ABSTRACT

Title of Thesis: EFFECT OF ACID ADAPTATION ON THE THERMAL INACTIVATION OF *LISTERIA MONOCYTOGENES* IN HEATING MENSTRUA HAVING VARIOUS COMBINATIONS OF PH AND WATER ACTIVITIES

Sharon G. Edelson-Mammel, Master of Science, 2003

Thesis Directed by: Professor Sam W. Joseph
Department of Cell Biology & Molecular Genetics

The impact of prior growth and food matrix conditions on thermal resistance of *L. monocytogenes*, a causative agent of the foodborne illness listeriosis, was evaluated in this study.

In an initial study (Main Study #1), acid induced and non-induced cells of thirteen strains of *Listeria monocytogenes* were placed into two heating menstrua: pH 3.0, water activity (A_w) 0.987 and pH 7.0, Aw 0.970. In twelve out of twenty-six combinations, non-induced cells were more heat resistant than induced cells. *L. monocytogenes* strain #201, in a follow-up study (Main Study #2) using a factorial design to test additional combinations of water activity and pH heating menstrua, non-acid-induced cells generally were more heat resistant than acid-induced cells although the

acid-induced cells showed greater thermal resistance in the heating menstrua having the lowest pH values.

An increase in thermal resistance could lead to underestimation of treatments necessary to eliminate potential contamination by *L. monocytogenes*.

EFFECT OF ACID ADAPTATION ON THE THERMAL INACTIVATION OF *LISTERIA MONOCYTOGENES* IN HEATING MENSTRUA HAVING VARIOUS COMBINATIONS OF PH AND WATER ACTIVITIES

by

Sharon G. Edelson-Mammel

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2003

Advisory Committee:

Professor Sam W. Joseph, Chair
Professor George Bean
Dr. Robert L. Buchanan
Professor Jianahong Meng
Dr. Richard R. Whiting

DEDICATION

I would like to dedicate this thesis to my husband Mark and my two children Samantha Rose and Isaac Anthony. Without their support I would have never been able to complete this project.

ACKNOWLEDGEMENTS

I would like to thank all of my co-workers at the FDA for their support and assistance. I would like to especially thank my supervisor Dick Whiting. I would like to thank my schoolmates and teachers at UMD. My special thanks go to Dr. Meng and Dr. Bean. I would like to give special thanks to my advisor Dr. Sam Joseph who has spent countless hours with me. I could not have finished this without your guidance. I would also like to thank Dr. Robert Buchanan who has been my supervisor, mentor, and friend. He helped me start on this educational journey and has been there the entire way. And last, but certainly not least, I would like to thank my family including my parents and siblings, and my in-laws. I give special thanks to my husband and children.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (CONT.)

LIST OF FIGURES

LIST OF FIGURES (CONT.)

LIST OF APPENDICES

I. INTRODUCTION

Listeria monocytogenes has been recognized as a cause of human illness for over sixty years, but only in the last fifteen to twenty years has food been identified as its primary means of transmission (Meng and Doyle, 1998). *L. monocytogenes* is transmitted in a variety of foods including milk, cheese, and other dairy products (Table 1). Although it causes fewer cases of illnesses than *Escherichia coli* O157:H7, the lethality rate is higher (Meng and Doyle, 1998). It is one of the few pathogenic bacteria that can pass the placental barrier (Doyle, *et al.,* 1997). *Listeria* spp. are also causative agents of fish, fowl and animal listeriosis (Farber & Pagotto, 1992).

Thermal treatments are the most widely used means by which food processors and consumers eliminate harmful microorganisms such as *L. monocytogenes* from foods. Cooking and related thermal processes are cost effective and easily applied. However, the extent of cooking required to achieve control of pathogenic microorganism must be balanced against the detrimental effects of thermal processing (e.g., loss of thermal sensitive nutrients, sensory alterations). This is particularly important with *L. monocytogenes* whose relative resistance is typically greater than other pathogens. While *L. monocytogenes* has been studied extensively over the past 20 years, there is surprisingly little data available on the effects that prior growth conditions or pH/water activity combinations have on the thermal resistance of the microorganism. The objective of the current study was to address a number of these deficiencies by (1) developing simple systems for inducing acid adaptation in *L. monocytogenes*, (2) assessing the effect of growing the microorganism in a moderately acidic environment on

its thermal resistance, and (3) assessing in a systematic manner the effect of various pH and water activity combinations in heating menstrua on thermal resistance.

II. LITERATURE REVIEW

A. Characteristics of *Listeria monocytogenes*

Listeria monocytogenes is a gram positive, facultatively anaerobic, non-spore forming rod-shaped bacterial species within the genus *Listeria*. The genus is part of the Clostridium subbranch which includes *Staphylococcus*, *Streptococcus*, and *Lactobacillus* (Doyle, 2001). When gram stained, the rods appear as small coccobacilli (1-2 um in length) occurring singly or in short chains. Although smooth colonies are seen on agar plates under many conditions, rough colony characteristics were shown to emerge under conditions of severe osmotic stress (Jorgensen, *et al.,* 1996). When grown on blood agar plates, the colonies are small, translucent, and gray, and form a narrow zone of beta (b)-hemolysis. The hemolysis can be enhanced by the CAMP test in which the organism is streaked in a line on a blood agar plate perpendicular to a streaked line of *Staphylococcus aureus.* CAMP factor secreted by *Listeria* produces a synergistic, bright zone of hemolysis with the hemolysin of the *S. aureus* (Diagnostic Microbiology, Koneman *et al.*, 1992).

L. monocytogenes has peritrichous flagella which enable it to be motile in the temperature range of 20 to 25°C. Conversely, at 37°C, it is not motile or is very weakly motile. Tumbling motility, an important aid in clinical identification, can be seen in a wet mount of organisms grown in a broth culture for 6 hr at 20°C. Motility can be demonstrated in a semisolid agar as an umbrella shaped pattern in the media after overnight incubation at room temperature (Diagnostic Microbiology, Koneman *et al.*, 1992).

The microorganism is catalase positive, Voges-Proskaur positive, urease negative and hydrolyzes esculin. The biochemical characteristics of *L. monocytogenes* include the ability to produce acid from amygdalin, fructose, salicin, mannose, maltose, rhamnose, and glycerol (Ryser and Marth, 1999). Acid production is not observed with mannitol, xylose, inositol, arabinose, and adonitol. *Listeria spp.* prefer glucose as a carbohydrate energy source (Pine *et al.*, 1989).

L. monocytogenes is a globally dispersed, saprophytic bacterium that can be found widely in soil, sewage and fresh water, and can grow on decaying vegetative matter. The organism is found in various aqueous environments such as the surface water of canals and lakes, and freshwater tributaries (Rocourt and Cossart 1997). It is able to persist at low pH and low water activity (CheroutreVialette *et al*., 1998, Palumbo *et al.,* 1993), particularly at refrigeration temperatures. It can survive for long periods of time in many environments, including survival in frozen foods for extended periods.

Listeria monocytogenes is psychrotropic; it can grow at refrigeration temperatures if other growth requirements are met (Stephens, *et al*., 1994). However, unlike many psychrotrophs, the bacterium has a broad temperature range; it has been reported to grow at temperatures as low as 1 to 4°C and as high as 45°C (Stephens, *et al.*, 1994; Augustin, *et al*., 1998). Like most mesophilic pathogens, the optimum growth temperature for *L. monocytogenes* lies between 30°C and 37°C. Because of the ability of the microorganism to grow in a broad temperature range, foods that contain *Listeria* and are stored at temperatures above freezing could allow cell multiplication (Jorgensen, *et al*., 1995, Smith, *et al*., 1991). *Listeria* is more resistant than many other vegetative

foodborne pathogens to many of the treatments commonly used to reduce or eliminate pathogenic bacteria. These and other characteristics of *L. monocytogenes,* such as its relatively high heat resistance, have made this foodborne pathogen difficult to control (Hansen and Knochel, 1996, Rocourt and Cossart, 1997).

L. monocytogenes was subdivided by Seeliger in 1961 into thirteen serovars based on O (somatic) and H (flagellar) antigens (McLauchlin, 1987). The thirteen serovars consist of 1/2a, 1/2b, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 based on agglutination reactions with rabbit antisera against the O and the H antigens (Seeliger & Hohne, 1979). Of the 13 serovars, 95% of the human isolates fall into three of these serovars- 1/2a, 1/2b and 4b.

B. Listeriosis

Listeria monocytogenes is an intracellular pathogen in humans and other animals. *Listeria monocytogenes* infections in humans, which are referred to as listeriosis, can affect the central nervous system (CNS), pregnant uterus, or bloodstream (McLauchlin, 1996). Current data indicates a morbidity of about 2500 cases of listeriosis per year and a mortality of 504 cases per year (Mead *et al.*, 1999). One serovar in particular, 4b, is responsible for 33 to 50% of sporadic human cases and a majority of the outbreaks of listeriosis since 1981 (Rocourt and Cossart 1997). Most often the isolates recovered from foods belong to serovar 1/2a (Doyle *et al*., 2001).

In non-pregnant animals, listeriosis typically manifests either as CNS diseases such as meningitis and encephalitis, or as septicemia. After at least 12 hours post-

exposure, individuals may experience gastrointestinal symptoms such as nausea, vomiting, and diarrhea. After a few days or up to three weeks, symptoms may progress to the more serious forms of illness. Those at higher risk for disease are the elderly, immunocompromised patients, and patients with AIDS.

In pregnant women, the disease typically manifests as mild flu-like symptoms. However, *L. monocytogenes* can pass the placental barrier and spread from the mother to the fetus, which can result in severe infections in the fetus and lead to miscarriages, stillbirths, spontaneous abortions or the birth of a severely ill infant. These surviving infants may suffer from meningitis or severe neonatal septicemia (McLauchlin, 1996).

L. monocytogenes has been recognized as a cause of human illness for over 60 years. The first reported case of listeriosis was a World War I soldier suffering from meningitis (Meng and Doyle, 1998, Rocourt and Cossart, 1997). Infections caused by *L. monocytogenes* are not as common as infections by other foodborne pathogens such as *Salmonella* and *Escherichia coli* O157:H7. However, the fatality rate observed with invasive (systemic) listeriosis (20-30%) is the highest among foodborne pathogens (Rocourt and Cossart, 1997).

L. monocytogenes after human consumption are internalized by resident macrophage where they can survive and replicate. They are then transported via blood to regional lymph nodes, from which they can reach the liver and spleen, where most *Listeria* are destroyed (Rocourt and Cossart, 1997). Infected animals and humans are found to have *L. monocytogenes* in their blood, milk, and feces and have a low potential to infect others who come into contact with these contaminated body fluids (Donnelly

and Briggs, 1986). Although exposure to *L. monocytogenes* is common, cases of listeriosis are rare (Rocourt & Cossart, 1997). A milder form of infection caused by *Listeria* is listerial gastrointestinal disease.

There are 3 general steps necessary for *Listeria monocytogenes* to cause invasive listeriosis:

1) exposure of the host through consumption of contaminated food.

2) invasion of the host via multiplication within and subsequent translocation through the intestinal lining.

3) the organism is transported to the target tissue such as regional lymph nodes, the brain, or the placenta by way of the blood.

The organism may be dispersed throughout the body via circulating blood, depending on the T cell response of the immune system (Rocourt & Cossart, 1997). Direct cell to cell spread allows the bacteria to move throughout the host and induce formation of infection loci, while being sheltered from host defenses such as circulating antibodies. This mechanism allows the microorganism to gain access to targets such as the placenta and CNS (Rocourt and Cossart, 1997, Ryser and Marth, 1999). Survival mechanisms of *L. monocytogenes* include the ability to invade and multiply in non-phagocytic cells; to survive and multiply within macrophage; and to induce engulfment by the host cells (Sherris, 1990). *Listeria monocytogenes* infections are associated with a set of virulence factors that enable invasion of the host cell and evasion of the host cell phagosome. The bacteria are able to move within the cytoplasm of the cell and spread to neighboring cells (Gahan and Hill, 1999). These virulence factors include internalin

(inlA), which is required for entry into the gastrointestinal lining; listeriolysin O (LLO) which is used to escape from the vacuole by forming a pore; and *act*A which enables the bacteria to move intracellularly by actin polymerization.

C. Treatment

Ampicillin and penicillin are the drugs of choice, but slow recovery may be observed because of the ability of *L. monocytogenes* to survive within cells thus avoiding the action of the antibiotic. Use of bacteriostatic drugs such as chloramphenicol and tetracycline can result in high rates of failure (Southwick and Parich,1996). Trimethoprim-sulfamethoxazole (TMP-SMZ) which readily enters cells and kills *L. monocytogenes*, may be the most effective treatment (Armstrong and Fung, 1993). Treatment with one part TMP to five parts SMZ for four to six weeks may be necessary to clear the infected cells, especially in immunosuppressed patients (Armstrong, 1995).

D. Susceptible Populations

Listeriosis is rarely a problem for healthy adults or children (McLauchlin, 1996). The populations most at risk for infection are the elderly, the immunocompromised, and pregnant women. Immunocompromised individuals most at risk are those who are deficient in their T-cell mediated immunity (Rocourt and Cossart, 1997), for example AIDS patients, neonates, elderly, and cancer patients (Rocourt, 1996). Others at risk are those who have received organ transplants and are receiving immunosuppressive therapy (McLauchlin, 1996). Chronic conditions can increase an individual's susceptibility to

infection: therapy causing the blockage or neutralization of gastric acid that could increase the chances of *L. monocytogenes* surviving passage through the stomach (Rocourt, 1996). Underlying conditions can increase the severity of the illness. Mortality can increase 38 to 40% among immunocompromised and elderly patients (Rocourt and Cossart, 1997). Pregnant women are themselves not at increased risk of serious *L. monocytogenes* disease; however their fetuses are at risk for listeriosis, particularly during the third trimester (Rocourt, 1996, Rocourt and Cossart, 1997). When the microorganism causes disease in non-pregnant women at risk, it usually presents as meningitis or septicemia. One of the first outbreaks due to *Listeria* took place in Massachusetts in 1983, where 49 cases of listeriosis were reported with 14 deaths (29%)(Bradshaw, *et al*., 1985). Of the 49 cases, 7 patients were infants and 42 patients were immunosuppressed adults.

E. Epidemiology

Because of the widespread distribution of *L. monocytogenes*, there are numerous opportunities for this pathogen to contaminate foods and thus pose a risk to consumers (McLauchlin, 1996, Rocourt and Cossart, 1997). The bacteria can easily get into a food processing plant via soil on the animal's carcass, on vegetative matter, or on a worker's hands or shoes. Once inside a plant, the microorganism attaches readily to a variety of surfaces and is often found in moist areas of the plant such as floor drains (Rocourt and Cossart, 1997). These surfaces include plastic, glass, or metal (McLauchlin, 1996). Although many of the outbreaks are due to cross contamination, such as transfer of the

microorganism from a worker's hands or from improperly cleaned equipment to an already cooked food product. *Listeria monocytogenes* is a hardy microorganism and has been isolated from plants, where good manufacturing practices (GMPs) were implemented (Budo-Amoako, *et al.*, 1992).

One of the first documented outbreaks associated with *L. monocytogenes* occurred in 1981 in Canada, where the implicated food was coleslaw (Rocourt and Cossart, 1997). In 1983, an outbreak in Massachusetts was suspected of being associated with milk. Dairy cows infected with *Listeria* were identified at the farm, from which the implicated milk had been shipped. There was no evidence that faulty pasteurization had occurred at the dairy plant, where the milk was processed (Bunning, *et al.*, 1986, Donnelly, *et al.*, 1987). Confirmed outbreaks of listeriosis in North America have been linked to foods of both animal and plant origin (Beuchat, *et al.*, 1986, Budo-Amoako, *et al.*, 1992). There have been several outbreaks of listeriosis associated with dairy foods, although it was unclear if contamination of the products was due to prior contamination and pathogen survival of the heat treatments or to post-pasteurization contamination. (Boyle, *et al.*, 1990, Casadei, *et al.*, 1998). However, post-pasteurization recontamination is generally assumed to be the case since standard pasteurization is sufficient to inactivate the microorganism. A number of other foodborne outbreaks of listeriosis have occurred in the United States and around the world (Table 1).

F. Infectious Dose

The number of organisms required to cause an immune response is known as the infective dose. The infectious dose itself is variable and depends on three main factors: the host, the pathogens, and the effects of the food matrix (Buchanan and Lindqvist). The infectious dose necessary for *Listeria* to cause an infection depends on the individuals and their immunologic status. A lower dose (i.e., number of organisms) would be sufficient for populations who are more susceptible to infections such as the elderly and the immunocompromised (individuals with compromised T-cell immunity) (Rocourt, 1996, Rocourt and Cossart, 1997). The pathogenicity of the microorganism affects the infective dose, an organism with more virulence factors may require a smaller dose for infection to occur.

The infectious dose is also influenced by the characteristics of the food ingested. The type and amount of food consumed and the concentration of the pathogen in the food affect the likelihood of infection (McLauchlin, 1996). One potential factor is the pH of the food consumed. Exposure of *L. monocytogenes* to foods that have a moderately acidic pH may enhance the ability of the microorganism to survive in the acidic environment of the stomach, thereby reducing the number of organisms required to cause an infection.

G. Foods with Greater Susceptibility to Contamination

Foods associated with outbreaks have largely been refrigerated, processed, ready-to-eat foods, *i.e.* consumed without prior cooking or reheating (Doyle, *et al.*,

1997) and that supported the growth of *L. monocytogenes* during refrigerated storage. These foods are often heat treated, thereby eliminating competing microorganisms, which extends shelf life at refrigeration temperatures (McLauchlin, 1996, Rocourt and Cossart, 1997). The extended storage time affords *L. monocytogenes,* when present, the opportunity to grow to high numbers. Marginal temperature abuse (e.g. 8 to 12°C), during refrigerated distribution and storage may accelerated the growth of the microorganism (Beuchat, *et al.*, 1986). These fluctuations in refrigerated storage temperatures have been found to commonly occur (Chawla, *et al.*, 1996).

The ability of a food to support the growth of a microorganism is dependent on an array of intrinsic and extrinsic characteristics. Intrinsic factors are those that are inherently associated with the food such as pH and water activity (Cheroutre Vialette, *et al.*, 1998), whereas extrinsic factors are those determined by the conditions of storage, such as temperature or gaseous environment. Care must be taken to consider what the microorganism is experiencing and not the composite characteristics of the food in evaluating the potential for a food to support the growth of *L. monocytogenes*. A food will often contain multiple micro-environments, any of which may support the growth of bacteria present in the food (Montville, 1997). There has been some speculation that changes in food processing and distribution, particularly the extension of shelf life through refrigerated storage, may have contributed to the emergence of *L. monocytogenes* as a foodborne pathogen (Rocourt and Cossart 1997).

L. monocytogenes has been found in a wide variety of retail, ready-to-eat foods including milk and dairy products, meats (e.g., sausage, pate), fresh produce and fish (i.e,

finfish, bivalves, crustaceans), reflecting the increasingly cosmopolitan nature of the marketplace (Rocourt and Cossart, 1997, Yen, *et al.*, 1991). In developing their *L. monocytogenes* risk assessment, FDA/FSIS reviewed exposure data for 23 ready-to-eat food categories which represented more than 400,000 samples (DHHS/USDA, 2003). The microorganism was isolated from the different food categories at a mean frequency that ranged from 0.2 CFU for ice cream to 12.9 CFU for smoked seafood. The presence of *L. monocytogenes* has also been reportedfor other foods not covered by the risk assessment such as liquid egg product, although transmission to humans from that product has not been documented (Schuman and Sheldon, 1997).

The risks in each category of ready-to-eat foods are not equal. In the deli meats category, there is more of a problem with contamination in poultry versus other meats. Roast beef and summer sausage appear to support the least growth of the microorganism. It has also been found that *L. monocytogenes* can strongly attach to the surface of raw meat from which it is difficult to remove.

With seafood, shellfish are at high risk for transmitting *Listeria,* because it has been found in mollusks, fresh and frozen mussels, clams, and oysters. But the types of seafood posing the highest risk are raw fin fish and lightly preserved fish products such as salted, marinated, fermented or cold-smoked products (Huss *et al.*, 2000).

Susceptible foods also include fermented sausage which relies upon the low water activity of the preserved sausage (CheroutreVialette, *et al.*, 1998), low pH and competition from the commonly occurring bacteria needed to ferment the sausage to eliminate the potential threat from bacteria such as *Listeria.* The normal process of

fermenting and drying the sausage may prevent the growth of *Listeria* but may not eliminate the organism from the finished product (Glass and Doyle, 1989). To reduce the risk of *Listeria* growth during the fermentation process of beaker sausage (uses a glass beaker as a casing), an important step was the addition of a lactic starter culture (Glass and Doyle 1989). The addition of a lactic starter culture was found to reduce the number of *Listeria* by 1 to 2 $\log_{10}$ CFU/g during the fermentation process of beaker sausage (Glass and Doyle, 1989).

Some of the food safety problems with *Listeria* and other bacteria may be due to cross contamination from workers or food contact surfaces which may re-introduce the organism into the product after it has been processed (Dorsa, *et al.*, 1993). *Listeria* species has also been found in processed lobster, but the contamination could have been due to under-cooking or post-cooking contamination from the packing environment (Budo-Amoako, *et al.*, 1992).

Currently the FDA has a zero tolerance policy which means that ready-to-eat food samples must be negative for *L. monocytogenes* in two 25g samples of the food product (Meng and Doyle, 1998). Measures are needed to prevent such post-pasteurization contamination from occurring in products such as processed dairy products (Donnelly and Briggs 1986).

H. Industry Procedures for Prevention of *Listeria* Contamination

L. monocytogenes is found widely in the environment (i.e., soil, plant material, water), where it is able to survive for long periods of time. In addition, the

microorganism can survive for long periods of time in foods and in food processing facilities. There are many avenues for the microorganism to gain entry into the food processing plant and thus provide a means for the consumer to be exposed to contaminated food if the organism is not eliminated from the food product. Examples of how the microorganism enters food processing plants are via soil on workers' clothes and shoes, transport equipment and raw plant foods. Animals can excrete the microorganism and contaminated hides and hooves may be a source of contamination (Food Microbiology Fundamentals and Frontiers, 2001). Once in the processing plant, the organism thrives in locations that are nutrient rich and have a high humidity, such as floor drains, condensed and stagnant water, floors, and processing equipment (Cox *et al.*, 1989).

