READ(*,*)Z
      WRITE(*,6010)
6010  FORMAT(' TYPE THE NUMBER OF THERMOCOUPLES(1 TO 8): '\)
      READ(*,*)NTEMP
      WRITE(*,6020)
6020  FORMAT(' ENTER THE FILE NAME CONTAINING DATA: '\)
      READ(*,'(A)')FNAME
      OPEN(8,FILE=FNAME,STATUS='OLD')
      DO 55 I1=1,600
```

```
      READ(8,*,END=60) TIME(I1),(TEMP(I1,J),J=1,NTEMP)
   55 CONTINUE
      WRITE(*,6030)
 6030 FORMAT('0*** WARNING *** ALL THE DATA WAS NOT READ
INTO'
     1   ,' PROGRAM'/)
   60 N=I1-1
      I1=0
      IREF=REF
      WRITE(*,6090)IREF,IREF,IREF,IREF,IREF,IREF,IREF,IREF
 6090 FORMAT(1H1,////,3X,'TIME',8(3X,'F(',I3,')',))
      DO 150 J=1,NTEMP
      F(J)=0
  150 LO(J)=10.**((TEMP(1,J)-REF)/Z)
      DO 200 I=2,N
      I1=I1+1
      DO 175 J=1,NTEMP
      LH(J)=10.**((TEMP(I,J)-REF)/Z)
      F(J)=F(J)+(TIME(I)-TIME(I-1))*(LO(J)+0.5*((LH(J)-LO(J))))
  175 LO(J)=LH(J)
      WRITE(*,6175) TIME(I),(F(J),J=1,NTEMP)
 6175 FORMAT(1X,F6.2,8(1X,F8.4))
      I1=0
  190 CONTINUE
  200 CONTINUE
  900 STOP
      END
```

APPENDIX B: ALUMINUM TUBES

The TDT experiment was originally performed with aluminum tubes instead of glass. The results were inconclusive and are presented below.

Experimental Design

Aluminum tubes obtained from Industrial Supply Corporation were used as the thermal death time vessel. Tubes were 1/8" x 1 1/2" Schedule 40 aluminum nipples threaded at both ends. Tubes were sealed with a transparent food grade film, Seal View™ , and then wrapped with Teflon tape. The seal was insured to be intact. Using the aluminum tubes allowed for quick come up time of the contents. Each tube contained 1.5 ml of milk. The tubes had an approximate I.D. of 7 mm and O.D. of 9 mm. The experiment was performed exactly as previously stated previously.

Results

Results using this method were inconclusive. Aluminum tubes were originally used because of the negligible lag factor. The results were very consistent over all trials but were unexplainable. Figure 1 is an example of the results obtained.

Conclusions

The organisms were allowed sufficient time to recover from any injury that may have been caused by the heating. The tubes were completely submerged and completely filled. The area of greatest concern is the seal of the tubes. The Seal View may have leaked causing an air space to form at the top of the tubes. This air space would have been an area where the heating would not have been uniform. This seal may have been the cause of the erratic data. Another acceptable form of sealing the tubes was not available at the time.