There are three separate avenues through which a processor can reduceor eliminate the threat from *L. monocytogenes.*

1) Reduce the chances of the microorganism entering the processing plant by washing the soil off of workers' clothes and transport equipment. Make sure there is adequate clean up of animal excrement.

2) Identify the problem locations in plants and apply more stringent cleaning routines and standards.

3) Post processing re-contamination is avoided by separating unclean zones from clean zones.

A food processor using a Hazard Analysis and Critical Control Point (HACCP) plan studies these questions (USDA/FSIS, 1999):

1) Knowing potential levels of *L. monocytogenes* on a surface material for products - Do they fluctuate depending on supplier?

2) What do validation results tell you about the efficacy of kill steps used in your process?

3) If your products are exposed to an environment not known to be *L. monocytogenes* free, what post kill steps are employed to prevent contamination or re-contamination? What do records reveal about *L. monocytogenes* in the facility?

4) Does the food product support the growth of *L. monocytogenes*?

5) Does finished product testing reveal microbial contamination?

I. Food Processing and Hurdle Approach

Many methods are used to enhance the flavor and texture of the food. These include cooking, salting, reducing pH, drying etc. These steps are also used to reduce or eliminate the presence of a microbial population or decrease its ability to grow. This approach, which uses a series of sublethal steps rather than one extreme step, is referred to as the "hurdles" technique. The technique is a way to alter several factors to assure that overall, growth will not occur (Fennema, 1996).

J. Thermal Processing, General

Though thermal processing is one of the oldest methods used to process foods (e.g., canning, pasteurization) or to eliminate pathogenic microorganisms prior to

consumption (i.e., cooking), it remains the most effective and widely used preservation technology (Farkas, 1997). It is also used to impart specific sensory characteristics to food such as the development of flavors, aromas, or textural changes. However, excessive thermal processing can also lead to the development of undesirable changes such as the development of off-flavors, changes in texture, the loss of desirable sensorial factors and the loss of nutritional food characteristics. The ideal thermal process is one that is sufficient to reduce the probability that pathogenic microorganisms of concern would survive, maximize the development of desirable organoleptic characteristics, and minimize the development of deleterious changes in the food (Buchanan and Edelson, 1999b).

Pasteurization, one of the most well known thermal methods, consists of mild heat treatments that inactivate enzymes and destroy pathogenic vegetative microorganisms (e.g., non-spore forming bacteria). The method can extend the shelf life of a product provided that the packaging prevents re-contamination. The inactivation of microorganisms can be achieved using different time-temperature combinations and can be selected to optimize the positive and negative attributes associated with thermal processing. For example, high temperature short time (HTST) pasteurization of milk has largely replaced traditional pasteurization techniques because it provides effective microbial inactivation while causing less damage to the sensory and nutritional characteristics of the product. Some thermally resistant microorganisms and most spore-formers can survive the pasteurization, therefore foods should be kept at low temperatures to prevent their growth (Farkas, 1997).

Sterilization is the complete destruction of microorganisms potentially present in the food. This preservation method is divided into two categories: the sterilization of the food in their final packaging and sterilization of the foods followed by aseptic transfer of the product to sterile packages. Appertization, also known as canning, is the sterilization of foods by thermal means in closed containers (Farkas, 1997).

The use of a microwave to heat treat foods is a method based on internal heat generation. A difficulty with this method is the lack of uniformity of the heat as it is applied to the entire food product (Farkas, 1997). During the microwave treatments, if a temperature of 70°C is reached and maintained for at least 2 min., there will be a substantial reduction in the number of *L. monocytogenes*. Survival of any of the organisms is most likely due to uneven heating (presence of cold spots in foods) during the heat treatment. To reduce this possibility the temperature should be measured at different locations on the food to check for cold spots (Coote, *et al.*, 1991).

The minimal temperature and time required to kill *Salmonella* is often used as the measure of minimal thermal conditions, but the thermal resistance of *L. monocytogenes* was found to be much higher than that of *Salmonella* (Schoeni, *et al.*, 1991). Current pasteurization processes are sufficient to destroy any *L. monocytogenes* that may be present in milk (Bradshaw, *et al.*, 1985, Bradshaw, *et al.*, 1987). The microorganism was incapable of surviving heating at the times and temperatures recommended by the FDA milk pasteurization guidelines, i.e, minimum temperature of 72°C for a minimum of 15 seconds (Donnelly and Briggs, 1986). However, this requires that all parts of the milk achieve adequate thermal treatment, and difficulties may arise with certain pasteurization

technologies. For example, splashing of milk during vat pasteurization can allow cells to remain above the main milk level, thereby allowing them to be insufficiently heated to assure destruction. This potential problem could be eliminated by using airspace heaters to ensure adequate heating of every particle of milk present in a milk vat (Donnelly, *et al.*, 1987).

K. Resistance to Thermal Processing

Listeria monocytogenes is generally considered to be more heat resistant than other non-spore forming foodborne pathogens. For example, in ground beef *Listeria* was found to be four times more heat resistant than *Salmonella* when placed in pouches and submerged in a water bath (Schoeni, *et al.*, 1991).

In general, *L. monocytogenes* is more thermally resistant when grown at elevated temperatures (e.g., 35° to 40°C) as compared to cells grown at room temperature or below. *Listeria* may respond to the increases in temperature by synthesizing a specific set of heat shock proteins that facilitate the repair of damaged cells and protect against further injury (Jorgensen, *et al.*, 1995). These proteins appear to be analogous to the proteins synthesized by *Salmonella* during heat or acid shock. However, it is important to note that while the microorganism is somewhat more heat resistant than many of the gram negative foodborne pathogens, most thermal processes used in the production and preparation of foods appear to be sufficient to inactivate *L. monocytogenes*.

The heat resistance of a microorganism is influenced by many factors such as

strain variation, growth stage, growth temperature, food matrix and composition of the growth media (Buchanan and Edelson, 1999b, Casadei, *et al.*, 1998, Doherty, *et al.*, 1998, Rowan and Anderson, 1998; Golden, *et al.*, 1988). Environmental factors such as treatment temperature are also important factors that influence the heat resistance of a microorganism such as *L. monocytogenes* and must be considered when using heat inactivation (Anderson, *et al.*, 1991, Pagan, *et al.*, 1998). For example, in one study there was no increase in the thermal resistance of intracellular bacteria versus bacteria in free suspensions of milk (Bunning, *et al.*, 1986). Another study by Bradshaw *et al.*, 1987, found that the thermal tolerance of *Listeria* was increased if the food, in which it was contained, was heat sterilized. The increased thermal tolerance may have been due to lack of competition from other bacteria or due to changes in the food matrix due to the sterilization process.

The growth stage of a spore former is a very important factor in the thermal resistance of a microorganism. Many vegetative cells of spore formers are as heat sensitive as other vegetative cells, whereas the microorganism's spores have substantial thermal resistant (Farkas, 1997). Reducing the growth temperature under both anaerobic and aerobic conditions eventually was found to decrease the thermal resistance of *L. monocytogenes* strain Scott A (Smith, *et al.*, 1991). The decrease in the thermal resistance of the organism may be caused by the unsaturated fatty acid content of the organism, which increases as the growth temperature is lowered. The increase in the levels of unsaturated fatty acids in the phospholipids may create a more fluid, less viscous cytoplasmic membrane (Smith, *et al.*, 1991). Stationary phase bacterial cells are

typically found to be more resistant than exponential phase cells (Buchanan and Edelson, 1999a, Buchanan and Edelson, 1999b).

Increasing the concentration of solutes such as NaCl in foods can affect the thermal resistance of *Listeria*. Yen *et al.*, 1991 found that a 3% increase in the NaCl concentration reduced the thermal resistance of *Listeria* in ground pork. In the same study, the addition of a mixture used to cure meat which contained NaCl, dextrose, and sodium phosphates increased the thermal resistance of *Listeria* in ground pork. The pH of the food also influences the thermal resistance of an organism (Pagan, *et al.*, 1998).

Prior exposure of the bacterial cells present in different foods to sub-lethal environmental stresses can increase bacterial thermal tolerance. These stresses include temperature, water activity (CheroutreVialette, *et al.*, 1998), starvation, pH and the atmosphere (Farkas, 1997, Lou and Yousef, 1996, Pagan, *et al.*, 1997). If the length of time that the bacteria were starved was increased, the greater the increase in the bacterial thermal resistance (Lou and Yousef, 1996). Bacterial cells that were adapted to an environment of increased salinity prior to receiving heat treatments showed a 10-fold increase in their thermotolerance when heated in minced beef and a 22- fold increase when heated in tryptic phosphate broth (Jorgensen, *et al.*, 1996). An increase in the thermal tolerance of *Listeria* has also been seen after heat shocking the cells prior to heating (Augustin, *et al.*, 1998, Fedio and Jackson, 1989, Pagan, *et al.*, 1997). Cells are heat shocked by being exposed to sub-lethal temperatures for a period of time. Heat shocking the bacterial cells can cause them to synthesize heat shock proteins (HSP), which might help the bacterial cells to cope with stress-induced damage by promoting

the degradation of abnormal proteins (Farber and Pagotto, 1992, Rowan and Anderson, 1998). These HSP may also help to protect the cells against further damage during thermal treatment. The amount of protection that can be provided by prior heat shocking depends on the temperature and the duration of the heat shock. Farber and Brown, 1990, found that the heat resistance of *L. monocytogenes* was greatly increased if the heat shock was between 1-2 hours, while Hansel and Knochel, 1996 found that heat shocking *L. monocytogenes* cells for 30 minutes at 46°C was sufficient to increase the thermal death time 2 to 4 fold.

Under certain thermal processing conditions, microorganisms may be injured but not killed. In the proper environment, the organisms may repair themselves and begin to grow and replicate (Smith and Buchanan, 1990). The media used to test food samples for the presence of bacteria may only enumerate uninjured cells leaving a population of cells undetected. In one study by Rowan and Anderson, 1998, enrichment media were used to recover *L. monocytogenes* survivors from milk after high temperature, short term (HTST) pasteurization.

L. Molecular Mechanisms of Survival Under Various Types of Stresses

Many proteins are expressed by *L. monocytogenes* during times of stress. For example, *L. monocytogenes* is commonly exposed to salt during food processing and food preservation and it has been shown that during salt concentration, 40 proteins were either repressed or induced at a higher rate by this microorganism (Duche *et al.*, 2002).

The protein sB regulates the expression of numerous genes during stress

conditions and upon entry into stationary phase. The sB acts in a role similar to rpoS in gram negative bacteria responding to general stress. sB also contributes to oxidative stress resistance and carbon starvation resistance. Heat stress was found to be partially independent of sB in *L. monocytogenes*. Acid resistance, however, was found to be sB dependent and pH dependent (Ferreira *et al.,* 2001).

DsigB strains (sB mutants) are less resistant to Brain Heart Infusion broth (pH 2.5) or synthetic gastric fluid (pH 2.5) than the wild type strain. The functioning sB protein is necessary for full resistance of *L. monocytogenes* to lethal acid treatments (Ferreira *et al.,* 2003).

Proteins whose synthesis is induced by heat shock and other stress conditions include proteases and ATP dependent chaperones. *L. monocytogenes* contains genes for Clp ATPases including ClpC, ClpP, and ClpE that are required for stress survival and intracellular growth. CtsR is a stress response regulon that controls a class III heat shock gene (Nair *et al.,* 2000). Expression of GAD - glutamate decarboxylase activity by *L. monocytogenes* is required for survival in the acidic environment of the stomach (Cotter *et al.,* 2001).

M. Acid Resistance

Listeria and other bacteria show an increased resistance or tolerance to acidic conditions after prior exposure to acidic conditions. Acid resistance, also known as acid habituation, occurs after the organism is exposed to moderate non-lethal acid conditions. The bacterium is then able to withstand formerly lethal pH conditions as low as pH 2.5

(Buchanan and Edelson 1999a, Buchanan and Edelson 1999b, Phan-Thanh, *et al.,* 2000). Acid tolerance is the enhanced survival of bacteria at a pH range of between 2.4 and 4.0 after exposure to moderate acidic conditions (Buchanan and Edelson, 1999, Gahan and Hill, 1999). Cells with increased acid resistance, also known as the acid tolerance response (ATR), have also been shown to have increased resistance to other stresses such as ionizing irradiation and thermal stress (Buchanan and Edelson, 1999a, Montville, 1997). While *Listeria,* following acid adaptation, did show an increase in its acid tolerance, the virulence of the pathogen was not altered (Gahan and Hill, 1999).

During the processing of certain foods there is ample opportunity for the bacteria to be exposed to sub-lethal acid conditions such as through the introduction of starter cultures to produce a quick drop in the pH or due to naturally occurring acid producing bacteria (Farber and Pagotto, 1992).

N. Acid Resistance and Increased Thermal Resistance

Cells that have been acid-adapted show an increase in their thermal resistance in addition to increased acid resistance compared to non -acid-adapted cells. This form of cross-protection has been seen to occur with *E. coli* (Buchanan and Edelson, 1999a, Farber & Pagotto 1992, Jorgensen, Hansen, et al. 1999). There are conflicting opinions on whether *L. monocytogenes* also show increased thermal resistance after acid-adaption of the cells. Phan-Thanh et al., 2000 and Mazzotta, 2001 found that *L. monocytogenes* cells which have been exposed to acidic conditions have also shown increased resistance to other stresses such as thermal inactivation, alcohol stress, osmotic stress and

antimicrobial agents. Conversely, Cole *et al.*, 1993 found that prior stress with a low pH led to decreased heat resistance.

In the Mazzotta, 2001 study, four strains of *L. monocytogenes* were grown overnight in Tryptic Soy broth (TSB) modified to pH 5.0 with HCl. A composite of all four strains were then used in thermal experiments. The thermal resistance of the composite cells was measured in three different fruit juices with a pH of 3.9 and TSB modified to pH 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0. In the Phan-Thanh *et al.*, 2000 study, the cells were exposed for two hours at pH 5.5 before the thermal resistance of the cells was determined at 52°C. In the Cole et al., 1993 study, the cells were only exposed to the various pH's (pH 4.24 to pH 7.0) of the heating menstruum for twenty minutes before being injected into the heating coil.

O. Resistance Modeling

Mathematical models have been used for a number of years by food processors to predict critical food safety margins (Cole, Davies, et al. 1993) and to predict the outcome of different processing conditions on the survival of microorganisms during thermal processing (Peleg & Cole 1998).

Some models do not take into consideration the contribution that the heating rate or prior stresses have on the total thermal process (Stephens, Cole, et al. 1994). Increased thermal tolerance as a result of prior stresses create thermal death time curves that may contain both a shoulder and a tail section (Pagan, Condon, et al. 1997). Currently there are models that are more accurate than others; i.e. those that allow for

variability of heat sensitivity throughout a cell population (Stephens, Cole, et al. 1994). These models have the ability to describe non-linear thermal inactivation (Farkas 1997). Examples of these are logistic curves, which can be a better fit for certain types of data because they allow for the possibility of survivors (Augustin, Carlier, et al. 1998), (Cole, Davies, et al. 1993). The use of a logistic curve may provide a good general model in order to predict the death of *L. monocytogenes* under a wide variety of environmental conditions during procedures such as thermal processing (Cole, Davies, et al. 1993). There is also the Gompertz equation which is an asymmetrical sigmoidal curve or S curve which fits many bacterial growth curves and does not assume a constant rate of growth (Augustin, Carlier, et al. 1998), (Chawla, Chen, et al. 1996). The Gompertz equation also considers more data points and therefore may provide a more accurate estimate of thermal resistance of the microorganisms (Bhaduri, Smith, et al. 1991). Another non-linear model used by Doherty *et al.*, 1998, used a Gauss-Newton curve fitting program, where the model included an initial shoulder and two populations. The main part of the curve consisted of the major population, while the sub-population comprised the tail of the curve.

Ultimately these more advanced equations and models will allow food scientists and processors to better predict the processes needed to eliminate potential pathogen contamination of a food product. A good example, available from the United States Department of Agriculture, is the Pathogen Modeling Program (PMP) (USDA/ARS, 2001), a useful tool for the food processor to estimate inactivation or elimination of foodborne pathogens. Models are also included on the growth and survival of several

food borne pathogens.

Mathematical tools can be used to estimate the effect of multiple hurdles used by the food processor; i.e. the ability of an organism to repair itself or bacterial response to the food ecosystem (Chawla, Chen, et al. 1996). These tools can also be used as an aid when designing and implementing a Hazard Analysis and Critical Control Point (HAACP) program.

P. Linear vs. Non-Linear Resistance Curves

Thermal inactivation curves have been typically represented by log-linear models. These models assume that all the organisms in a population are equally sensitive to heat (Stephens, Cole, et al. 1994). Another assumption is that while there may be a distribution of heat sensitivities within a cell population, all the cells will receive the same amount of heat. Based on this assumption, the dose response curve at a given time and at a given temperature will be a measure of the dose received (Cole, Davies, et al. 1993). Most thermal curves are plots of the number of survivors versus the thermal exposure time. However there have been deviations from these straight line survivor curves seen during studies on the effects of mild processing temperature on vegetative bacterial cells (Stephens, Cole, et al. 1994). A number of studies showed deviations such as the presence of shoulders and tails, especially during milder thermal treatments (Bhaduri, Smith, et al. 1991, Farkas 1997, Stephens, Cole, et al. 1994). Data analyzed from such studies revealed the potential for the presence of heat resistant sub-populations (Doherty, McMahon, et al. 1998).

Q. Predictive Food Microbiology

Food processors need information to better determine the death of certain bacteria under mild heating conditions in a wide range of environmental conditions. Predictive food microbiology is able to provide quantitative data on the impact that different parameters, both intrinsic and extrinsic, have on the growth and decline of certain bacteria (Buchanan & Bagi 1997). Unlike the canning process, today's milder thermal processing methods have much smaller margins of safety and therefore require more accurate and precise predictions (Cole, Davies, et al. 1993, Stephens, Cole, et al. 1994).

R. Water Activity (A_w)

Water activity or water availability or water potential (CheroutreVialette, Lebert, et al. 1998) is the amount of free water available in a food for potential use by a microorganism. The A_w is a measure of the vapor pressure of the free water in a food over the vapor pressure of pure water (Farkas 1997), the ratio is then expressed as a fraction (Jay, 1996). Buchanan and Bagi, 1997, found that frequently the duration of growth phase and lag phase increase proportionally as the A_w declines. Decreased A_w can also reduce the time needed to kill a bacterium at a given temperature during thermal processing. The A_w in a food can be altered by the addition of a humectant or by the removal of the water vapor itself. The primary humectant currently used in the food industry is NaCl (Buchanan & Bagi 1997). Dehydration is the removal of the water vapor available for microbial growth. Gram negative bacteria typically require higher A_w

levels than do gram positive organisms or fungi which are less sensitive to reduced A_w levels (Farkas 1997). The ability to remove the water vapor from a food depends on several factors: the moisture content and composition of the food, temperature and the moisture content of the surrounding environment (Farkas 1997).

S. Experimental Thermal Resistance Studies

Some of the variation in the literature regarding the thermal resistance of *Listeria* may be due to experimental conditions, protocols, recovery media and methods used (Doherty, McMahon, et al. 1998). Experimental methods include sealed tube, test tube, differential scanning calorimetry and a submerged coil. Differential Scanning Calorimetry has been used to heat the bacteria in order to determine thermal death times and has been used to compare thermal denaturation curves (thermographs) (Anderson, Hedges, et al. 1991). In another study conducted by Donnelly *et al.*, 1987, that compared the use of test tubes and sealed tubes, survivors were consistently found when using the test tubes, where none were found under the same conditions in the sealed tubes (Donnelly, Briggs, *et al*., 1987). An advantage of both of these methods is the ability to use both liquid samples and small solid samples during the thermal studies. The submerged coil can only handle microbiological media and foods that are in the liquid state (Buchanan & Edelson 1999). There are a number of advantages in using the submerged coil. Samples have a short temperature equilibration time and the variable time intervals allow easily reproducible thermal inactivation determinations (Buchanan & Edelson 1999, Cole & Jones 1990). The equipment is also simple to operate and use,

therefore there are fewer problems due to multiple user error (Cole & Jones 1990). Studies on the submerged coil by Cole and Jones, 1990 have shown that there is no carry-over from one sample to the next and there is complete separation of the sample and the sterile water used to flush or move the sample out of the coil.

The decimal reduction time, or D value, is the rate at a specific temperature of the reduction of the bacterial population by 90% and is inversely related to the destruction rate. D value determination, which describes the death kinetics of an organism, is usually done by plotting the $\log_{10}$ of the survivors versus the inactivation time (Casadei, De Matos, et al. 1998). The D value is then obtained by taking the negative reciprocal of slope of the best fit line (Donnelly, Briggs, et al. 1987, Lou & Yousef 1996, Rowan & Anderson 1998). The rate of death is constant at any given temperature and is independent of the initial cell culture (Farkas 1997). A thermal death curve, which is the D value logs versus the exposure temperature, shows the relative resistance of the organism at different temperatures. The slope of the thermal death curve is expressed as Z value or the temperature needed to alter the log D value by one unit (Casadei, De Matos, et al. 1998, Farkas 1997, Stephens, Cole, et al. 1994). The problem with the D value is that the number is calculated from the linear sections of the thermal death curve, not the entire curve. Usually the shoulder and/or the tail section of the curve is eliminated from the calculation, this could lead to an underestimation of the time required to reduce or eliminate the pathogen (Rowan & Anderson 1998).

The media, either selective or non-selective, used to recover cells during a thermal study can also determine which cells are recovered. The correct non-selective

media will generally recover most of the injured and non-injured cells, while the correct selective media will mostly recover only uninjured cells (Chawla, Chen, et al. 1996, Smith & Buchanan 1990). The difference in plate counts between a non-selective medium and the selective medium will reflect the degree of injury experienced by the organism. In a previous study by Smith and Buchanan, 1990, modified Vogel Johnson (MVJ) agar, used as the selective agar for *Listeria*, did not allow for the repair and subsequent colony formation of injured *Listeria* cells. A non-selective agar should always be used, since this medium is more likely to reveal the total number of cells, both injured and non-injured. This subtle delineation is very important since injured or non-culturable cells on selective agar have the potential to repair themselves in the food or when ingested by susceptible human populations and could lead lead to potential health problems (Lou & Yousef 1996, Rowan & Anderson 1998).

T. Statement of the Problem

The goal of this study was to gain a better understanding of the impact that prior growth conditions and food matrices have on the thermotolerance of *L. monocytogenes*. Previous research has been performed on the effect of increased acid resistance of *L. monocytogenes* and concomitant increase in the resistance of the microorganism to additional environmental stresses such as acid and heat (Farber and Pagotto, 1992), (Gahan & Hill, 1999), (Mazzota, 2001). However, there has been little research to determine if induced acid resistance increases either the resistance or sensitivity of the microorganism to stresses such as heat, acid, and reduced water activity under

concurrent well-defined conditions.

The data obtained from such experiments would then be used to develop improved mathematical models. Such models could be used to aid food processors in the design of more efficient heating systems, which could reduce the loss of the sensory properties of the food while still ensuring a safe food product for the consumer. The information would also provide a better understanding of the ability of the microorganisms to become more resistant to an environmental stress. Overall the information derived from these experiments will enable the food processing industry to promote a safer and tastier food product.

In previous experiments by other investigators, cells were either grown in a defined pH, or subsequently exposed to a low pH for a period of time before being thermally stressed. In addition most cells were exposed to only moderate acidic conditions such as pH 5.0-pH 5.5. The effects of the heating menstruum on the thermal tolerance of the microorganism were not usually tested adequately. Chhabra *et al.*, (2002) observed the effect of different combinations of milk fats on the resistance of the cells during the thermal process. Previous experiments also used different methods to test the thermal tolerance of unheated cells, e.g. utilizing open flasks immersed into the water bath. The heating menstruum was placed into the flask and allowed to equilibrate before the addition of the tested suspension of organisms (Fedio and Jackson, 1989), (Golden et al, 1988). Also, when composite or cocktails of several different strains were used, the results would be skewed because the cells of the most thermal resistant strain(s) masked the less resistant strains in the cocktail. (Budu-Amoako *et al.*, 1992,

Glass and Doyle, 1989, Mazzotta, 2001).

In this research, unlike previous experiments, where the prior growth conditions consisted of growth in either an acidogenic or non-acidogenic media, the cells in either medium were gradually exposed to an acidic environment. The pH in the acidogenic medium dropped from about pH 7.0 to a pH of about 4.25 in about 12 hr due to the utilization of the glucose by the cells. The cells were then allowed to remain at this low pH for about 6 hr before the start of the thermal trials. In Main Study #1, thirteen strains were exposed to 58°C in two different heating menstrua. In Main Study #2, a single strain, #201 (F4258), was exposed to the same temperature in additional heating menstrua. All of the heating menstrua used in these studies were Brain Heart Infusion broth (BHI), altered in pH using either concentrated or 1.0M HCl and water activity (Aw) using saturated NaCl. The heating menstruum with the cells were injected into the submerged heating coil apparatus (Fig. 1) set at 58°C to test the thermal resistance of the organism. Both studies tested the thermal resistance of the microorganism under varying combinatorial conditions of altered pH and A_w.

III. MATERIAL AND METHODS

A. Microorganisms

The thirteen *L. monocytogenes* strains used in this research were obtained from the culture collection maintained by the FDA/CFSAN microbiology laboratories (Table 3). Strains 200 through 209 were obtained from the stock culture collection from the Microbial Food Safety Research Unit of the USDA/ARS Eastern Regional Research Center (Wyndmoor, PA). Strains 210, 211, and 212 were obtained directly from the American Type Culture Collection (ATCC, Manassas, VA). When the strains were obtained, they were streaked on Oxford agar (Fig 2) and checked for typical growth. They were then Gram stained and examined for motility and hemolysis. After confirmation to genus, the species was identified by API Listeria tests (bioMerieux Vitek, Inc., Hazelwood, Missouri), CAMP test reaction, and hemolytic activity. The thirteen strains were used in Main Study #1, while *L. monocytogenes* strain #201 (F4258) was used for Main Study #2 and for the preliminary studies.

Permanent frozen stock cultures of each strain were prepared by growing the strains in Brain Heart Infusion broth (BHI) (Becton Dickinson Diagnostic Systems (Difco), Sparks, MD), combining the BHI culture with glycerol at a ratio of 85:15, and then storing at -70°C. Working stock cultures of the strains were kept in a -20°C lab freezer. Cultures were activated by transferring a 1.0 ml aliquot of a -20°C working stock culture to a test tube containing 10 mL of BHI and incubating overnight at 37°C. This BHI culture was then used to inoculate the overnight (O/N) cultures used for the thermal resistance trials (see below).

B. API Biochemical Identification

1. Inoculation

The *Listeria* strains were grown on TSA blood agar plates for 24 hr at 37°C, examined, and the hemolysis noted. Several colonies from the TSA blood agar plate were then transferred to the API suspension medium (demineralized water), and adjusted to a concentration equivalent to a McFarland 7 standard. This suspension was then used to fill the twelve reaction tubes of the API strip. The incubation tray was covered and incubated for 18-24 hr at 36°C.

2. Interpretation

After the tray was removed from the incubator, and the proper reagents added, the reactions were recorded as + or – on the results sheet, along with hemolysis.

3. Identification

Identification was made using a numerical profile. The tests were separated into groups of three and a value 1, 2 or 4 was assigned to each. A 4 digit profile was determined by adding the values together to provide a code number and then was compared to the list of *Listeria* spp codes that were included with the test package. The digital code could differ slightly but still be identified as *L. monocytogenes* (Appendix A).

C. Preliminary Studies:

1. Preliminary Study #1 Acid Resistance of Induced Cells vs. Non-Induced Cells

To determine if acid-induced cells were more acid resistant than non-induced cells, and to determine if glutamine has any effect on the acid resistance, cells were exposed to two different acid conditions, pH 2.5 and pH 3.0. To accomplish this purpose, strain #201 was grown in 4 different modifications of Tryptic Soy broth (TSB) (18 hr at 37°C) emended as follows: 1% glucose, 1% glutamine (++), 1% glutamine (-+), 1% glucose(+-), absence of glucose and glutamine (--). Before beginning the experiment sterile TSB was modified using HCl to provide a final pH of either 3.0 or 2.5. The broths were then re-sterilized by passage through Nalgene filters (0.2 μm pore size) . The solutions were then aseptically aliquoted in 10 ml portions to sterile test tubes. The day before the experiment, labeled tubes were placed into a 37°C incubator. Eighteen hours before the start of the experiment, 0.1 ml of strain #201 was transferred to each of the 4 overnight cultures. The cultures were then placed into the 37°C incubator. At the start of the experiment the overnight culture containing 1% glucose and 1% glutamine was removed from the incubator, 0.1 ml portions were transferred to the pH 2.5 replicate set for this overnight culture. The zero hour sample was plated on duplicate BHIA and MVJ agar plates using the spiral plater (Spiral Biotech, Bethesda MD) (Fig. 3). The sample hour was recorded and the remaining replicate tubes were placed back into the incubator. The pH 3.0 tubes were removed from the incubator and the process repeated (Fig. 4). The entire procedure was repeated with the other three

overnight cultures. At the 2 hr, 4 hr, and 6 hr sample times, the appropriate tube was removed from the incubator. The 10^0 and 10^2 dilutions were then plated similarly to the zero hour sample. All plates were held at room temperature until the end of the experiment day. The plates were then incubated at 37°C for 48 hr. Colonies on the plates were counted using the spiral counter (Spiral Biotech, Bethesda MD)(Fig. 5) and the averages and log values calculated using Lotus 1-2-3.

2. Preliminary Study #2 L. monocytogenes Growth Phase

To determine the growth curve of strain #201, 0.1 ml, of an 18 hr stock culture was transferred to two 10 ml tubes of TSB, containing either 1% glucose and 1% glutamine or TSB containing 1% glutamine, 18 hours before the start of the experiment, then placed into a 37°C incubator. Three different sized inocula from the two different media were then used to study the growth curves of *L. monocytogenes* #201 over a 9 hr period to determine the effect that prior growth had on the cells reaching exponential growth phase. Therefore the acidogenic culture (grown in the presence of 1% glucose and glutamine) was removed from the incubator and 0.1 ml transferred to a tube containing 9.9 ml BHI and labeled tube A, the zero time recorded and the appropriate dilution spiral plated on BHI agar (BHIA) plates. Before returning the A tube (10^{-2} dil) to the incubator, 0.1 ml of the sample was transferred to a tube containing 9.9 ml BHI which was labeled tube E (10^{-4} dil), the zero time was recorded and the appropriate dilution spiral plated on BHIA. Tube E was then placed in the 37°C incubator. An additional 1.0 ml of the overnight culture was then transferred to a tube containing 9.0

ml of BHI broth. From this tube 0.1 ml was transferred to a tube containing 9.9 ml of BHI labeled tube C (10^{-3} dil), the zero time was recorded and the appropriate dilution was spiral plated on BHIA. The tube was then placed into the 37°C incubator. The entire procedure was repeated using the overnight culture grown in the TSB without glucose except that the tubes were labeled B, F and D (Fig 6). To achieve the growth curves of strain #201, at the 1st, 2nd, 3rd, 4th, and 7th experimental hr, the tubes were removed from the incubator and samples taken and spiral plated on BHIA as described above. After the last sample hour was taken, the pHs of the cultures were recorded. All plates were held at room temperature until the end of the experimental day and were then placed into the 37°C incubator for 48 hr. The colonies were then counted using the spiral counter.

3. Preliminary #3 Thermal Study Using Log phase Cells #201 in Heating Menstruum pH3, Aw 0.960

Test tubes of TSB containing 1% glucose and 1% glutamine and TSB containing 1% glutamine were inoculated with 0.1ml of strain #201's 18 hr stock culture and placed into a 37°C incubator for 21 hours before initiating the thermal study of the preliminary experiment. Three hours before the start of the thermal study, 0.1 ml of the 18 hr overnight culture was transferred to a fresh tube containing 9.9 ml of BHI which was then placed into the 37°C incubator. The pH was then recorded and the appropriate dilution of the 18 hr overnight culture was spiral plated on BHIA. At the start of the study the three hr old culture was utilized in the thermal study (Fig. 7). Following the

regular procedure (described in section D.1), a thermal study using a pH 3, A_w 0.960 heating menstruum was performed using 16 sec sampling intervals. The dilutions were plated on BHIA and placed into the 37°C incubator. The pH was also recorded and the appropriate dilution was made of the 3 hr overnight culture which was spiral plated on BHIA. After 48 hr, the colonies on the plates were enumerated using the spiral counter.

4. Preliminary #4 pH Decline of Stationary Phase Cells Grown with and without Glucose

To determine the decline of the pH in TSB as the cells passed into stationary phase over a 24 hr period, the sample was divided into 2 sets. Set 1 was sampled on day 1 and set 2 sampled on day 2, which allowed for sample times over a 24 hr time period. At the beginning of the experiment, the + glucose overnight culture was removed from the incubator and 0.1 ml was transferred to each tube of the replicate + glucose set, labeled A + Glu. The zero hour tube was removed and the sample time recorded. The appropriate dilution was spiral plated on duplicate BHIA plates, and the pH of the sample then measured. The remaining sample tubes were returned to the 37°C incubator. Using the -glucose culture, the procedure was repeated with the replicate tube set labeled A - Glu. The + glucose and - glucose procedure was repeated with replicate B and C. The overnight cultures were placed into the 4°C refrigerator, and then were used to inoculate the set two samples at the end of day one. At the appropriate sample times of day one, the correct tube was removed, the time recorded, the sample was plated on duplicate BHIA plates, and the pH recorded. After the last sample hour of the

day, the overnight cultures were removed from the refrigerator. Following the same procedure used for set one, a replicate set of tubes were inoculated, sample time recorded, and the zero sample was removed to plate and determine the pH (Fig 8). The tubes were placed into the incubator and samples removed at the correct times to plate and record the pH. All of the plates were held until the end of the sample day. Plates were then incubated for 40 hours before being counted using the spiral counter.

D. Main Studies

1. Heating Menstrua:

Only two combinations of pH and A_w heating menstruum were used in Main Study #1: A_w 0.987, pH 3.0 and A_w 0.960, pH 7.0. In addition, the effects of the prior growth conditions of the overnight culture (acidogenic and non-acidogenic) on the thermal resistance of *L. monocytogenes* was tested. The heating menstrua for both studies were several combinations of pH and A_w. The pH of BHI was modified using concentrated HCl, and the water activity was altered using saturated NaCl.

In Main Study #2, however, a fractional factorial design (Table 2) was established to examine the effect of the water activity (0.987, 0.980, 0.970, 0.960) and pH (3.0, 3.5, 4.0,4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) of the heating menstruum, along with the effect of prior growth conditions of the overnight culture (acidogenic and non-acidogenic), on the thermal resistance of *L. monocytogenes.* The fractional factorial design enables the researcher to gather a broad range of information on the effects of the heating menstrua without having to sample each pH and A_w combination.

***2. Strains utilized*:**

All thirteen strains of *L. monocytogenes* were examined in Main Study #1 (Table 3). For Main Study #2 only one of the thirteen *L. monocytogenes* strains, #201 (F4258), was examined. This strain did not show either extreme (thermal sensitivity or resistance) in the two heating menstrua used in Main Study #1. Therefore, #201 was an ideal representative because it had a median response to thermal conditions after exposure to the varying pH and A_w levels. Further it is serotype 4b, which is most commonly associated with foodborne outbreaks (Table 1)

3. Water Activity and pH of Heating Menstrua:

Modified BHI was used to represent the effects of the usual food matrix. Therefore, BHI was modified to have specific water activities and pH to create two heating menstrua as follows: Menstruum #1 was BHI acidified to pH 3.0 through the addition of concentrated hydrochloric acid. The sodium chloride (NaCl) content of the BHI was unaltered, thus menstruum #1 had an a_w of 0.987. Menstruum #2 consisted of BHI to which additional NaCl was added to achieve an $a_w = 0.960$; the pH remained unaltered at pH = 7.0. The pH and water activities of the two BHI solutions were verified using a pH meter (model 8005 by VWR Scientific) and a water activity meter (Aqua Lab model CX-2, Decagon Devices Inc). Both BHI preparations were then filtered sterilized into a sterile Wheaton bottle and stored at 4°C until used as a heating menstruum. The BHI preparations were used within fourteen days of preparation. The

two menstrua selected for Main Study #1 were at the extreme ends of the heating menstrua that would be used for Main Study #2.

4. Preparation of Culture:

Approximately eighteen hours before beginning a heating trial, one culture tube containing 10 ml of Tryptic Soy broth (TSB) without dextrose (Difco #0155-17-4, Sparks, MD)+ 1% glutamine (Gibco, BRL) and a second tube containing 10 ml of TSB + 1% glutamine + 1% glucose were inoculated for each strain being tested. TSB + 1% glutamine is non-acidogenic, allowing the growth of *L. monocytogenes* without acid production due to fermentation of glucose. Conversely, TSB + 1% glutamine + 1% glucose is acidogenic, supporting growth of the microorganism with an accompanying decrease in pH. For consistency, the cultures grown with glucose also contained glutamine. The tubes were incubated at 37°C for eighteen hours.

5. Thermal Experiments of Preliminary Study #3 and Main Studies #1 and #2:

The thermal resistance of the thirteen strains were measured using a submerged heating coil apparatus (model 2, Sherwood Instruments, Lynnfield, MA)(Fig 1). The heating coil was equilibrated to 58°C and programmed to dispense ten samples within the time frame of the experiment over the course of a heating trial. The heating coil allowed for accurate and easily reproducible results (Cole and Jones, 1990).

Two fifteen mL aliquots of the heating menstruum being utilized in the Main Studies were transferred to sterile 50 mL centrifuge tubes and allowed to equilibrate to room temperature. A 0.9 mL portion of a acidogenic culture of the *L. monocytogenes* strain was transferred to one of the centrifuge tubes. Immediately following a rapid mixing, 0.4 mL was transferred to a pre-cooled (4°C), 3.6-mL dilution blank of sterile 0.1% peptone water, which served as the 0-time sample. The remainder of the inoculated heating menstruum was injected into the heating coil and 0.4 ml samples were dispensed at the appropriate times into individual sterile vials. The samples were then transferred to pre-cooled 3.6 ml dilution blanks. As soon as the heating trial with the acidogenic culture was completed, the second centrifuge tube of heating menstruum being utilized was inoculated with the same strain grown under non-acidogenic conditions.

Samples were diluted further as necessary using 0.1% peptone water and surface plated on duplicate Brain Heart Infusion Agar (BHIA) (Becton Dickinson Diagnostic Systems (Difco), using a spiral plater. All plates were incubated at 37°C for 40 hr. The plates were then enumerated using an automatic plate counter. Each strain was tested once under each of the four conditions.

6. Data Analysis:

The slope and lag (shoulder) time of each thermal inactivation trial was determined by fitting the data to a 2-phase inactivation model (Buchanan and Edelson, 1996b) using the curve fitting software ABACUS (Damart, 1994). D-values were calculated by taking the negative reciprocal of the slope of the thermal inactivation curve. The time to achieve a 4 $\log_{10}$ inactivation (t_{4d}) was used to provide an integrated measure of the shoulder and exponential phases of the inactivation curve (Buchanan and Edelson, 1999b). The t_{4d} value was calculated using the following equation:

$$t_{4d} = t_{lag} + 4D$$

where t_{lag} is the duration of the shoulder of the thermal inactivation curve.

7. Additional Data Analysis

Additional analysis of the data was carried out in the Division of Mathematics Department of FDA. SAS statistical software was used to develop a second-order polynomial of the two variables, A_w and pH to provide estimates of the t_{4d}.

IV. RESULTS

A. Microorganisms

All thirteen strains utilized showed typical biological *L. monocytogenes* characteristics: gram positive, tumbling motility in motility agar at 23°C, and beta hemolytic activity. All thirteen were confirmed as *L. monocytogenes* by *Listeria* API tests, but due to some variation in their chemical reactions had different profiles (Table 4). This is a good example of strain variation within a species.

B. Acid Resistance of Induced Cells vs Non-Induced Cells

Induced cells were more acid resistant than non-induced cells. Glutamine did not affect the resistance. At both pH 2.5 and pH 3.0 the +glucose cells showed less than one log decline after 6 hr of exposure (Fig 10 and 11). Although at the 6 hr sampling some injury of the cells could be observed as indicated by the lower counts obtained on the MVJ plates, in which only uninjured cells were able to grow. Non-induced cells, those grown without glucose, had a 4 log reduction at the 2 hr sampling after exposure to pH 2.5 and a 2 log reduction after exposure to pH 3.0. By the 4 hr sampling, cells exposed to pH 2.5 were below the level of detection (Fig 10). Cells exposed to pH 3.0 were low but still detectable at the 4 and 6 hr samplings (Fig 11).

C. *L. monocytogenes* Growth Phase

A growth period of approximately 3-5 hr was necessary for both the acid induced cells and non-acid-induced cells to reach exponential growth phase (Table 6). Growth

was minimal in all the cultures before 3 hr, but there was approximately 1.5 log increase in growth between 3-5 hr for all six culture experimental tubes. The growth curves of all six cultures are shown in Fig. 10.

D. Thermal Study Using Log phase Cells F4258 in Heating Menstruum pH3, Aw 0.960

Exponential phase cells were much less resistant to the thermal conditions cells in the stationary phase cells. The 5D value of the logarithmic phase cells was 202.4 sec. The average 5D value of the stationary phase cells in the same heating menstruum was 371.25 sec (Table 8). The exposure of exponential phase cells in heating menstruum pH 3.0, A_w 0.960 to 58°C showed increased thermal sensitivity when compared to stationary phase cells.

E. Decline of pH by Stationary Phase Cells of *L. monocytogenes* strain #201 grown with and without 1% added glucose

There was a gradual decrease in the pH over a 14 hr period of cell growth (Table 9). Acid induced cells showed a larger decrease from about pH 7.0 to pH 4.5. Non-induced cells also decreased their pH, but only by about 0.5. The pH of both overnight cultures showed minor reductions in their pH for the remaining 10 hr of incubation. Also by the 10th hr, the cell counts of both cultures increased by about 3.5 logs. By 24 hr there were only slight increases, about 0.5 log, over the 10 hr sampling.

F. Main Study One

Presented in Table 10 (a) and 10(b) are the D, t_{lag}, and the t_{4D} values for all of the strains in both heating menstrua. In the heating studies that utilized menstruum #1, eight out of the thirteen strains grown under acidogenic conditions had higher D-values and survived longer than cells grown under non-acidogenic conditions. However, in menstruum #2, the results were just the opposite with six out of the thirteen strains grown in glucose having higher D-values than those not grown in glucose. Fig 13 is an example of a survivor curve where the cells grown in glucose exhibit more thermal resistance, while Fig 12 illustrates a survivor curve where the cells grown in the absence of glucose display more thermal resistance. All of the survivor curves for thermal menstrua #1 and #2 are in Appendix A.

The pH range of the overnight cultures grown in the presence of glucose, ranged from 4.16 to 5.59 with a mean of 4.67 and standard deviation of 0.36. For the cultures grown in the absence of glucose, the pH range was 6.65-6.92 with a mean of 6.78 and standard deviation of 0.06.

The effect of overnight growth in glucose was not consistent among all strains; occasionally the D-values of cells grown without glucose were higher than that of cells grown with glucose. This inconsistency was also observed with strain #201, the 4b serovar strains.

In heating menstruum #1, the acidogenic cells from strains #209 and #205 exhibited the greatest thermal resistance, while the cells of strain #207 were the most thermal sensitive (Table 10a and Fig 14). Likewise, in the same heating menstruum, the

non-acidogenic cells of #209 displayed a similar pattern of greater thermal tolerance and strain #207, greater thermal sensitivity (Fig 14). In thermal menstruum #2, the acidogenic cells from strain #207 was the most thermal tolerant of the thirteen strains, while cells from strain #209 were the most thermal sensitive (Table 10b and Fig 15). Similarly, strain #207 grown in the absence of glucose was the most thermal resistant strain. However in the absence of glucose the most thermal sensitive strain was #210 in heating menstruum #2 (Table 10b and Fig 15). Overall both the acidogenic and non-acidogenic cells were more thermal resistant and therefore had higher D-values in heating menstruum #2 when compared to the cells in heating menstruum #1.

For all organisms grown in acidogenic media, those exposed to heating menstruum #1 had lower t_{4D} values, indicating lesser survival, than the organisms exposed to heating menstruum #2. In the non-acidogenic media, the organisms exposed to heating menstruum #1 also illustrated lower t_{4D} values. Heating menstruum #2 had a less acidic pH which reduced the environmental stress load on the cells as they were exposed to the thermal environmental stress. There was attempt to separate the effect of the pH from the effect of the A_w.