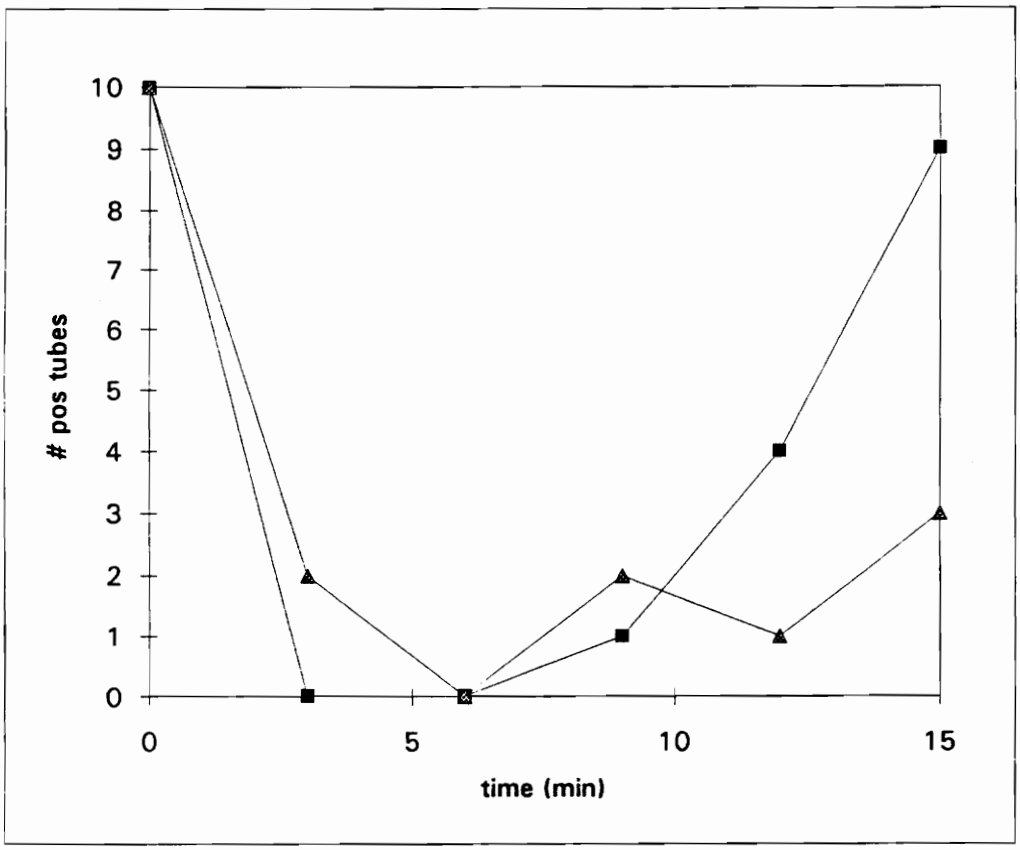

Figure 1. Survival of Salmonella typhimurium at 60°C. D-tubes contain inoculum plus a sterile o-ring.

APPENDIX C: Program for Campbell Micrologger

I. Turn on datalogger and wait for display to read 1111.11.
II. Punch in program (numbers or characters in bold) in following sequence. Follow each entry with A.

*1
5 (second interval)

01: P17 Panel Temperature
01:33 Loc :

02: P20 Set Port
01: 1 Set high
02: 1 Port Number

03: P87 Beginning of Loop
01:0 Delay
02:20 Loop Count --- (Number of Thermocouples Used)

04: P22 Excitation with Delay
01:1 EX Chan
02:1 Delay w/EX (units = .01sec)
03:0 Delay after EX (units = .01sec)
04:5000 mV Excitation

05: P14 Thermocouple Temp (SE)
01:1 Rep
02:1 5 mV slow Range
03:4 IN Chan
04:1 Type T (Copper-Constantan)
05:33 Ref Temp Loc
06:1C Loc: (Will display as 1--)
07:1 Mult (degrees Centigrade) (1.8 if degrees F)
08:0 Offset (degrees Centigrade) (32 if degrees F)

06: P95 End

07: P86 Do
01:10

08: P77 Real Time

01:0011 Day, Hour-Minute, Second

09: P71 Average
01:20 Reps (Number of Thermocouples Used)
02:1 Loc.

10: P96 Serial Out (To Printer)
01:11 Printer, ASCII, 1200 Baud
A

*A
40 (Changes display from 28 to 40)
A (Should read 64)

*5 (Set clock)
(95) Year (not necessary - will not print out)
(111) Day of Year (not necessary - will not print out)
0000 Hrs:Min (Sets to zero)

*0 (Should read LOG1)
Printer will start printing in a few seconds.

*6 To view readings on display. Advance (A) to go between channels.