For each heating menstruum, analysis of variance did not reveal a difference between the t_{4D} mean values of the acidogenic and non-acidogenic. With the same glucose conditions, a difference between the t_{4D} values of the two heating menstrua was observed ($p<0.05$).

Exposure of the thirteen strains in this study to acid, achieved by overnight growth in glucose, did not show consistent effects after thermal treatments. In each of the two

menstrua, approximately one half of the strains showed increased heat tolerance after acid exposure. Many of the strains displayed a different effect in each of the two menstrua. The serovar 4b strains also presented inconsistent results upon exposure to acidic conditions. In only three out of six replicates did the acidogenic strains show increased thermal tolerance. Strain number 201, a 4b serovar, did not display extreme thermal resistance or thermal sensitivity and would therefore represent the majority of 4b strains. When selecting a single strain for further studies of the thermal tolerance of *L. monocytogenes*, strain number 201 would be a good choice for representing the characteristics of all *L. monocytogenes* species.

Variance analysis of the t_{4D} values for acidogenic condition versus the non-acidogenic condition used the null hypothesis suggesting that there was no difference between the means of the two groups. In heating menstruum #1, the mean t_{4D} value among the thirteen strains was 205.6 with a standard deviation (SD) of 123.6 for the acidogenic cells and a mean of 193.3 and SD 168.7 for the non-acidogenic cells. The calculated F (analysis of variance) value was 0.0448 and the F distribution value was 4.2597 ($a = 0.05$), therefore the null hypothesis was not rejected. In heating menstruum #2, the mean t_{4D} value among the strains was 1331.0, with a SD of 502.9 for the acidogenic cells and a mean of 1192.8, with a SD 582.3 for the non-acidogenic cells. The calculated F value was 0.4194 therefore the null hypothesis was not rejected. In comparing the two heating menstrua, using the acidogenic cells, the calculated F value was 61.3982 and the F distribution value was 4.2597, therefore the null hypothesis was

rejected. Comparing the two heating menstrua, but using the non-acidogenic cells, the calculated F value was 35.3921 thus also rejecting the null hypothesis.

G. Main Study Two

In this study using a single strain, results of the t_{4D} values under the different conditions are presented in Tables 11 and 12 and in Figures 16, 17, 18, and 19. Using cells grown overnight with glucose and plated onto BHIA, at each A_w value the t_{4D} values peaked as the pH rose to 6.0 - 6.5 and declined at higher pHs. In general, as the A_w decreased at a fixed pH, the t_{4D} value increased. For those grown without glucose, the pattern was similar except that the t_{4D} value at Aw 0.980 and pH 4.0 was 1100, which was approximately twice the value (551) observed at A_w 0.960. In the cells grown without glucose and plated to BHIA, the t_{4D} values were higher than in those with glucose when the pH conditions were above 3.5. Using cells grown overnight with glucose and plated to MVJ media, the peak t_{4D} values were seen at pH 5.5 to 6.5. Decreasing the A_w again increased the t_{4D} values. For those without glucose, the t_{4D} values peaked in the range of pH 6 to 7.5 and were generally higher as the A_w decreased. Cells grown in the presence of glucose showed higher t_{4D} values than those grown without glucose when tested in the low pH conditions (3 and 3.5) but at less acidic conditions, the cells grown without glucose had higher t_{4D} values. The t_{4D} values of the organisms plated to MVJ were less than those on BHIA. In addition for samples plated on BHIA, large lag times were often seen (>100 sec), but not seen when plated on MVJ. The MVJ media is only able to grow uninjured cells.

Some of the most striking changes in the t_{4D} values occurred at pH 5.5 and 6.5 at A_w of 0.970. The cells grown in the presence of glucose and plated to BHIA in these conditions yielded much lower t_{4D} values than those without glucose. The same effect was seen in the same conditions using MVJ media. In addition, the MVJ plated cells showed a larger drop in t_{4D} value at A_w 0.980 and pH 6.0 when grown without glucose compared to the same conditions with glucose.

In the samples grown in glucose and plated on BHIA, large lag times were usually observed (>100 sec), but not when plated on MVJ. In samples grown without glucose, the only large lag times were seen on samples exposed to a pH of either 4.0 or 4.5 and plated on to BHIA.

In figure 20, *L. monocytogenes* #201 cells subjected to A_w of 0.960 at pH values ranging from 3.0 to 7.0 showed higher resistance to thermal stress at higher pH values, peaking at pH 6.0, grown in the absence of glucose than cells grown in the presence of glucose. The t_{4D} values of the cells grown without glucose were approximately twice those with glucose at pH > 4.0.

1. Mathematical Model

Statistical analyses of the data were performed through the courtesy of Jung Lee from the Division of Mathematics at the FDA to provide an equation for predicting D values for other combinations of pH and A_w. A second-order polynomial of the variables pH and A_w was fitted to the D-values for each combination of media and glucose: BHIA plus glucose, BHIA without glucose, MVJ plus glucose, and MVJ without glucose.

Unfortunately, when the adequacy of the fitted function was checked, the test showed significance ($p<0.05$) indicating that the results must be viewed with caution.

For predicting D-values adjusted for a lag time, the equation is:

$$Y = 7.234 - 0.304\ mda - 0.149\ g - 8.404\ A_w + 1.205\ pH - 0.084\ (pH*pH)$$

with R^2 (the percent of the variation about the mean accounted for by the fitted model) = 86%, where mda = 1 for the BHIA media and mda=2 for MVJ, g=1 for glucose added and g=0 for no glucose added. Y represents the logarithm (base 10) of the D-value, to calculate the D-value, calculate 10^Y. For predicting D-values unadjusted for lag time:

$$Y = 6.924 - 0.316\ mda - 0.109\ g - 7.716\ Aw + 1.091\ pH - 0.075\ (pH*pH)$$

with $R^2 = 86\%$. From the fitted model, we can determine that the maximum D-values within the pH range of 6.8 to 7.5 is at $A_w = 0.960$. The minimum D-values are at A_w=0.987 and pH = 3.0 which is the boundary point of the experiment. The fitted model should not be used to predict beyond this boundary of the experimental region.

V. DISCUSSION

Previous studies have outlined various effects of environmental stresses on the heat tolerance of foodborne pathogens. For example, previous research on *E. coli O157:H7* has shown that when the organism is grown in an acidogenic environment, thermal resistance is always greater than that for the same organism grown in a non-acidogenic culture (Buchanan and Edelson, 1999b). Earlier research on the thermal resistance of *Listeria* has suggested that the resistance of the microorganisms is enhanced by prior exposure to environmental stress (Donnelly *et al*., 1987). The results from this study illustrated that prior exposure to an environmental stress can alter the thermal resistance *L. monocytogenes*. Depending upon the strain tested and the environment in which the bacteria are heated, resistance may either increase or decrease after exposure to an acidogenic environment.

Farber and Pagotto, 1992, suggested that exposing particular strains of *L. monocytogenes* to acid resulted in an initial increase in D-values. Mazzotta, 2001a, found that the thermal resistance of *Listeria* decreased as the environment became more basic. Taormina and Beuchat, 2001, found that exposure to an alkaline environment increased the thermal resistance of a single strain of *L. monocytogenes*, serotype 4b. In that same study when the strain of *L. monocytogenes* was exposed to a chlorine environment, thermal resistance decreased.

Salt concentration is another factor that has been shown to affect the thermal response of *L. monocytogenes*. Increased salt concentrations used in the curing of pork had been found to increase the thermal resistance of *Listeria monocytogenes* (Ryser and

Marth, 1999). Another study found that osmotic shocking of the cells by different NaCl concentrations increased the thermal resistance of *Listeria* (Farkas, 1997). After a five hour adaptation to TSB supplemented with 15% NaCl, *L. monocytogenes*, was found to have increased heat resistance in surimi-based imitation crab (Mazzotta, 2001b). Salt concentration can also affect the organisms resistance to other stresses such as nisin, a bacteriocin, used in the food industry as a preservative. De Martinis *et al*. 1997, found that low salt concentration (2 to 3.5%) protects *L. monocytogenes* against the bactericidal effects of nisin.

Thermal resistance may also be influenced by components present in the heating menstruum. These components can protect the bacterial cells from thermal damage by stabilizing membranes or other cellular structures by stimulating the production of stress related proteins (Rocourt and Cossart, 1997).

Preliminary studies reported herein were conducted on the acid resistance of *L. monocytogenes* strain #201, (F4258) after prior exposure to acidogenic environment. The results from these studies indicated that prior exposure to moderate acidic conditions increased the resistance of the microorganism to more severe acidic conditions. Kroll and Patchett, 1992, found that growth at a pH of 5.0 compared to pH 7.0 increased the resistance of *L. monocytogenes* to low pH. Besides increasing the ability of the microorganism to survive the acidic environment of some foods, this type of stress response may enable the organism to better survive in the acidic environment of the stomach or within a macrophage phagosome (O'Driscoll *et al*., 1996). The increased resistance may be due to the ability of the microorganism to increase protein synthesis

during the stress of acid adaptation. Phan-Thanh and Mahouin, 1999 found that cells of *L. monocytogenes* exposed to pH 5.5 for 2 hr showed increased expression of constitutive proteins and synthesis of novel proteins. The results from this study also indicate that some of the strains of *L. monocytogenes* tested showed an acid tolerance response (ATR). According to Farber, 1995, ATR is enhanced survival of an microorganism to acidic conditions (pH 2.5 to pH 4.0) after exposure to moderate acidic conditions. Buchanan and Edelson, 1999b, saw similar results with seven strains of *Escherichia coli* O157:H7. In that study, after cells were acid induced they became more resistant to BHI acidified with either organic acid or HCl. In the preliminary study of this investigation, strain #201 also appeared to have an inducible pH dependent state of acid resistance showing increased resistance to BHI acidified with HCl.

Virulence may also be influenced by prior exposure to an environmental stress. Wiedmann *et al*., 1998 sequenced a region of *L. monocytogenes* which was identified as the alternative sigma factor σ^B. This factor has been linked with the ability of *L. monocytogenes* to tolerate and survive acid stress after prior exposure to acidic conditions (Ferreira *et al*., 2003). Interestingly, Gahan and Hill, 1999 found that, while prior exposure to acidic conditions increased the acid resistance of *L. monocytogenes*, it did not increase the virulence of the microorganism. Conversely in a study by Conte *et al*., 2000, acid adapted cells of *L. monocytogenes* were better able to adhere to and enter Caco-2, enterocyte-like cells, which would translate to an increased ability to pass through the stomach and invade the intestinal tract.

One of the objectives of the preliminary research was to determine when the cells of strain #201 reached the exponential growth phase. The reduction in the pH during exponential growth was also monitored until the cells reached the stationary growth phase. Exponential phase cells of strain #201 were then utilized in a thermal resistance study. The T_{4D} values from this study were then compared to the T_{4D} values of stationary phase cells utilized in the Main Study #2 under the same thermal conditions. The T_{4D} values of the exponential phase cells were almost half of the T_{4D} values of strain #201 stationary phase cells. Studies by Lou and Yousef, 1996 also found that exponential phase cells were more thermally sensitive than stationary phase cells. On the other hand studies conducted on *L. monocytogenes* by Jørgensen *et al.*, 1999 found that heat shocked exponential phase cells showed a greater increase in thermal resistance as compared to heat shocked stationary phase cells.

In Main Study #1, the influence of the growth condition on the thermal resistance of the thirteen strains was not consistent. In heating menstruum #1 (pH 3.0, A_w 0.987) strain 207 was the most thermal sensitive and strain 209 was the most thermal resistant. But opposite observations were made in experiments with heating menstruum #2 (pH 7.0, A_w 0.960), where strain 209 was the most thermal sensitive and strain 207 was the most thermal resistant. This provocative change described from highest to lowest sensitivity in these two strains as the menstruum condition changes is intriguing, but seemingly unexplainable. In a recently published review Doyle *et al*, 2001, reviewed the heat resistance of nineteen strains of *L. monocytogenes* tested under similar conditions. Several strains showed thermal resistance that were more than 2.5 to 3 times greater

than other strains; the authors attributed these responses to strain variation. Beuchat *et al*., 1986 found that one strain of *L. monocytogenes,* Scott A, had increased thermal resistance in the same heating condition versus strain LCDC 81-861. Bradshaw *et al*., 1985 also observed that one strain of *L. monocytogenes* had higher thermal resistance than other strains. The differences observed in the thermal resistance of the thirteen strains utilized in Main Study #1, in the same heating conditions, was also most likely due to strain variation. This could have been due to the ability of some strains to synthesize stress proteins, as compared with other strains. Otherwise, the difference noted may have been due to simple strain variation in membrane composition. Based on the moderate resistance and sensitivity shown by strain #201 to both heating menstrua, the strain was selected for use in Main Study #2.

In Main Study #2, the pH of the heating menstruum had a strong effect on the thermal tolerance of *Listeria*. The t_{4D} values in acidic heating menstrua were lower thus indicating that cells have poorer survival in low pH, as expected. This was also seen in a thermal study by Beuchat *et al*., 1986, in which cabbage juice was used as the heating menstruum. In the study, two strains of *L. monocytogenes* had increased thermal resistance in the heating menstruum of pH 5.6 cabbage juice in comparison to the pH 4.6 cabbage juice heating menstruum.

The water activity also showed an overall effect on the survival of the bacteria tested. Lower water activity conditions increased the t_{4D} value indicating increased survival. The increased survival times allowed the effects of A_w to become more apparent in the heating menstrua as they became less acidic. In Casadei *et al*., 1998 the

thermal resistance of two strains of *L. monocytogenes* was higher in a heating menstruum of butter versus a heating menstruum of tryptic soy broth.

Glutamine-based medium vs. glucose plus glutamine-based medium provides a simple means of studying pH-dependent stationary phase acid resistance in *L. monocytogenes*. The cells grown in the presence of glucose were then exposed to an acidic environment created by the bacteria themselves prior to the heating trials. Whether plated to BHIA or MJV, the cells grown in an acidic environment showed less heat tolerance than those grown without the presence of glucose when tested in pH of greater than 4.0. The prior exposure tends to help the cells survive further acidic conditions but compared to cells grown without glucose, it did not appear to enhance the thermotolerance of the cells in less acidic conditions.

According to Bergey's Manual (Seelinger and Jones, 1986), catabolism of glucose proceeds by the Embden-Meyerhof pathway both aerobically and anaerobically. This pathway is also referred to as glycolysis, in which glucose is catabolized to pyruvate and is then further reduced to lactic acid (Stryer, 1995). The lactic acid is released from the cell, thus acidifying the tryptic soy broth environment surrounding the bacteria. In some of the experiments described herein, an alternate carbon source, glutamine, was identified as a substrate that would not acidify the surrounding environment when catabolized. Glutamine is hydrolyzed to glutamate and then catalyzed by oxidative deamination using glutamate dehydrogenase to produce α-ketoglutarate which then enters the tricarboxylic acid cycle to produce energy without releasing acidic products into the environment.

Listeria monocytogenes inactivation kinetics was log-linear when the cells were grown in a non-acidogenic medium and became non-log-linear when grown in acidogenic medium. The t_{4D} values of cells grown in a non-acidogenic medium were greater than those of corresponding cells grown in acidogenic conditions medium. Overall thermal resistance is more complex due to non-log-linear inactivation kinetics.

Shoulders, or lag times, were not consistently seen in the analysis of the data recorded during the thermal inactivation curves. They were only seen when the cultures were plated on BHIA agar, a non selective agar, thus demonstrating non-linear kinetics. The shoulders were not present when the cultures were plated on MVJ agar, a selective agar. The selective agar will allow most of the uninjured cells to grow, while not allowing the injured cells to form colonies. The lack of the shoulder on the curves based on MVJ agar colony counts shows that the cells were injured but not being destroyed initially.

The data obtained from both experiments could be utilized to enhance the Pathogen Modeling Program. In this program, the models are based on experimental data obtained from many experiments. The program is updated periodically as new data and models are obtained. Models included in the current program included those on growth and thermal inactivation of *L. monocytogenes*. An example of a thermal decline curve is shown in Fig 9. The model shows the time required for *L. monocytogenes* to decline in "meat gravy" with a pH of 6.0 and an A_w of 0.970.

The results also indicate that further research is needed on the thermal resistance of strain #201. In Main Study #2, the thermal resistance of both heating menstrua A_w

0.960, A_w 0.970, and A_w 0.980 increased as the pH became more basic but then peaked. At the most basic pH values, pH 7.0 and pH 7.5, cells in both heating menstrua showed decreases in their thermal resistance. This was observed in cells grown in either the presence or the absence of glucose. But in the heating menstruum A_w 0.987 no true peak was observed as the pH increased and became more basic. Therefore additional thermal studies should performed in heating menstrua with A_w 0.987 and pH values of 8.5 and 9.5 with cells grown both in the presence and absence of glucose.

Main Study #2 should also be repeated using additional strains from Main Study #1. This effort would provide additional information on the effects of strain variation. Also fatty acid membrane analysis should be carried out on the thirteen strains to determine if this cellular component had any role on the variation of the thermal resistance observed.

The mathematical model provides a way of predicting bacterial survival under varying conditions. The predicted minimum D-values occurred at A_w=0.987 and pH = 3.0 which is on the boundary of the experimental regions. Further experimentation may identify the stationary point of A_w and pH levels by testing pH below 3.0 and A_w above 0.987 where lower D-values may be obtained.

VI. CONCLUSIONS

This research showed that the growth conditions and the heating menstrua affected the thermal resistance of *L. monocytogenes*. It provides important information on one of the most utilized methods to reduce potential pathogen contamination of food products, i.e., thermal processing.

Although the effects of prior growth conditions were not consistent, they should be taken into consideration, since some strains of *L. monocytogenes* can achieve enhanced thermal resistance based on prior exposure to acidogenic conditions. If not considered, the process utilized to prepare the food product may not eliminate potential contamination of the food product. Therefore the food processors should ensure that the process they utilize will eliminate the most resistant forms of the pathogen.

The effects of the heating menstrua must also have an important role in the thermal process. As the pH of heating menstrua became more basic, the thermal tolerance of strain #201 increased, which would mean that the food processor would have to increase the time a food is heated in order to eliminate the potential pathogen.

All of the findings obtained from this research will help to enhance programs such as the Pathogen Modeling Program. The programs are limited only by the data on which the models are currently based. Addition of more reliable data will enable improvements in the accuracy of the programs. Therefore the processor can make the thermal process more precise for the elimination of potential contamination *by Listeria monocytogenes.* These improvements would result in reduction of the development of off-flavors, which occur due to over-processing, thereby creating an improved food product. While safety

is an important factor to the food processor, making a product that the consumers will purchase is ultimately the raison d'etre for manufacturing the food products.

VII. Tables

Table 1. Worldwide foodborne outbreaks of invasive listeriosis- (US DHHS/USDA, 2003)[a]

Year	**Place**	**No. of Cases (deaths)**	**Implicated (or likely vehicle)**	**% of perinatal cases**	***L. monocytogenes* serotype**
1978-1979	Australia	12(0)	raw vegetables	unknown	unknown
1979	Massachusetts	20 (3)	(Cheese or Raw Vegetables)	0	4b
1980	New Zealand	22(6)	Raw seafood	100	1b
1981	Nova Scotia, Canada	41 (18)	Coleslaw	83	4b
1981	England	11(5)	Dairy Products	unknown	1/2a
1983	Massachusetts	32 (14)	Pasteurized milk	22	4b
1985	California	142 (48)	Mexican-style cheese	66	4b
1983-1987	Switzerland	122 (34)	Soft cheese	53	4b
1986	Austria	28(5)	Unpasteurized milk, organic vegetables	86	unknown
1986-1987	Pennsylvania	36(0)	Ice cream, salami, brie cheese	44	4b, 1/2b, 1/2a
1986-1987	California	2(0)	Raw eggs	unknown	4b
1987	California	11(0)	Butter	unknown	unknown

1987-1989	England	355(94)	Pâté and meat spreads	52	4b
1989	Connecticut	10 (0)	(Shrimp)	33	4b
1989-1990	Denmark	23(0)	Semi-soft cheese (blue)	unknown	4b
not spec.	Texas	7(0)	Frozen vegetables	43	4b
1990	Australia	11(6)	Pâté and meat spreads	100	1/2a
1991	Tasmania, Australia	4(0)	Smoked mussels	0	1/2a
1992	New Zealand	4(0)	Smoked mussels	0	1/2
1992	France	279 (85)	Pork tongue in jelly	0	4b
1993	France	38(11)	Rillettes	82	4b
1993	Italy	18 (0)	Rice Salad	0	1/2b
1994	Illinois	48 (0)	Pasteurized chocolate milk	0	1/2b
1994-1995	Sweden	9(2)	Smoked Seafood	33	4b
1995	France	33(4)	Soft Ripened Cheese (>50% moisture)	45	4b
1997	France	14(0)	Pon l'Eveque cheese	unknown	4b

1998[b]	Multi-state, US from a single source	101 (21)	Hot Dogs	unknown	4b
1998-1999	Finland	25(6)	Butter	0	3a
1999	CT, MD, NY	11(0)	Pâté	18	1/2a
1999-2000	France	26(7)	Pigs tongue in aspic	unknown	unknown
2000	10 states	29(7)	Deli Turkey meat	28	unknown
2000-2001	North Carolina	12(5)	Mexican-style cheese	83	unknown
2002	8 North eastern states	63(7)	Deli turkey meat	5	unknown

[a]Compiled from Risk assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods, USDA (86).

Table 2. Fractional factorial design used for Main Study #2.

pH	A_w	# of Replicates
3.0	0.980	2
4.0	0.980	1
5.0	0.980	3
6.0	0.980	1
7.0	0.980	2
3.5	0.987	3
4.5	0.987	1
5.5	0.987	1
6.5	0.987	3
3.0	0.960	3
4.0	0.960	1
5.0	0.960	1
6.0	0.960	1
7.0	0.960	3
3.5	0.970	2
4.5	0.970	1
5.5	0.970	1
6.5	0.970	2

Table 3. *Listeria monocytogenes* strains used in the preliminary studies and Main Studies #1 and #2.