APPENDIX D: Method for caluculting F-value from bulk residence times

Tube Constants For VPI UHT/HTST Lab-25 with Preheater.

Section	Description	Constant
1	Preheater	6932
2	Transit Preheater to Jumper Panel	5028
3	Transit Jumper Panel to Final Heater	4490
4	Final Heater	13509
5	Transit Final Heater to Jumper Panel	4669
6	Transit Jumper Panel to Hold Tubes	2115
7	Beverage Hold Tube	3000
8	EH1	12000
9	EH2	12000
10	EH3	6000
11	EH4	6000
12	Transit Hold Tube to Jumper Panel	1509
13	Transit Jumper Panel to Cooler	3179
14	Cooler	13509

Flow Rate = 400 mls/min

Sect.	Constant	**Residence Time** (sec)	Cum. Time (sec)	Cum. Time (min)	Temp. °C
0	0.001	0	0	0	26.1
1	6932	17.33	17.33	0.28	76.8
2	5028	12.57	29.9	0.49	76.3
7	3000	7.5	37.4	0.62	74
12	1509	3.7725	41.1725	0.69	73.8
13	3179	7.9475	49.12	0.82	73
14	13509	33.7725	82.8925	1.38	11.4

Formula: **Residence Seconds = C/Flow**

.

**To calculate an F-value using the computer program the Cum. time (min) and Temp °C are the only two columns that need to be used.

Flow Rate = 1800mls/min

Sect.	Constant	Residence Time (sec)	Cum. Time (sec)	Cum. Time (min)	Temp. °C
0	0.001	0	0	0	20.4
1	6932	17.33	17.33	0.06	74.8
2	5028	12.57	29.9	0.11	74.4
7	3000	7.5	37.4	0.14	73.6
12	1509	3.7725	41.1725	0.15	72.6
13	3179	7.9475	49.12	0.18	72.3
14	13509	33.7725	82.8925	0.31	22.6

Flow Rate = 2200mls/min

Sect.	Constant	Residence Time (sec)	Cum. Time (sec)	Cum. Time (min)	Temp. °C
0	0.001	0	0	0	21.1
1	6932	17.33	17.33	0.05	74.8
2	5028	12.57	29.9	0.09	74
7	3000	7.5	37.4	0.11	73.6
12	1509	3.7725	41.1725	0.12	72.6
13	3179	7.9475	49.12	0.15	72.3
14	13509	33.7725	82.8925	0.25	22.6

F-value:

The values for residence times are those for the bulk fluid flow. The fluid flowing through the lines is considered to be laminar flow. The thermocouples are placed in the center of the lines and are measuring the temperature of the fastest moving particle not the bulk fluid flow. To obtain the F-value for the bulk fluid the principles of Laminar flow are considered. The fluid at the center moves twice as fast as the fluid on the outside. Safety is achieved when the slowest particle has achieved the desired heat. The F-value obtained using the above bulk residence times will need to be divided in half to reflect the lethality given the slowest moving particle.

example: using the above data the f-value program yields an f-value of 1.65 min using 71.7°C as reference temperature and 6.7°F as z-value.