Laboratory Strain Number	Donor's Identification Number	Serovar or Poly O Type (1 or 4)[a]	Original Source
200	Brie L	4	Brie Cheese
201	F4258	4b	Clinical, CDC
202	GV6-V-S	1	Ground Veal [b]
203	F2-LPM-G	1	Flounder[b]
204	H4-VJ-G	1	Ground Beef[b]
205	Murray-B	4	Clinical, CDC
206	S9-NMB-S	1	Sausage[b]
207	ATCC 7644	1	Human Isolate
208	MF2-L-P	1	Monk Fish[b]
209	RMI	4	Bovine Tissue, FDA
210	ATCC 43256	4	Mexican Style Cheese from California
211	ATCC 51776	4b	Belgium Cheese
212	ATCC 51414	4b	Raw milk associated with an outbreak of listeriosis in

[a] Vilasini Suktankar (FDA, Atlanta, GA)

[b] (Buchanan et al.,1989)

Table 4: API numerical profiles of the 13 *Listeria monocytogenes* strains studied.

FST Strain #	Profile	API Profile ID
200	6410	*Listeria monocytogenes*
201	6510	*Listeria monocytogenes*
202	2510	*Listeria monocytogenes*
203	6450	*Listeria monocytogenes*
204	6510	*Listeria monocytogenes*
205	6450	*Listeria monocytogenes*
206	6110	*Listeria monocytogenes*
207	6450	*Listeria monocytogenes*
208	6450	*Listeria monocytogenes*
209	6510	*Listeria monocytogenes*
210	6510	*Listeria monocytogenes*
211	6510	*Listeria monocytogenes*
212	6410	*Listeria monocytogenes*

Table 5: Starter culture counts and pH of strain #201 after 18 hr of incubation at 37°C [a].

Sample	Average pH	Log [cfu/ml]	
Tube	$\bar{x}$	BHIA	MVJ
with glucose and glutamine	4.32	9.05	9.12
with glucose only	4.31	9.06	9.06
with glutamine only	7.17	8.12	8.03
without glucose and glutamine	7.18	8.17	7.92

[a]The cells were then utilized in the preliminary study on the acid resistance of acid induced cells vs. non acid induced cells.

Table 6. Results of various dilutions of cultures of strain #201 inoculated into TSB with 1% glucose (+Glu) or without 1% glucose (w/o Glu) to determine when cells are in the exponential phase of growth[a].

Sample Hr	Tube A (+Glu)[b] (10^{-2} Dil'n)	Tube B (w/oGlu)[c] (10^{-2} Dil'n)	Tube C (+Glu) (10^{-3} Dil'n)	Tube D (w/o Glu) (10^{-3} Dil'n)	Tube E[a] (+Glu) (10^{-4} Dil'n)	Tube F (w/o Glu) (10^{-4} Dil'n)
0	7.38[d]	6.27	6.50	5.72	5.59	4.30
1	7.64	6.47	6.54	5.50	5.55	4.41
2	7.66	7.04	6.54	5.81	5.57	5.25
3	8.07	7.57	7.02	6.33	6.00	5.49
5	8.80	8.34	8.04	7.31	7.09	6.60
7	9.17	8.89	8.80	8.39	8.13	7.68

[a]Cells grown with glucose were transferred only to tubes containing glucose. Conversely, cells grown in the absence of glucose were transferred to tubes without glucose.
[b]strain #201 + Glu 18 hr culture count 9.01 Log (cfu/ml), Avg. pH 4.27
[c]strain #201 w/o Glu 18 hr culture count 8.25 Log (cfu/ml), Avg. pH 7.15
[d]Results counts are shown in [Log(cfu/ml)].

Table 7. Overnight and Starter Culture Counts strain #201 after 18 hr of incubation at 37°C in the presence (+Glu) or absence (w/o Glu) of glucose[a].

Sample	Log [cfu/ml]	Average pH
O/N +Glu	8.61	4.35
O/N w/o Glu	8.31	7.20
3hr +Glu	8.29	6.13
3hr w/o Glu	8.02	6.30

[a]The exponential phase cells were utilized in the preliminary study of the thermal resistance of strain #201 in heating menstrum pH 3.0 A_w 0.960.

Table 8. D Value calculations of strain #201 stationary growth phase cells in comparison to strain #201 exponential growth phase cells[a].

Strain F4258 at different stages of growth.	Y_0	T_L	M	RMS	D Value	5 D
8/11/00 with Glucose (exponential phase cells)	6.42 *	0.0*	-0.02472	0.6279	40.486	202.43
7/24/00 with Glucose[b] (stationary phase cells)	7.82	197.997	-0.02180	0.2089	46.992	432.96
3/13/00 with Glucose[b] (stationary phase cells)	7.26	158.693	-0.03315	0.3397	30.167	309.53

[a]Experiments were performed at different times. The heating menstruum was the same combination for each experiment - pH 3.0, A_w 0.960.
[b]Replicate experiments.

Table 9. The average pH and CFU/ml of strain #201 grown at 37°C for 24 hr either in the presence (+Glu) or absence (w/o Glu) of glucose.[a]

	+ Glu		w/o Glu	
Time (hr)	pH	CFU/ml	pH	CFU/ml
0	7.22±0.09	5.52±0.38	7.29±0.07	5.34±0.12
2	7.09±0.00	5.97±0.04	7.14±0.02	5.89±0.08
4	7.10±0.02	6.81±0.04	7.15±0.00	6.78±0.01
6	6.56±0.01	8.11±0.02	7.09±0.01	7.96±0.06
8	6.33±0.02	8.82±0.03	6.97±0.00	8.50±0.02
9.5	5.40±0.03	8.89±0.02	6.96±0.01	8.53±0.02
14	4.43±0.02	9.11±0.09	6.97±0.01	8.70±0.03
15	4.37±0.03	9.19±0.09	6.91±0.00	8.72±0.01
16	4.34±0.01	9.37±0.03	6.93±0.01	8.69±0.05
17	4.28±0.00	9.22±0.10	6.94±0.00	8.77±0.05
18	4.24±0.01	9.31±0.04	6.94±0.00	8.75±0.12
20	4.21±0.01	9.42±0.18	6.93±0.00	8.72±0.08
22	4.18±0.00	9.41±0.08	6.95±0.03	9.00±0.06
23.5	4.14±0.03	9.33±0.05	7.00±0.01	8.71±0.16
23.75	4.14±0.01	9.41±0.00	6.96±0.00	9.09±0.00

[a]See Appendix Tables 18a and 18b for original data.

Table 10(a). The thermal resistance of 13 strains of *L. monocytogenes* grown under acidic (+ Glu) and non-acidic (w/o Glu) conditions and then heated at 58°C in Main Study #1 heating menstruum pH 3.0 A_w 0.987[a].

Strain Number	Acidogenic (+Glu)			Non-Acidogenic (w/o Glu)		
	D[b]	t lag[c]	t_{4D}[d]	D	t lag	t_{4D}
200	20.49	0.00	81.97	24.53	0.00	**98.12**[e]
201	28.23	0.00	112.92	45.30	20.80	**202.00**
202	44.90	30.81	**210.41**	40.52	0.00	162.08
203	41.58	0.00	**166.32**	23.45	15.70	109.50
204	26.32	0.00	105.28	22.74	36.74	**127.70**
205	76.34	108.66	**414.02**	62.11	0.00	248.44
206	28.30	0.00	113.20	31.75	0.00	**126.98**
207	12.66	0.00	**50.64**	11.86	0.00	47.44
208	87.80	0.00	**351.20**	38.79	0.00	155.16
209	79.62	103.87	422.35	178.80	13.80	**729.00**
210	45.14	0.00	**180.56**	44.19	0.00	176.76
211	64.81	0.00	**259.24**	31.94	14.91	142.67
212	40.67	42.07	**204.75**	46.88	0.00	187.52

[a]+ Glu mean 205.6, SD 123.6, and coefficient of variation (CV) 60.1%
w/o Glu mean 193.3, SD 168.7, and CV 87.3%

[b]D Value- time required for a 1 log reduction of a microorganism at a given temperature.

[c]T_{lag}- the duration of the shoulder of the thermal inactivation curve

[d]T_{4D}- The heat process at a given temperature to reduce the population by 10^-4, equivalent to 4 times the D value + the lag time if present.

[e]Bold = more thermal resistant cells.

Table 10(b) The thermal resistance of 13 strains of *L. monocytogenes* grown under acidic (+ Glu) and non-acidic (w/o Glu) conditions and then heated at 58°C in Main Study #1 heating menstruum pH 7.0 A_w 0.960[a].

Strain Number	Acidogenic (+Glu)			Non-Acidogenic (w/o Glu)		
	D[b]	t lag[c]	t_{4D}[d]	D	t lag	t_{4D}
200	389.11	0.00	**1556.40**[e]	181.82	0.00	727.28
201	328.69	46.94	**1361.70**	333.33	20.08	1353.40
202	431.03	0.00	**1725.12**	211.86	0.00	847.44
203	256.62	0.00	1026.48	236.02	107.49	**1051.57**
204	427.90	0.00	1711.60	455.27	15.78	**1836.86**
205	162.60	20.33	670.73	224.37	11.31	**908.78**
206	392.00	0.00	**1568.00**	201.14	119.54	924.10
207	529.35	0.00	2117.40	619.58	0.00	**2478.32**
208	295.12	307.24	**1487.72**	283.61	0.00	1134.44
209	71.35	19.76	305.16	124.71	0.00	**498.84**
210	364.17	45.29	**1501.97**	77.88	141.18	452.70
211	380.23	0.00	1520.92	384.62	0.00	**1538.48**
212	118.06	277.84	750.08	438.60	0.00	**1754.39**

[a]+ Glu mean 1331, SD 502.9, and coefficient of variation (CV) 37.8%
w/o Glu mean 1192.8, SD 582.3, and CV 48.8%

[b]D Value- time required for a 1 log reduction of a microorganism at a given temperature.

[c]T_{lag}- the duration of the shoulder of the thermal inactivation curve

[d]T_{4D}- The heat process at a given temperature to reduce the population by 10^-4, equivalent to 4 times the D value + the lag time if present.

[e]Bold = more thermal resistant cells.

Table 11. Thermal analysis of *L. monocytogenes* strain #201 grown in the presence of 1% glucose for 18 hr at 37°C and then tested in various heating menstrua.

Thermal menstruum[a]		Agar medium	Time Lag (TL)	D value (sec) ± SD	4D value (sec) ± SD
A_w 0.960	pH 3.0	BHIA	178.35	36.75 ± 6.89	325.32 ± 69.33
		MVJ	50.42	34.14 ± 3.90	186.96 ± 32.33
	pH 4.0	BHIA	134.54	104.27 ± 10.02	551.61 ± 32.71
		MVJ	18.29	65.07 ± 4.60	278.56 ± 0.13
	pH 5.0	BHIA	225.29	303.49 ± 38.06	1439.25 ± 147.99
		MVJ	23.71	226.43 ± 47.12	929.44 ± 163.06
	pH 6.0	BHIA	0.00	1554.73 ± 179.6	6218.91 ± 718.37
		MVJ	0.00	787.74 ± 37.85	3150.97 ± 151.40
	pH 7.0	BHIA	465.05	868.23 ± 361.70	3937.97 ± 868.35
		MVJ	0.00	553.87 ± 80.51	2215.49 ± 322.05
A_w 0.970	pH 3.5	BHIA	83.83	58.26 ± 26.02	316.87 ± 6.81
		MVJ	0.00	51.78 ± 2.07	207.11 ± 8.29
	pH 4.5	BHIA	251.13	104.54	669.30
		MVJ	39.67	84.67	378.35
	pH 5.5	BHIA	499.71	361.06 ± 107.41	1943.96 ± 257.49
		MVJ	91.83	370.20 ± 96.19	1572.62 ± 293.79
	pH 6.5	BHIA	0.00	812.98 ± 205.88	3251.90 ± 823.53
		MVJ	24.68	353.44 ± 60.39	1438.46 ± 241.55
	pH 7.5	BHIA	0.00	624.68	2498.72
		MVJ	NA	NA	NA

(cont'd)

[a]0.9 ml of the culture was added to 15 ml of the thermal menstruum.

Table 11 (cont'd). Thermal analysis of *L. monocytogenes* strain #201 grown in the presence of 1% glucose for 18 hr at 37°C and then tested in various heating menstrua.

Thermal menstruum[a]		Agar medium	Time Lag (TL)	D value (sec) ± SD	4D value (sec) ± SD
A_w 0.980	pH 3.0	BHIA	123.25	46.59 ± 3.27	309.59 ± 3.42
		MVJ	14.13	29.03 ± 6.96	130.25 ± 21.75
	pH 4.0	BHIA	89.93	93.24	462.87
		MVJ	0.00	67.19	268.74
	pH 5.0	BHIA	153.19	276.16 ± 46.94	1257.83 ± 286.33
		MVJ	0.00	183.67 ± 34.57	734.68 ± 138.29
	pH 6.0	BHIA	287.12	1086.96 ± 122.75	4347.83 ± 491.00
		MVJ	0.00	286.92	1147.67
	pH 7.0	BHIA	451.58	489.24 ± 78.12	2408.52 ± 621.11
		MVJ	0.00	252.79 ± 69.61	1011.16 ± 278.43
A_w 0.987	pH 3.5	BHIA	198.04	50.23 ± 13.05	398.96 ± 75.12
		MVJ	11.49	35.91 ± 12.35	155.13 ± 33.13
	pH 4.5	BHIA	178.95	109.86 ± 15.04	618.38 ± 100.71
		MVJ	51.78	50.48 ± 16.01	253.68 ± 37.69
	pH 5.5	BHIA	237.97	261.67 ± 21.68	1284.65 ± 361.58
		MVJ	71.52	117.91 ± 0.86	543.16 ± 97.70
	pH 6.5	BHIA	508.67	323.22 ± 89.89	1801.55 ± 160.07
		MVJ	18.83	203.96 ± 97.39	834.66 ± 371.44
	pH 7.5	BHIA	326.53	612.03	2774.63
		MVJ	NA	NA	NA

[a]0.9 ml of the culture was added to 15 ml of the thermal menstruum.

Table 12. Thermal analysis of *L. monocytogenes* strain #201 grown in the absence of 1% glucose for 18hr at 37°C and then tested in various heating menstrua.

Thermal menstruum[a]		Agar medium	Time Lag (TL)	D value (sec) ± SD	4D value (sec) ± SD
A_w 0.960	pH 3.0	BHIA	9.41	47.27 ± 6.12	198.48 ± 35.85
		MVJ	0.00	30.98 ± 6.27	123.91 ± 25.10
	pH 4.0	BHIA	100.24	112.69 ± 20.54	551.00 ± 7.67
		MVJ	0.00	84.26 ± 13.17	337.04 ± 52.70
	pH 5.0	BHIA	0.00	763.65 ± 28.20	3054.60 ± 112.80
		MVJ	67.50	180.02 ± 45.83	787.57 ± 250.84
	pH 6.0	BHIA	0.00	3061.85±1223.76	12247.4±7895.05
		MVJ	0.00	670.47 ± 72.16	2681.86 ± 288.64
	pH 7.0	BHIA	0.00	1758.71 ± 416.79	7034.82±1667.15
		MVJ	0.00	632.26 ± 40.61	2529.03 ± 162.44
A_w 0.970	pH 3.5	BHIA	14.64	49.70 ± 8.56	213.43 ± 57.22
		MVJ	0.00	33.04 ± 14.27	132.15 ± 57.18
	pH 4.5	BHIA	231.14	111.56 ± 10.28	677.40 ± 318.16
		MVJ	0.00	65.49 ± 46.48	261.98 ± 184.38
	pH 5.5	BHIA	0.00	1731.60 ± 471.20	6926.41±1884.78
		MVJ	0.00	594.35 ± 268.56	2377.41±1074.26
	pH 6.5	BHIA	0.00	2246.18 ± 733.29	8984.73±2933.18
		MVJ	0.00	598.53 ± 5.04	2394.13 ± 20.14
	pH 7.5	BHIA	0.00	1001.60	4006.41
		MVJ	NA	NA	NA

(cont'd)

[a]0.9 ml of the culture was added to 15 ml of the thermal menstruum.

Table 12 (cont'd). Thermal analysis of *L. monocytogenes* strain #201 grown in the absence of 1% glucose for 18hr at 37°C and then tested in various heating menstrua.

Thermal menstruum[a]		Agar medium	Time Lag (TL)	D value (sec) ± SD	4D value (sec) ± SD
A_w 0.980	pH 3.0	BHIA	10.823	38.21 ± 15.014	163.65 ± 71.91
		MVJ	0.00	19.73 ± 11.78	78.90 ± 47.10
	pH 4.0	BHIA	149.26	237.61	1099.69
		MVJ	3.835	88.50	357.82
	pH 5.0	BHIA	0.00	665.34 ± 171.45	2661.34 ± 658.84
		MVJ	1.94	256.33 ± 55.69	1027.25 ± 219.43
	pH 6.0	BHIA	0.00	1385.04 ± 179.60	5540.17 ± 718.39
		MVJ	0.00	274.37	1097.46
	pH 7.0	BHIA	0.00	1124.35 ± 42.70	4497.41 ± 170.82
		MVJ	0.00	402.28	1609.13
A_w 0.987	pH 3.5	BHIA	4.22	64.66 ± 3.84	262.86 ± 10.42
		MVJ	0.00	36.63 ± 2.08	146.51 ± 8.31
	pH 4.5	BHIA	214.26	162.98 ± 14.34	866.18 ± 72.04
		MVJ	0.00	78.08 ± 17.19	312.31 ± 68.76
	pH 5.5	BHIA	0.00	920.56 ± 236.75	3682.22 ± 947.01
		MVJ	0.00	301.08 ± 13.86	1204.33 ± 762.53
	pH 6.5	BHIA	0.00	992.06 ± 482.66	3968.25±1930.64
		MVJ	0.00	371.06 ± 118.17	1484.23 ± 472.66
	pH 7.5	BHIA	0.00	1021.03	4084.13
		MVJ	NA	NA	NA

[a]0.9 ml of the culture was added to 15 ml of the thermal menstruum.

VIII. Figures

Figure 1. Photograph of heating coil apparatus used in all thermal studies.

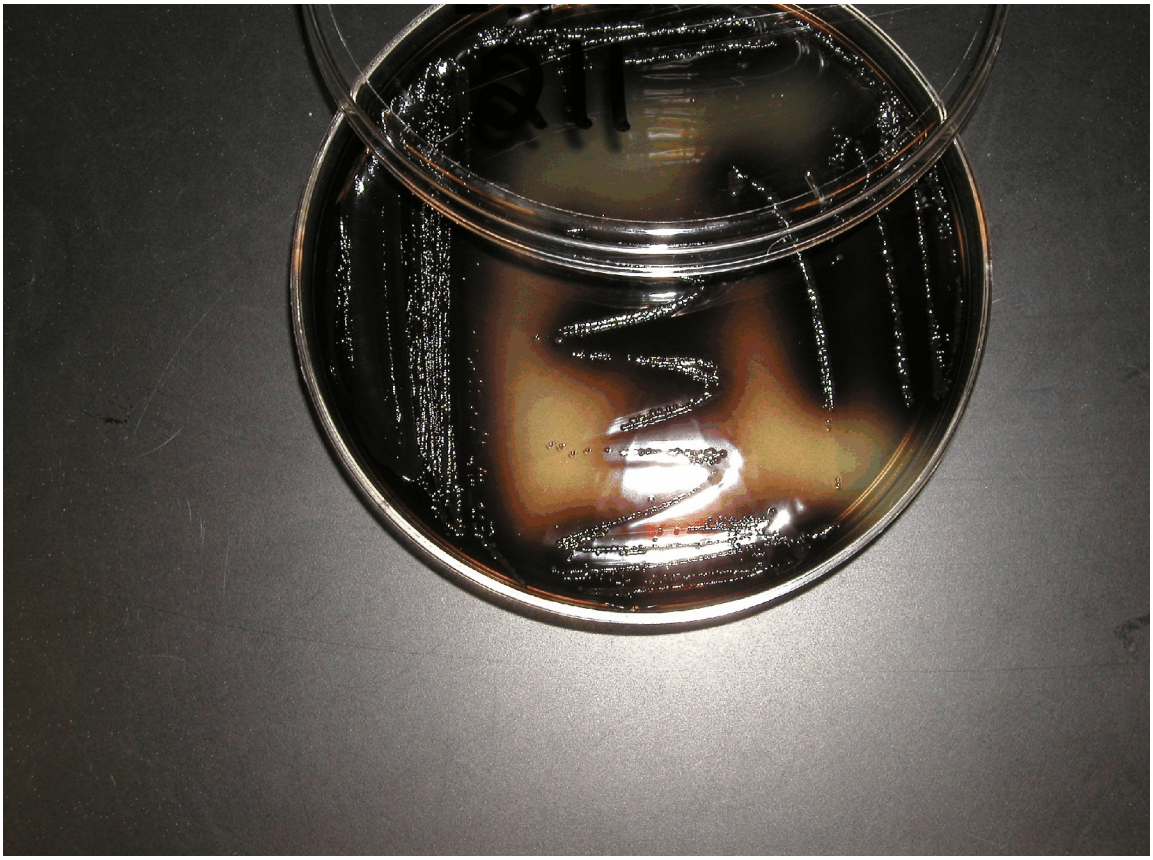


Figure 2. Growth of *Listeria monocytogenes* strain #201 on Oxoid agar

Figure 3. Photograph of Spiral Plater (Spiral Biotech, Bethesda, MD)

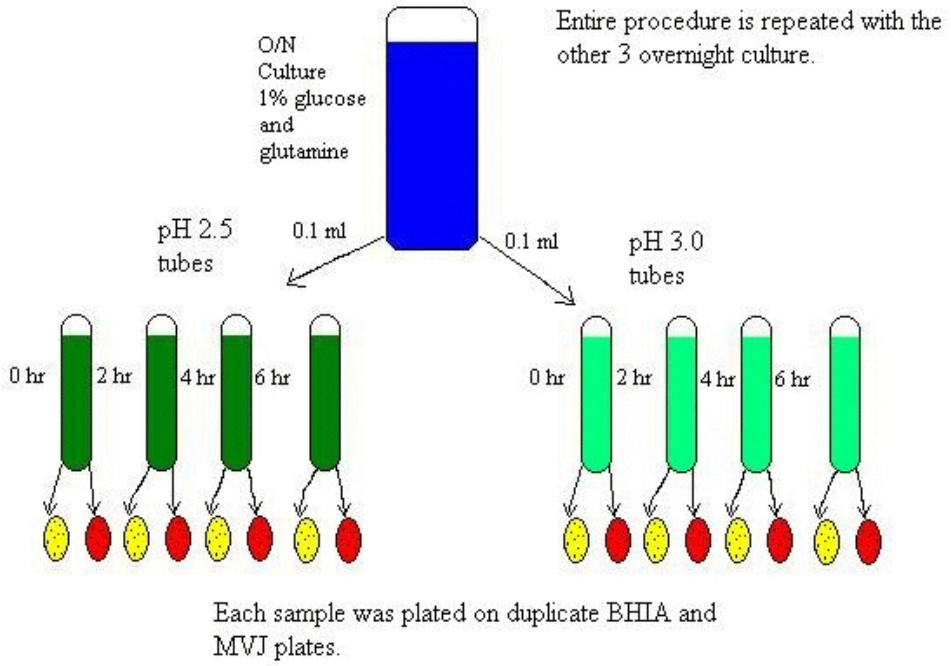


Figure 4. Diagram of preliminary study #1 acid resistance of induced cells vs non-induced cells of strain #201

Figure 5. Photograph of Spiral Counter (Spiral Biotech, Bethesda, MD)

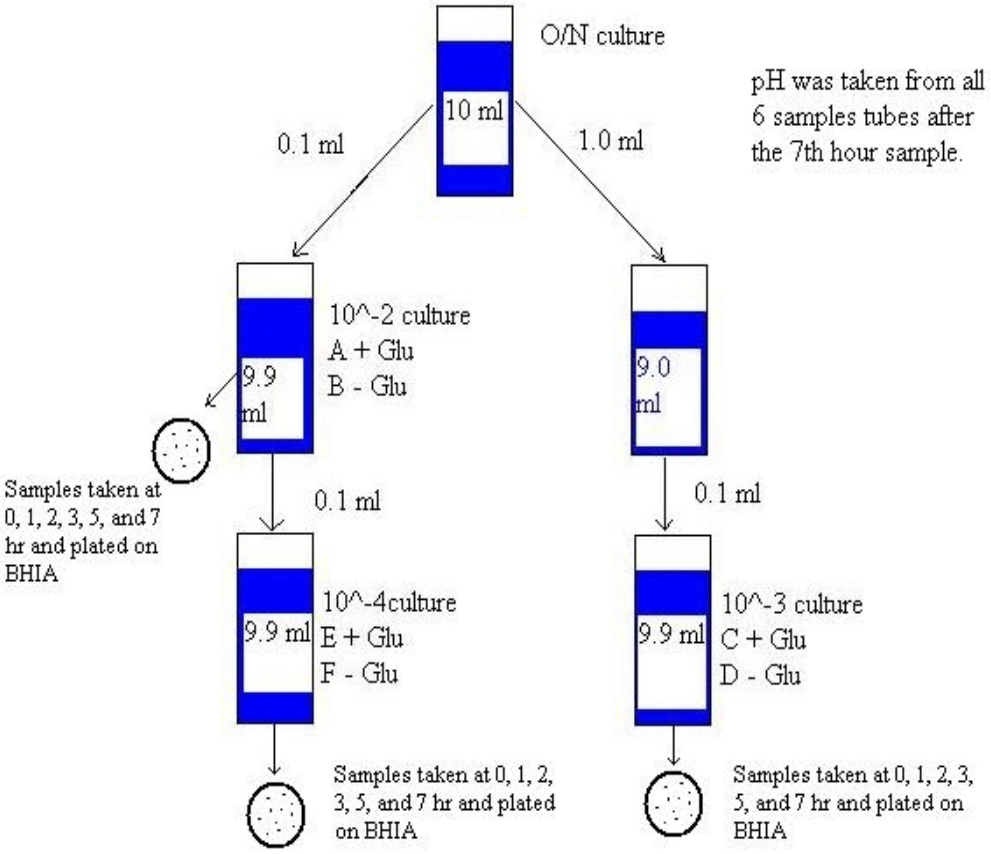


Figure 6. Diagram of preliminary study #2 procedure for *L. monocytogenes* growth phase.

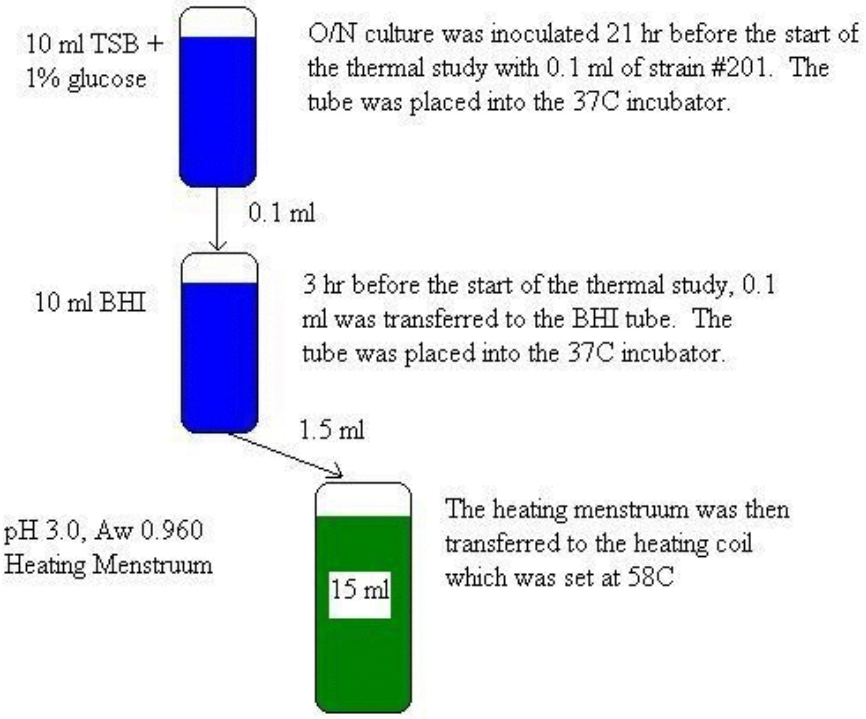


Figure 7. Diagram of procedure Preliminary #3 thermal study using log phase cells #201 in heating menstruum pH3, Aw 0.960

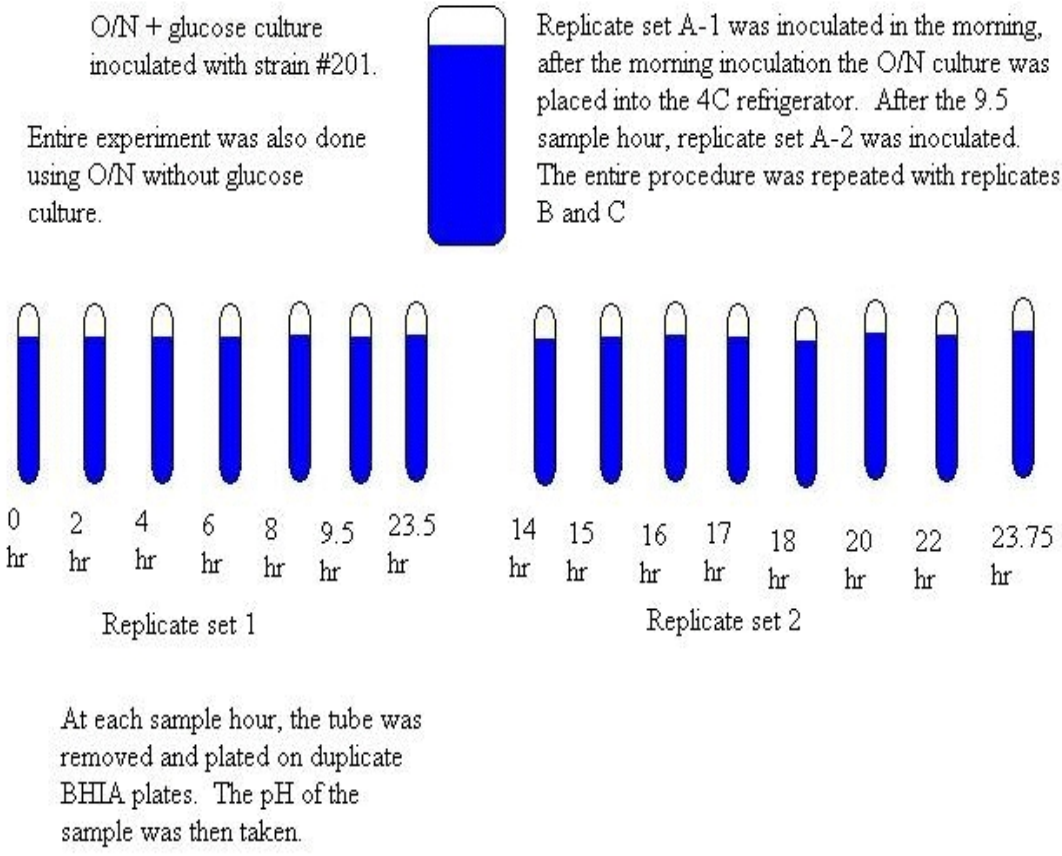


Figure 8. Diagram of procedure Preliminary #4 pH decline of stationary phase cells grown with and without glucose.

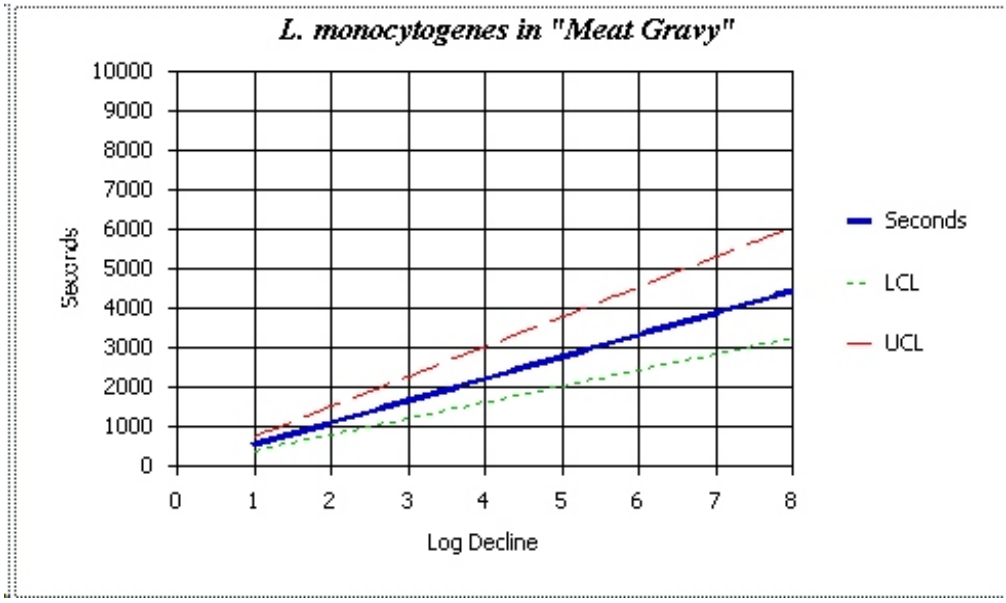


Figure 9. Thermal decline model showing the time required for *L. monocytogenes* to decline in "meat gravy" at a pH of 6.0 and an A_w of 0.970. (Pathogen Modeling Program, 2001.) LCL- lower confidence limit. UCL- upper confidence limit.

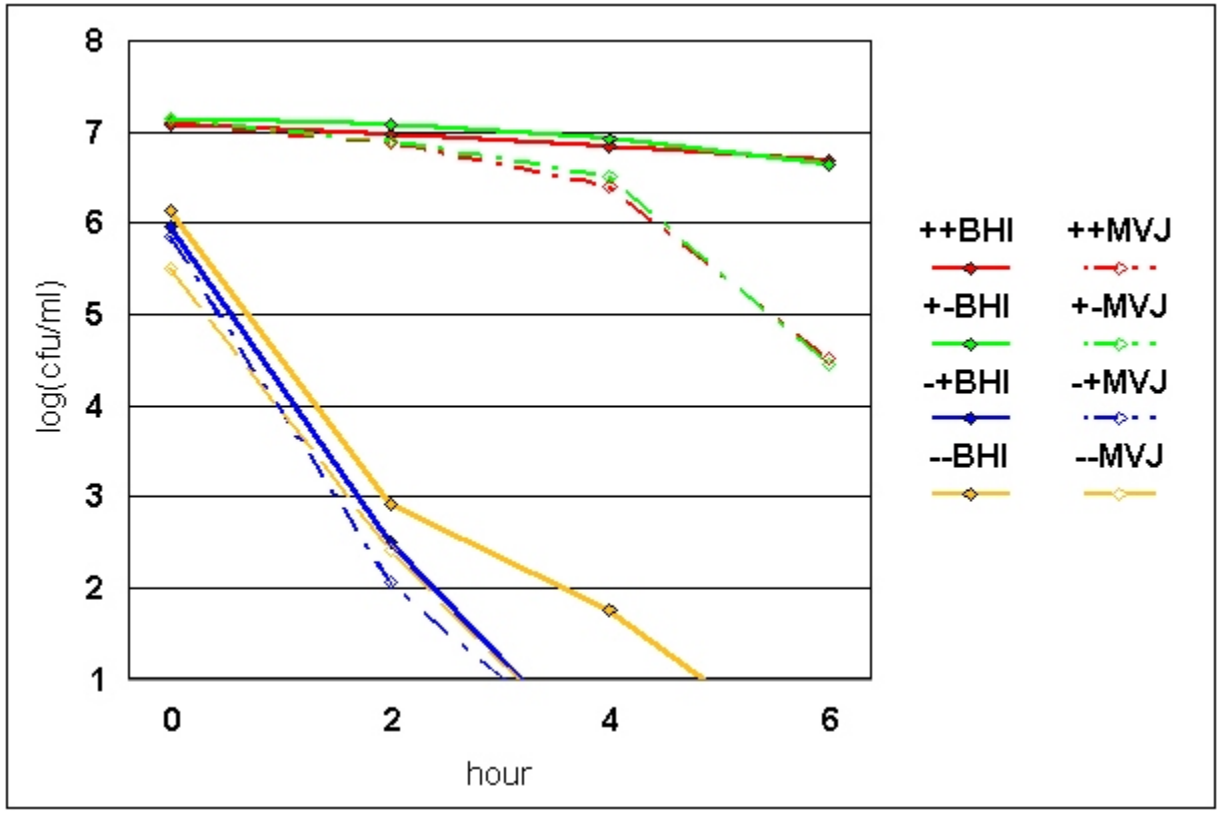


Figure 10. Acid exposure decline curve of strain 201 grown in TSB either with or without glucose or glutamine as a carbon source and then exposed to TSB adjusted to pH 2.5 using HCl for 0-6 hr. (++, glucose + glutamine; +-, glucose only; -+ glutamine only; --, absence of glucose + glutamine).

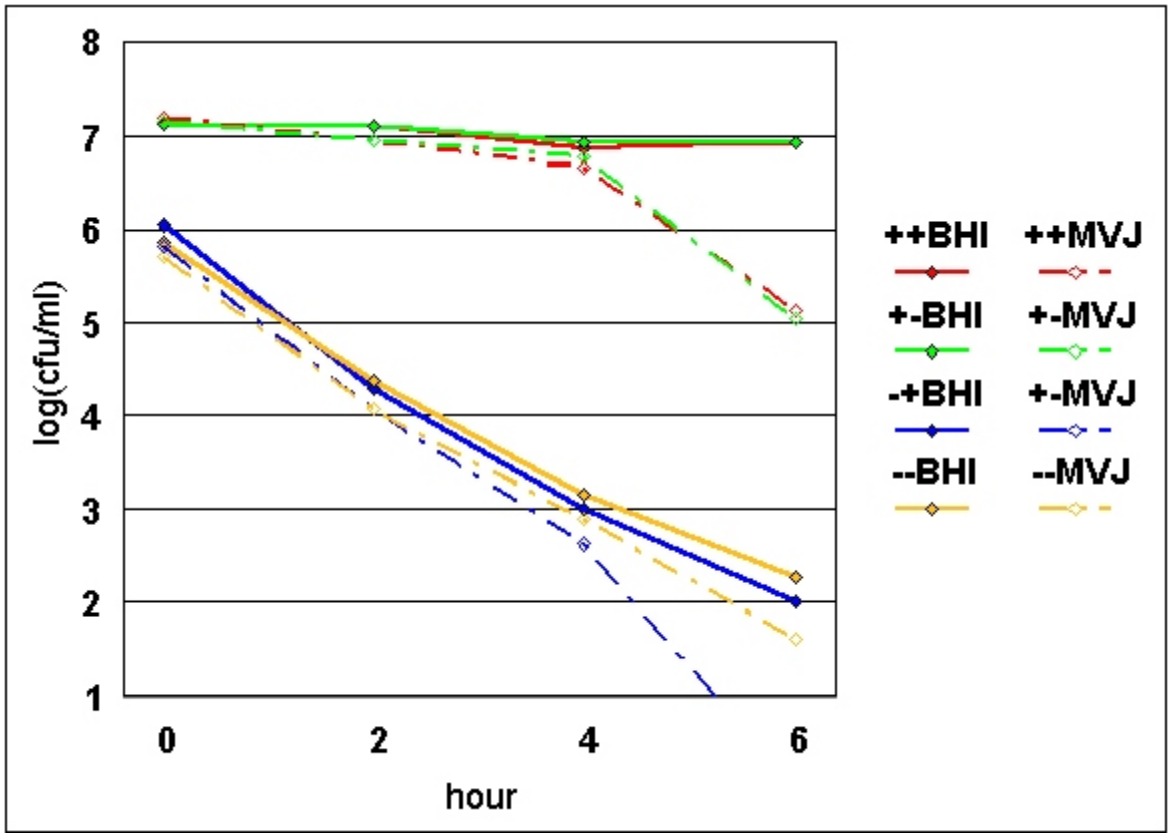


Figure 11. Acid exposure decline curve of strain 201 grown in TSB either with or without glucose or glutamine as a carbon source and then exposed to TSB adjusted to pH 3.0 using HCl for 0-6 hr. (++, glucose + glutamine; +-, glucose only; -+ glutamine only; --, absence of glucose + glutamine).

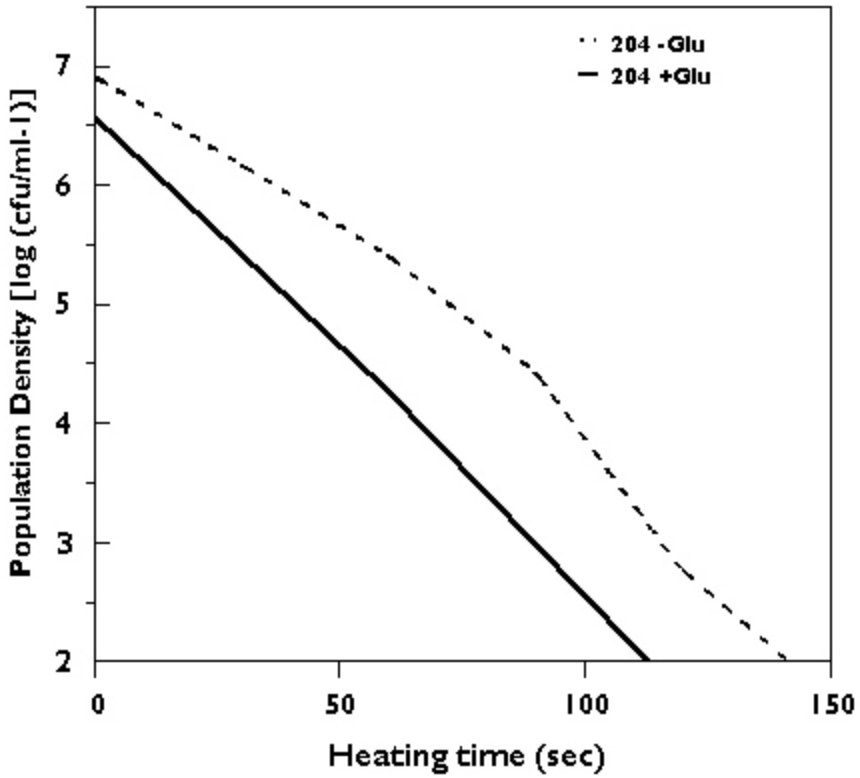


Figure 12. Thermal Decline Curve of strain #204 grown either in the presence or absence of glucose (Glu) when placed in the heating menstruum at pH 3.0, A_w 0.987, 58°C.

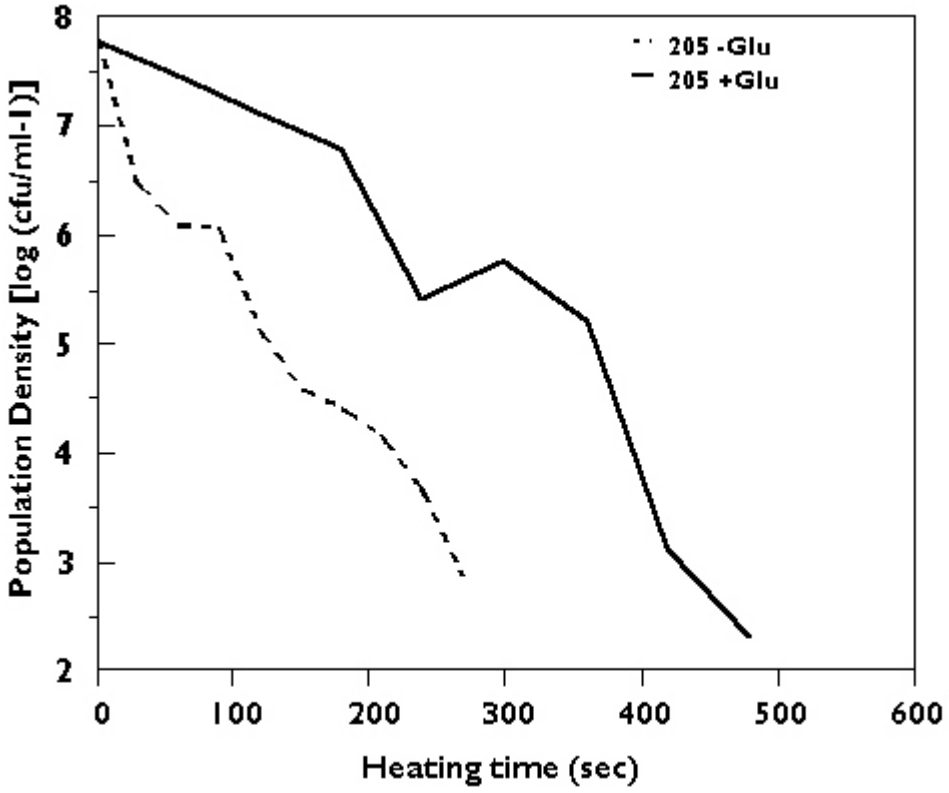


Figure 13. Thermal Decline Curve of strain #205 grown either in the presence or absence of glucose (Glu) when placed in the heating menstruum at pH 3.0, A_w 0.987, 58°C.