APPENDIX E
RAW DATA

time = equivalent time spent at given temperature (F-value)
mpn/10 tubes = 2.303/ 2ml *log (10/#neg. tubes) * 10*2ml
D-value = time/ [log initial number per 10 tubes - log mpn survivors per 10 tubes]

60°C Listeria

	treatment 1				control				treatment 2			
time	#pos tubes	mpn/ 10 tubeS	D-value	time	#pos tube	mpn/ 10 tubeS	D-value	time	#pos tube	mpn/ 10 tubeS	D-value
0	10	2400000		0	10	2400000		0	10	5800000	
3.36	9	23.03	0.67	3.4	9	23.03	0.67	3.4	7	12.04	0.59
6.5	3	3.56	1.12	6.5	9	23.03	1.3	6.5	0		
11.9	0			12	1	1.05	1.88	12	0		
18.5	0			18	0			18	0		
23.3	0			23	0			23	0		
0	10	2800000		0	10	2800000		0	10	320000	
3.36	10	2800000		3.4	10	2800000		3.4	6	9.16	0.74
6.5	7	12.04	1.21	6.5	10	2800000		6.5	1	1.05	1.19
11.9	0			12	0			12	0		
18.5	0			18	0			18	0		
23.3	0			23	0			23	0		
average		rep 1	0.89				1.28				0.59
D-value		rep 2	1.21				0				0.96

63°C Listeria

	treatment 1				control				treatment 2			
time	#pos tubes	mpn/ 10 tubeS	D-value	time	#pos tube	mpn/ 10 tubeS	D-value	time	#pos tube	mpn/ 10 tubeS	D-value
0	10	2600000		0	10	2600000		0	10	700000	
4.34	0			4.3	0			4.3	0		
8.07	0			8.1	1	1.05	1.26	8.1	1	1.05	1.39
13.6	0			14	0			14	0		
16.9	0			17	0			17	0		
22.5	0			22	0			22	0		
0	10	1660000		0	10	1660000		0	10	1100000	
4.34	0			4.3	1	1.05	0.7	4.3	0		
8.07	4	5.11	1.46	8.1	3	3.57	1.42	8.1	1	1.05	1.34
13.6	0			14	1	1.05	2.2	14	0		
16.9	0			17	0			17	0		
22.5	0			22	0			22	0		
average		rep 1	0				1.26				1.39
D-value		rep 2	1.46				1.44				1.34

67°C Listeria

treatment 1				control				treatment 2			
time	#pos tubes	mpn/ 10 tubeS	D-val	time	#pos tube	mpn/ 10 tubeS	D-val	time	#pos tube	mpn/ 10 tubeS	D-valu
0	10	4600000		0	10	4600000		0	10	210000	
3.38	0			3.4	0			3.4	0		
8.11	2	2.23	1.28	8.1	2	2.23	1.28	8.1	1	1.05	1.53
11	0			11	3	3.57	1.8	11	1	1.05	2.08
17	0			17	0			17	0		
26.7	0			27	0			27	0		
0	10	2400000		0	10	2400000		0	10	940000	
3.38	0			3.4	1	1.05	0.53	3.4	0		
8.11	2	2.23	1.34	8.1	2	2.23	1.34	8.1	1	1.05	1.36
11	4	5.11	1.94	11	4	5.11	1.94	11	1	1.05	1.85
17	0			17	0			17	0		
26.7	0			27	0			27	0		
average		rep 1	1.28				1.54				1.80
D-value		rep 2	1.64				1.27				1.61

Listeria summary

	D-values						average D-values			
temp	T1	logT1	C	logC	T2	logT2		T1	C	T2
60	0.89	-0.04939	1.28	0.1	0.59	-0.22855	60	1.05	0.64	0.78
	1.21	0.08352	0.00		0.96	-0.0165	63	0.73	1.35	1.36
63	0.00		1.26	0.1	1.39	0.1416	67	1.46	1.41	1.71
	1.46	0.16541	1.44	0.2	1.34	0.1272				
67	1.28	0.10854	1.54	0.2	1.80	0.25646				
	1.64	0.21585	1.27	0.1	1.6	0.20615				

60°C Salmonella

	treatment 1				control				treatment 2			