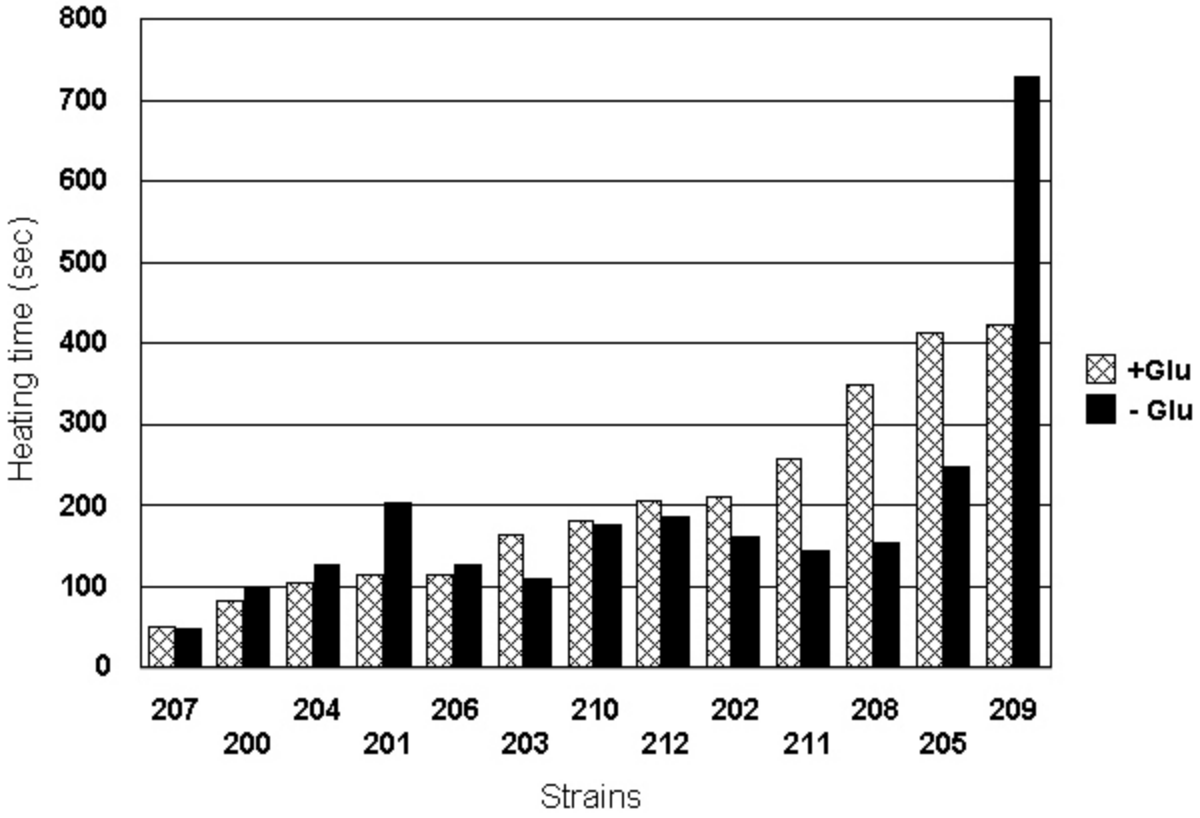


Figure 14. Comparison of the 4D Values of the strains in the heating menstruum pH 3.0, A_w 0.987, 58°C.

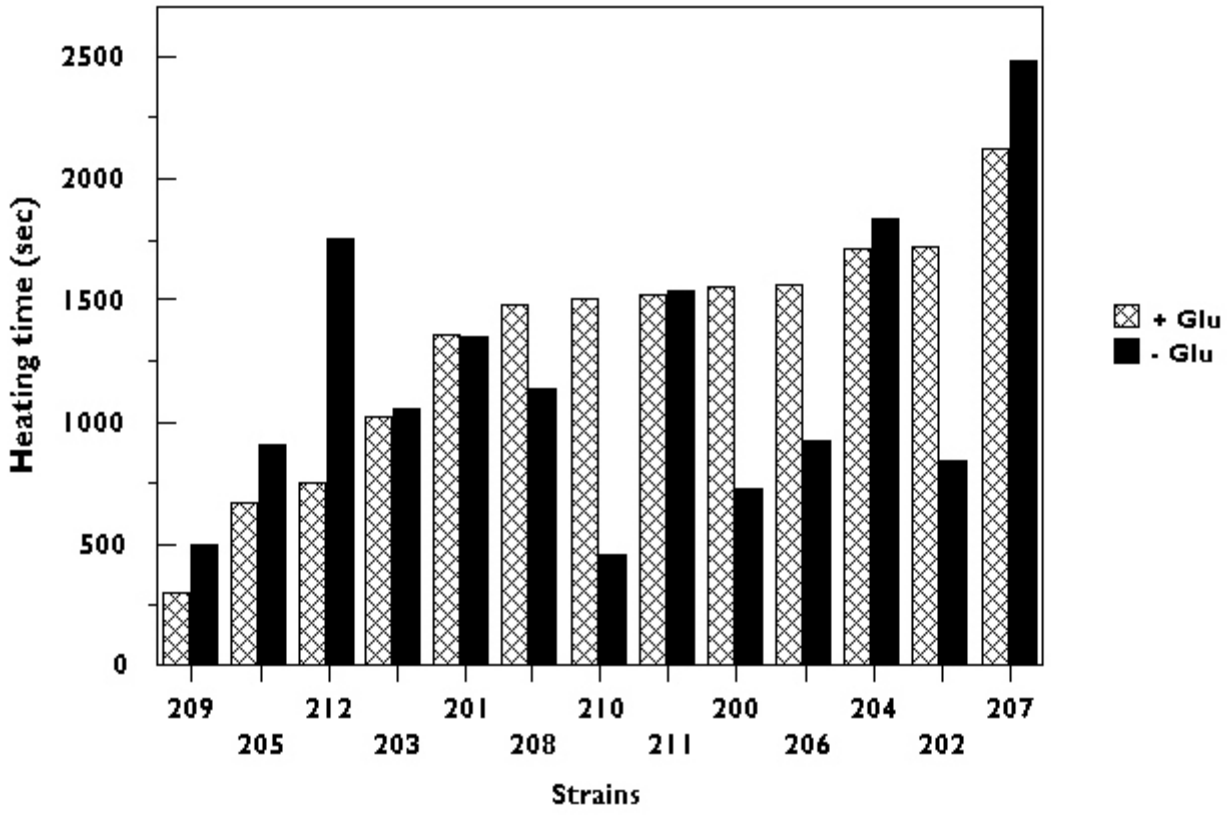


Figure 15. Comparison of the 4D Values of the strains in the heating menstruum pH 7.0, A_w 0.960, 58°C.

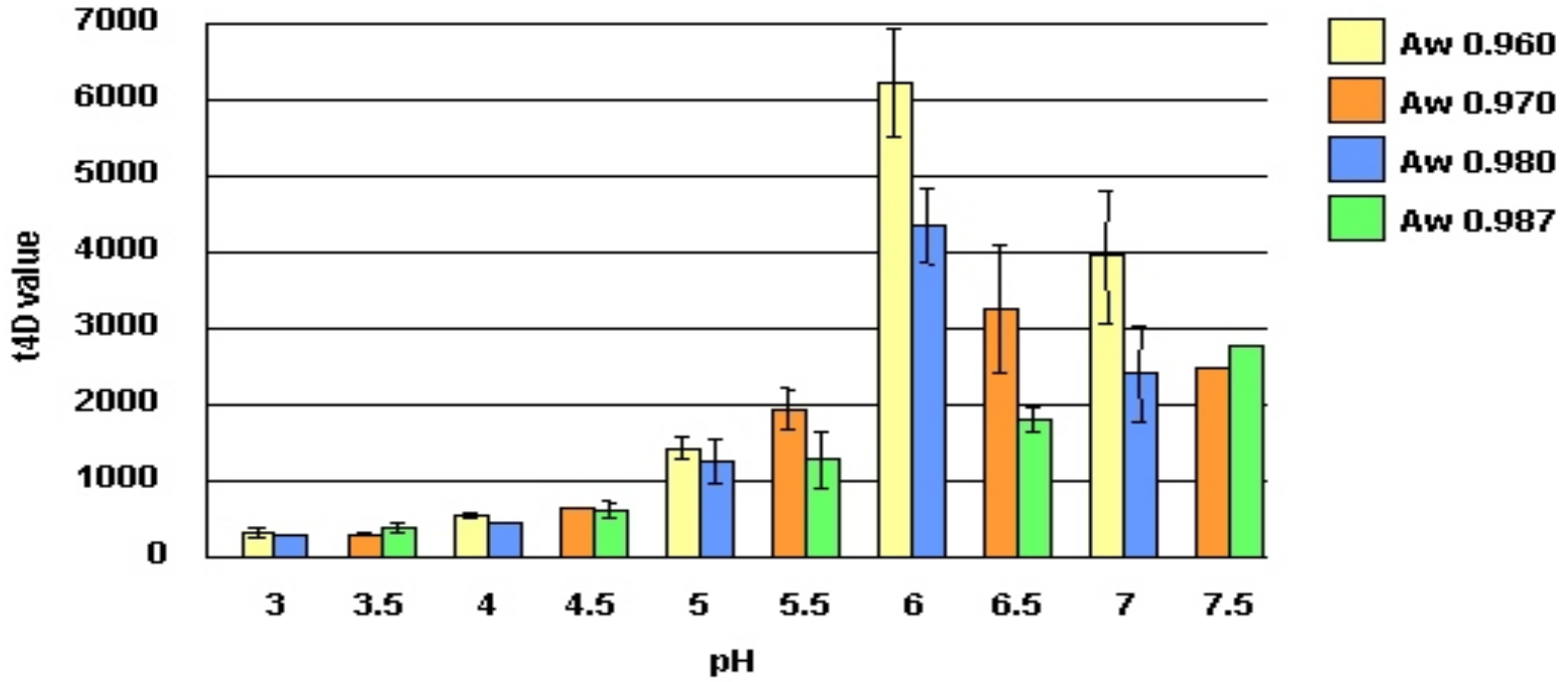


Figure 16. Comparison of the T_{4D} values of strain 201 grown in the presence of glucose and then exposed to 58°C in BHI in various combinations of pH and Aw heating menstrua.

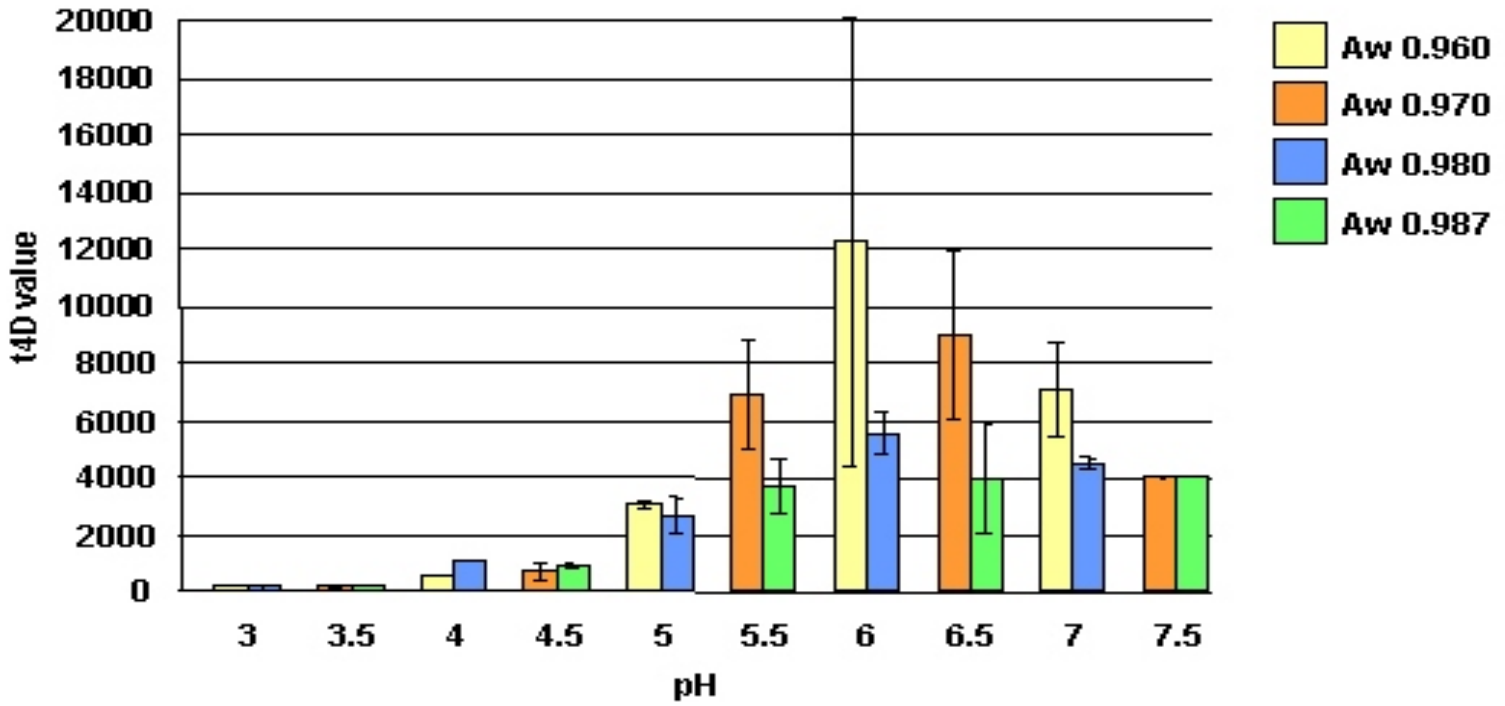


Figure 17. Comparison of the T_{4D} values of strain 201 grown in the absence of glucose and then exposed to 58°C in BHI in various combinations of pH and Aw heating menstrua.

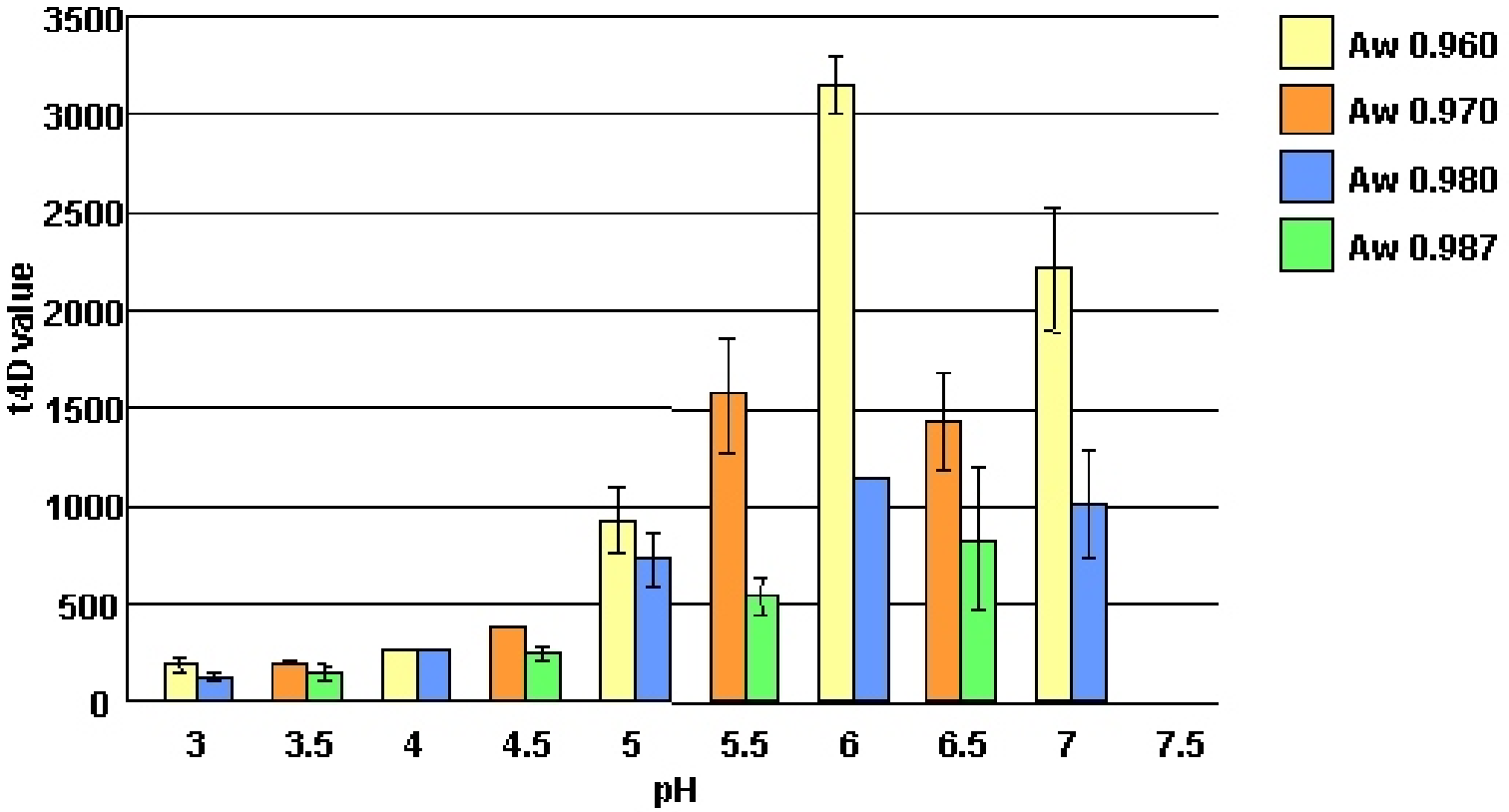


Figure 18. Comparison of the T_{4D} values of strain #201 grown in the presence of glucose and then exposed to 58°C in MVJ in various combinations of pH and Aw heating menstrua.

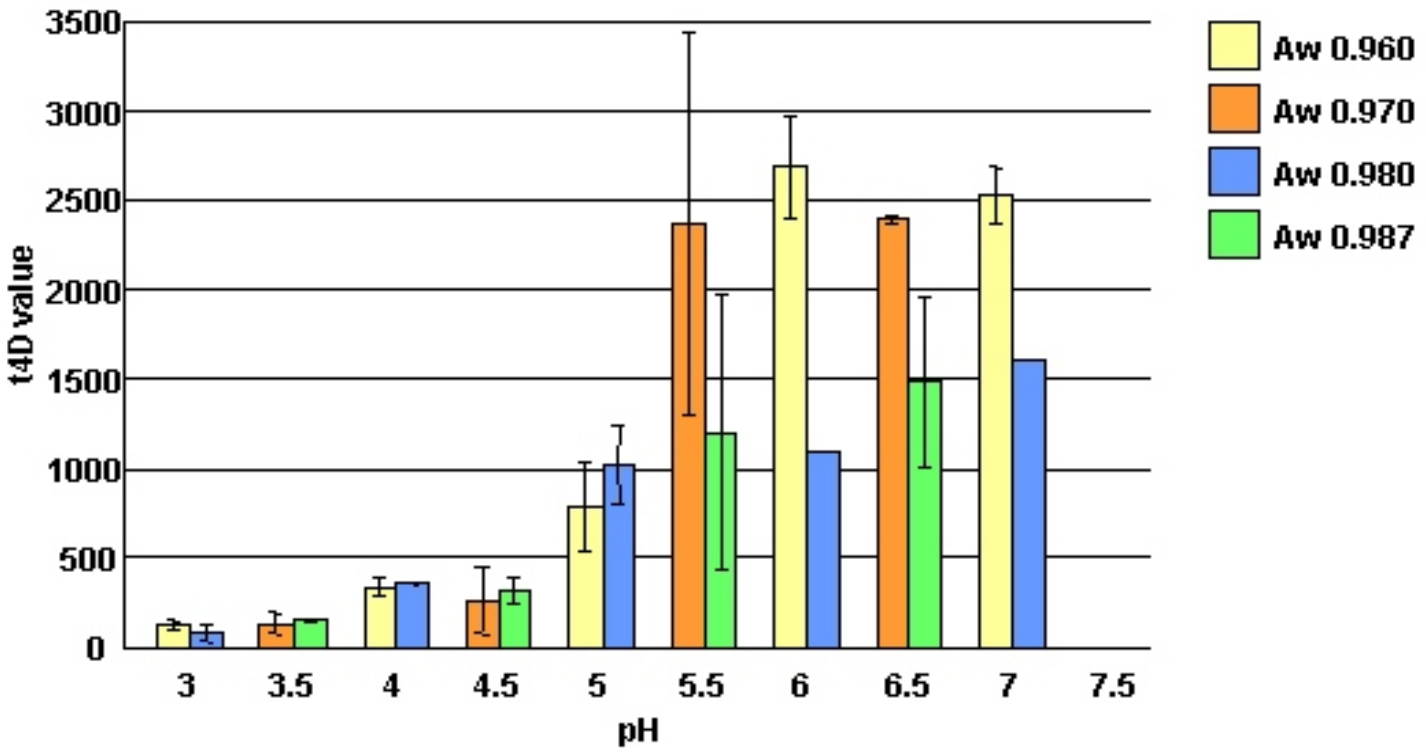


Figure 19. Comparison of the T4D values of strain #201 grown in the absence of glucose and then exposed to 58°C in MVJ in various combinations of pH and Aw heating menstrua.