time	#pos tubes	mpn/ 10 tubeS	D-value	time	#pos tubes	mpn/ 10 tubeS	D-value	time	#pos tubes	mpn/ 10 tubeS	D-value
0	10	3200000		0	10	3200000		0	10	1900000	
2.7	10	3200000		2.7	2	2.23	0.43	2.7	7	12.04	0.51
5.2	10	3200000		5.2	6	9.16	0.93	5.2	4	5.11	0.93
10	6	9.16	1.88	10	8	16.10	1.97	10	7	12.04	2
17	6	9.16	3.03	17	8	16.10	3.17	17	9	23.03	3.41
22	9	23.03	4.27	22	8	16.10	4.14	22	9	23.03	4.46
0	10	1200000		0	10	1200000		0	10	4500000	
2.7	10	1200000		2.7	10	1200000		2.7	9	23.03	0.5
5.2	4	5.11	0.96	5.2	6	9.16	1.01	5.2	3	3.57	0.85
10	1	1.05	1.72	10	4	5.11	1.94	10	2	2.23	1.65
17	5	6.93	3.2	17	6	9.16	3.28	17	4	5.11	2.82
22	9	23.03	4.65	22	5	6.93	4.19	22	7	12.04	3.94
average		rep 1	3.06				2.13				2.26
D-value		rep 2	2.64				2.61				1.95

63°C Salmonella

	treatment 1				control				treatment 2			
time	#pos tubes	mpn/ 10 tubeS	D-val	time	#pos tubes	mpn/ 10 tubeS	D-valu	time	#pos tubes	mpn/ 10 tubeS	D-valu
0	10	2000000		0	10	2000000		0	10	1000000	
3.9	5	6.93	0.71	3.9	2	2.23	0.65	3.9	3	3.57	0.71
7.1	2	2.23	1.19	7.1	5	6.93	1.29	7.1	1	1.05	1.18
12	1	1.05	1.97	12	4	5.11	2.21	12	2	2.23	2.18
15	0			15	0			15	1	1.05	2.52
21	0			21	0			21	0		
0	10	2000000		0	10	2000000		0	10	510000	
3.9	9	23.03	0.79	3.9	0			3.9	9	23.03	0.89
7.1	4	5.11	1.26	7.1	1	1.05	1.12	7.1	8	16.10	1.57
12	0			12	4	5.11	2.21	12	0		
15	1	1.05	2.4	15	5	6.93	2.76	15	6	9.16	3.17
21	0			21	0			21	0		
average		rep 1	1.29				1.38				1.65
D-value		rep 2	1.48				2.03				1.88

67°C Salmonella

treatment 1				control				treatment 2			
time	#pos tubes	mpn/ 10 tubeS	D-val	time	#pos tubes	mpn/ 10 tubeS	D-valu	time	#pos tubes	mpn/ 10 tubeS	D-valu
0	10	3200000		0	10	3200000		0	10	5300000	
2.9	0			2.9	2	2.23	0.48	2.9	4	5.11	0.49
7.6	5	6.93	1.35	7.6	1	1.05	1.18	7.6	2	2.23	1.2
9.9	4	5.11	1.71	9.9	1	1.05	1.53	9.9	4	5.11	1.65
17	1	1.05	2.63	17	0			17	2	2.23	2.67
28	0			28	0			28	0		
0	10	2400000		0	10	2400000		0	10	2600000	
2.9	2	2.23	0.49	2.9	2	2.23	0.49	2.9	3	3.57	0.5
7.6	4	5.11	1.34	7.6	3	3.57	1.31	7.6	1	1.05	1.19
9.9	2	2.23	1.64	9.9	2	2.23	1.64	9.9	2	2.23	1.63
17	0			17	1	1.05	2.68	17	1	1.05	2.67
28	0			28	0			28	0		

average	rep 1	1.89		1.06	1.5
D-value	rep 2	1.16		1.53	1.5

Salmonella summary

	D-values						average D-values			
temp	T 1	logT 1	C	logC	T2	logT2		T1	C	T2
60	3.06	0.485541	2.13	0.3	2.26	0.35503	60	2.85	2.37	2.11
	2.64	0.420824	2.61	0.4	1.95	0.29076	63	1.38	1.71	1.76
63	1.29	0.109765	1.38	0.1	1.65	0.2173	67	1.53	1.30	1.50
	1.48	0.170921	2.03	0.3	1.88	0.27378				
67	1.89	0.277555	1.06	0	1.50	0.1764				
	1.2	0.063763	1.53	0.2	1.5	0.17561				

VITA

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