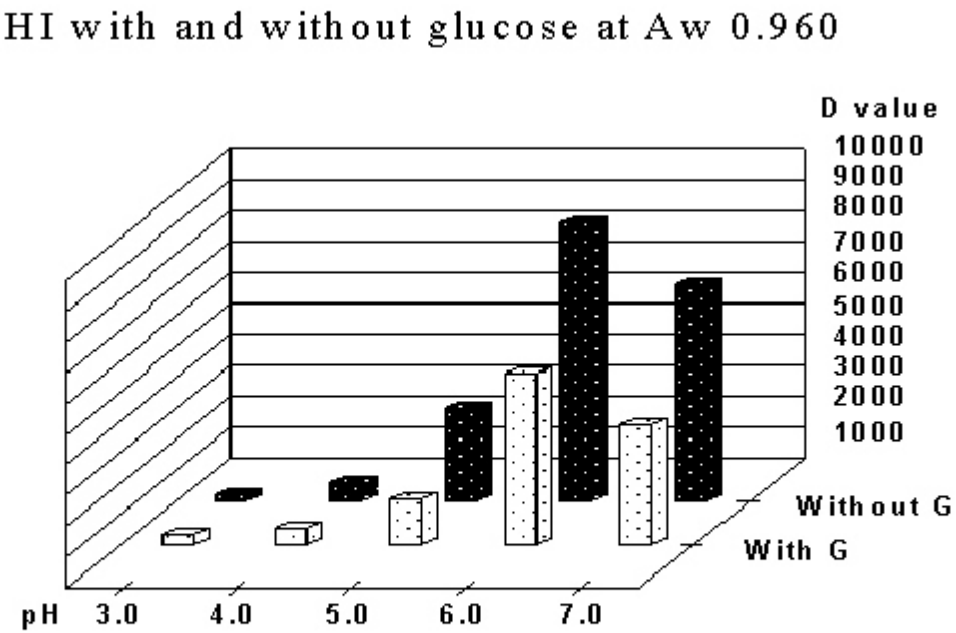


Figure 20. Comparison of the T4D values of strain 201 grown in the presence of glucose (+Glu) and added to various heating menstrua [pH (3.0 - 7.0) and A_w (0.960)] then exposed to 58°C in BHI with and without glucose (-Glu).

IX. Appendix

Appendix A

Identification by Listeria API Test

strains	DIM[a]	ESC	αMAN	DARL	XYL	RHA	MDG	RIB	G1P	TAG
#200	-	+	+	-	-	+	+	-	-	-
#201	-	+	+	+	-	+	+	-	-	-
#202	-	+	-	+	-	+	+	-	-	-
#203	-	+	+	-	-	+	+	-	+	-
#204	-	+	+	+	-	+	+	-	-	-
#205	-	+	+	-	-	+	+	-	+	-
#206	-	+	+	+	-	-	+	-	-	-
#207	-	+	+	-	-	+	+	-	+	-
#208	-	+	+	-	-	+	+	-	+	-
#209	-	+	+	+	-	+	+	-	-	-
#210	-	+	+	+	-	+	+	-	-	-
#211	-	+	+	+	-	+	+	-	-	-
#212	-	+	+	-	-	+	+	-	-	-

[a] DIM, Differentiation of *L. Innocue* and *L. monocytogenes*; ESC, esculin hydrolysis; αMAN, α-mannosidase; DARL, D-arabitol; XYL, D-xylose; RHA, L-rhamnose; MDG, methyl-αD-glucopyranoside; RIB, D-ribose; G1P, glucose-1-phosphate; TAG, D-tagatose.

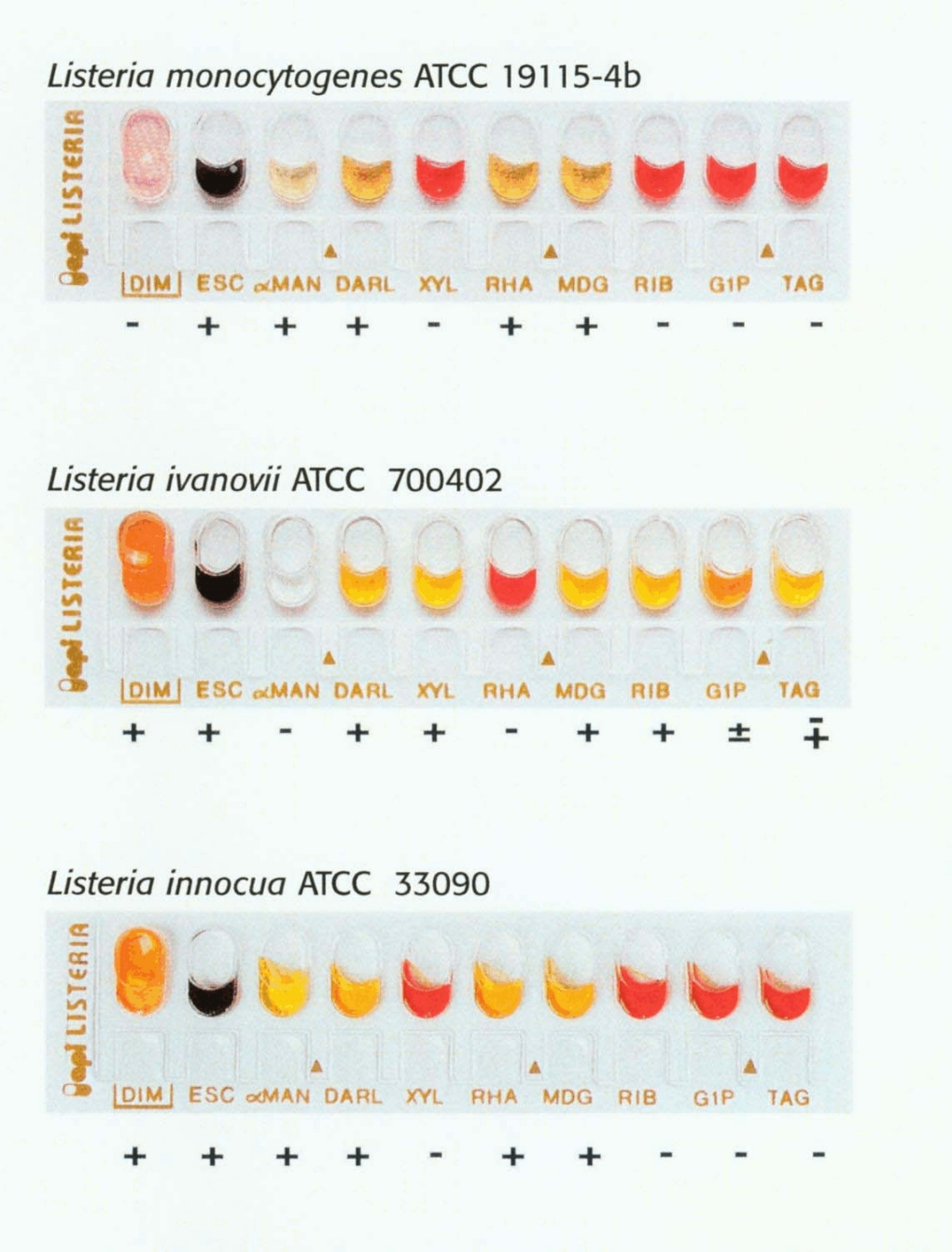
Appendix B

API numerical profiles of the thirteen *Listeria monocytogenes* strain utilized.

Strain #	Profile	API Profile ID
200	6410	*Listeria monocytogenes*
201	6510	*Listeria monocytogenes*
202	2510	*Listeria monocytogenes*
203	6450	*Listeria monocytogenes*
204	6510	*Listeria monocytogenes*
205	6450	*Listeria monocytogenes*
206	6110	*Listeria monocytogenes*
207	6450	*Listeria monocytogenes*
208	6450	*Listeria monocytogenes*
209	6510	*Listeria monocytogenes*
210	6510	*Listeria monocytogenes*
211	6510	*Listeria monocytogenes*
212	6410	*Listeria monocytogenes*

Appendix C

Photographs of the API Listeria test results of ATCC strains showing positive and negative reactions[a].



[a]Adapted from photographs provided by bioMérieux.

Appendix D

Standard plate counts of strain #201 grown in TSB with either glucose or glutamate as a carbon source with subsequent exposure to TSB adjusted to pH 2.5 using HCl.

Sample	(gluc & glut)[a]		(gluc only)		(glut only)		( ) neither	
Hr	BHIA	MVJ	BHIA	MVJ	BHIA	MVJ	BHIA	MVJ
0	7.10	7.12	7.16	7.17	5.97	5.85	6.15	5.50
2	6.98	6.89	7.09	6.90	2.51	2.08	2.93	2.43
4	6.84	6.41	6.93	6.51	<1.00	<1.00	1.78	<1.00
6	6.69	4.54	6.65	4.46	<1.00	<1.00	<1.00	<1.00

[a.] All sample were plated on both brain heart infusion agar (BHIA) and modified vogel johnson agar (MVJ)

Appendix E

Standard plate counts of strain #201 grown in TSB with either glucose or glutamate as a carbon source with subsequent exposure to TSB adjusted to pH 3.0 using HCl .

Sample	(gluc & glut)[a]		(gluc only)		(glut only)		( ) neither	
Hr	BHIA	MVJ	BHIA	MVJ	BHIA	MVJ	BHIA	MVJ
0	7.13	7.19	7.12	7.14	6.03	5.82	5.86	5.71
2	7.10	6.94	7.10	6.94	4.29	4.06	4.37	4.07
4	6.87	6.67	6.92	6.76	3.00	2.62	3.15	2.89
6	6.92	5.13	6.93	5.04	2.00	<1.00	2.28	1.60

[a.] All sample were plated on both brain heart infusion agar (BHIA) and modified vogel johnson agar (MVJ)

Appendix F

Thermal decline of strain #201 cells in logarithmic growth phase at 58°C followed by exposure to a heating menstruum of pH 3.0, A_w 0.960.

Sample	BHIA
Time (sec)	Log [cfu/ml]
0	6.42
16	5.15
32	4.60
48	4.25
64	3.88
80	3.85
96	3.54
112	3.51
128	3.08
144	3.08
160	2.85
176	2.48
192	2.30

Appendix G

Preliminary Study #4 growth of strain #201 and pH decline, Set A - early growth period.

	Rep A				Rep B				Rep C			
	+ gluc		w/o gluc		+ gluc		w/o gluc		+ gluc		w/o gluc	
Hour	pH	ct	pH	ct	pH	ct	pH	ct	pH	ct	pH	ct
0	7.14	5.42	7.28	5.17	7.25	5.17	7.35	5.36	7.32	5.38	7.40	5.25
2	7.09	5.97	7.11	5.77	7.09	6.01	7.14	5.93	7.10	5.92	7.17	5.96
4	7.08	6.76	7.15	6.78	7.11	6.84	7.16	6.77	7.12	6.84	7.15	6.80
6	6.55	8.11	7.07	7.87	6.57	8.14	7.10	8.00	6.57	8.09	7.10	8.01
8	6.36	8.78	6.97	8.47	6.32	8.82	6.97	8.52	6.32	8.85	6.97	8.51
9.5	5.38	8.91	6.95	8.50	5.38	8.86	6.96	8.55	5.45	8.91	6.97	8.55
23.5	4.11	9.36	6.99	8.54	4.12	9.25	7.00	8.92	4.18	9.37	7.01	8.66

Preliminary Study #4 growth of strain #201 and pH decline, Set B - late growth period.

	Rep D				Rep E				Rep F			
	+ gluc		w/o gluc		+ gluc		w/o gluc		+ gluc		w/o gluc	
Hour	pH	ct	pH	ct	pH	ct	pH	ct	pH	ct	pH	ct
0	7.10	6.34	7.19	5.55	7.16	5.42	7.25	5.40	7.32	5.39	7.25	5.31
14	4.43	9.15	6.98	8.74	4.41	8.98	6.98	8.69	4.45	9.20	6.96	8.67
15	4.39	9.10	6.91	8.71	4.38	9.15	6.91	8.71	4.33	9.31	6.90	8.73
16	4.35	9.40	6.92	8.64	4.33	9.32	6.92	8.76	4.33	9.38	6.94	8.68
17	4.27	9.08	6.93	8.70	4.28	9.32	6.94	8.81	4.28	9.27	6.94	8.81
18	4.23	9.35	6.94	8.65	4.25	9.31	6.93	8.67	4.24	9.26	6.94	8.92
20	4.22	9.47	6.93	8.79	4.21	9.18	6.93	8.75	4.20	9.60	6.94	8.61
22	4.17	9.32	6.92	9.06	4.18	9.40	6.94	9.01	4.18	9.52	6.99	8.92
23.7	4.15	9.45	6.95	9.02	4.14	9.35	6.97	8.85	4.13	9.43	6.95	9.39

X. Glossary of Terms

D Value- time required for a 1 log reduction of a microorganism at a given temperature.

T_{4D}- The heat process at a given temperature to reduce the population by 10^{-4}, equivalent to 4 times the D value + the lag time if present.

T_{lag}- the duration of the shoulder of the thermal inactivation curve

"Hurdle" Technology- manipulation of multiple environmental factors to inhibit microbial growth

Intrinsic Factors of Foods-pH, water activity, and nutrients

Extrinsic Factor of Foods-temperature, presence of other bacteria

A_w- Water Activity is the fraction of the vapor pressure of the free water in a food over the vapor pressure of pure water. The water activity represents the amount of free water available in a food for potential use by a microorganism.

HACCP- Hazard Analysis and Critical Control Point System

XI. REFERENCES

1. Armstrong, D. 1995. *Listeria monocytogenes*, In G.L. Mandell, J.E. Bennett, R. Dolin, eds. Mendell, Douglas, and Bennett's Principle and Practice of Infectious Disease. New York, NY: Churchill Livingstone, pp. 1880-1885.

2. Armstrong, R.W. and P.C. Fung. 1993.Brainstem encephalitis (rhombenephalitis) due to *Listeria monocytogenes*: case report and review. Clin Infect. Dis. 16:689-702.

3 . Anderson, W. A., N. D. Hedges, and M. V. Jones. 1991. Thermal inactivation of *Listeria monocytogenes* studied by differential scanning calorimetry. J. Gen. Microbiol. 137:1419-1424.

4. Augustin, J. C., V. Carlier, and J. Rozier. 1998. Mathematical modeling of the heat resistance of *Listeria Monocytogenes*. J. Appl. Microbiol. 84:185-191.

5. Beuchat, L. R. and R. E. Brackett. 1989. Observations on survival and thermal inactivation of *Listeria monocytogenes* in ravioli. Lett. Appl. Microbiol. 8:173-175.

6. Beuchat, L. R., R. E. Brackett, D. Y.-Y. Hao, and D. E. Conner. 1986. Growth and thermal inactivation of *Listeria monocytogenes* in cabbage and cabbage juice. Can. J. Microbiol. 32:791-795.

7. Bhaduri, S., P. W. Smith, and S. A. Palumbo. 1991. Thermal destruction of *Listeria monocytogenes* in liver sausage slurry. Food Microbiol. 8:75-78.

8. Boyle, D. L., J. N. Sofos, and G. R. Schmidt. 1990. Thermal destruction of *Listeria monocytogenes* in a meat slurry and in ground beef. J. Food Science 55:327-329.

9. Bradshaw, J. G., J. T. Peeler, J. J. Corwin, J. M. Hunt, J. T. Tierney, E. P. Larkin, and R. M. Twedt. 1985. Thermal resistance of *Listeria monocytogenes* in milk. J. Food Prot. 48:743-745.

10. Bradshaw, J. G., J. T. Peeler, J. J. Corwin, J. M. Hunt, and R. M. Twedt. 1987. Thermal resistance of *Listeria monocytogenes* in dairy products. J. Food Prot. 50:543-544.

11. Buchanan, R. and Lindqvist, R. Topic 1:Hazard characterization of *Listeria monocytogenes* in ready-to-eat foods.

12. Buchanan, R. L. and L. K. Bagi. 1997. Effect of water activity and humectant identity on the growth kinetics of *Escherichia coli* O157:H7. Food Microbiol. 14:413-423.

13. Buchanan, R. L. and S. G. Edelson. 1999a. Effects of pH dependent, stationary phase acid resistance on the thermal tolerance of *Escherichia coli* O157:H7. Food Microbiol. 16:447-458.

14. Buchanan, R. L. and S. G. Edelson. 1999b. pH dependent stationary phase acid resistance response of enterohemorrhagic *Escherichia coli* in the presence of various acidulants. J. Food Prot. 62:211-218.

15. Buchanan, R. L. and S. G. Edelson. 1999c. Effects of pH and acid resistance on the radiation resistance of enterohemorrhagic *Escherichia coli*. J. Food Prot. 62:219-228.

16. Buchanan, R.L. H.G Stahl, M.M. Bencivengo and F. Del Corral. 1989. Comparison of lithium chloride-phenylethanol-moxalactam and modified vogel johnson agars for detection of *Listeria* spp. in retail-level meats, poultry, and seafood. Appl. Environ.Microbiol. 55:599-603.

17. Budo-Amoako, E., S. Toora, C. Walton, R. F. Ablett, and J. Smith. 1992. Thermal death times for *Listeria monocytogenes* in lobster meat. J. Food Prot. 55:211-213.

18. Bunning, V. K., R. G. Crawford, J. G. Bradshaw, J. T. Peeler, J. T. Tierney, and R. M. Twedt . 1986. Thermal resistance of intracellular *Listeria monocyogenes* cell suspended in raw bovine milk. Appl. Environ. Microbiol. 52:1398-1402.

19. Casadei, M. A., R. E. De Matos, S. T. Harrison, and J. E. Gaze. 1998. Heat Resistance of *Listeria monocytogenes* in dairy products as affected by the growth medium. J. Appl. Microbiol. 84:234-239.

20. Chawla, C. S., H. Chen, and C. W. Donnelly. 1996. Mathematically modeling the repair of heat-injured *Listeria monocytogenes* as affected by temperature, pH, and salt concentration. Int. J. Food Microbiol. 30:231-242.

21. CheroutreVialette, M., I. Lebert, M. Hebraud, J. C. Labadie, and A. Lebert. 1998. Effects of a_w stress on growth of *Listeria monocytogenes.* Int. J. Food Microbiol. 42:71-77.

22. Chhabra, A.T., W.H. Carter, R.H. Linton, and M.A. Cousin. 2002. A predictive model that evaluates the effect of growth condition on the thermal resistance of *Listeria monocytogenes*. Int. J. Food Microbio. 78:235-243.

23. Cole, M. B., K. W. Davies, G. Munro, C. D. Holyoak, and D. C. Kilsby. 1993. A vitalistic model to describe the thermal inactivation of *Listeria monocytogenes*. J. Indust. Microbiol. 12:232-239.

24. Cole, M. B. and M. V. Jones. 1990. A submerged-coil heating apparatus for investigating thermal inactivation of micro-organisms. Lett. Appl. Microbiol. 11:233-235.

25. Conte, M.P., G. Petrone, A.M. Di Biase, M.G. Ammendolia, F. Superti, and L. Seganti. 2000. Acid tolerance in *Listeria monocyotgenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. Microbial Pathogenesis. 29:137-144.

26. Coote, P. J., C. D. Holyoak, and M. B. Cole. 1991. Thermal inactivation of *Listeria monocytogenes* during a process simulating temperatures achieved during microwave cooking. J. Appl. Bacteriol. 70:489-494.

27. Cotter, P.D, C.G.M. Gahan, and C. Hill. 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Molecular Microbiol. 40:765-775.

28. Cox, L., T. Kless, J. Cordier, C. Cordellana, P. Konkel, C. Pedrazzini, R. Beumer, and A. Siebenga. 1989. *Listeria* spp. in food processing, non-food and domestic enviroments. Food Microbiol. 6:49-61.

29. Damart,W.C. 1994. ABACUS: Interactive program for non-linear regression analysis. QCPE Bull 14:61.

30. De Martinis, E.C.P., A.D. Crandall, A.S. Mazzotta, and T.J. Montville. 1997. Influence of pH, salt, and temperature on nisin resistance in *Listeria monocytogenes*. J. Food Prot. 60:420-423.

31. Doherty, A. M., C. M. M. McMahon, J. J. Sheridan, I. S. Blair, D. A. Mcdowell, and T. Hegarty. 1998. Thermal resistance of *Yersinia Enterocolitica* and *Listeria monocytogenes* in meat and potato substrates. J. Food Safety 18:69-83.

32. Donnelly, C. W. 2001. *Listeria monocytogenes*: a continuing challenge. Nutrition Reviews 59:183-194.

33. Donnelly, C. W. and E. H. Briggs. 1986. Psychrotrophic growth and thermal inactivation of *Listeria monocytogenes* as a function of milk composition. J. Food Prot. 49:994-998.

34. Donnelly, C. W., E. H. Briggs, and L. S. Donnelly. 1987. Comparison of the heat resistance of *Listeria monocytogenes* in milk as determined by two methods. J. Food Prot. 20:14-17.

35. Dorsa, W. J., D. L. Marshall, M. W. Moody, and C. R. Hackney. 1993. Low temperature growth and thermal inactivation of *Listeria monocytogenes* in precooked crawfish tail meat. J. Food Science 56:106-109.

36. Doyle, M. P., L. R. Beuchat, and T. J. Montville. 2001. Food Microbiology, Fundamentals and Frontiers. ASM Press, Washington, D.C.

37. Doyle, M. E., A. S. Mazzotta, T. Wang, D. W. Wiseman, and V. N. Scott. 2001. Heat resistance of *Listeria monocytogenes*. J. Food Prot. 64:410-429.

38. Duche, O., F. Trémoulet, P. Glaser, and J. Labadie. 2002 Salt stress proteins in *Listeria monocytogenes*. Appl. Environ. Microbiol. 68:1491-1498.

39. Farber, J. M. and F. Pagotto. 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. 15:197-201.

40. Farkas, J. 1997. Physical Methods of Food Preservation, p. 497-519. *In* T. J. Montville (ed.), Food Microbiology, Fundamentals and Frontiers. ASM Press, Washington, D.C.

41. Fedio, W. M. and H. Jackson. 1989. Effects of tempering on the heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. 9:157-160.

42. Fennema, O.R.1996. Water and Ice, p. 83-84 in O. Fennema (ed.) Food Chemistry. Marcel Dekker, Inc, New York, NY.

43. Ferreira, A., C.P. O'Byrne, and K.J. Boor. 2001. Role of sB in heat, ethanol, acid, and oxidative streaa resistance and during carbon starvation in *Listeria monocytogenes*. Appl. Environ. Microbiol. 67:4454-4457.

44. Ferreira, A., D. Sue., C. P. O'Byrne, and K.J. Boor. 2003. Role of *Listeria monocytogenes* σ^B in survival in lethal acidic conditions and in the acquired acid tolerance response. Appl. Environ. Microbiol. 69:2692-2698.

45. Foster, J.W. 1995. Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. Crit. Rev Microbiol. 21:215-237

46. Gahan, C. G. M. and C. Hill. 1999. The relationship between acid stress responses and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. Int. J. Food Microbiol. 50:93-100.

47. George, S. M., L. C. C. Richardson, I. E. Pol, and M. W. Peck. 1998. Effect of oxygen concentration and redox potential on recovery of sublethally heat-damaged cells of *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes*. J. Appl. Microbiol. 84:903-909.

48. Glass, K. A. and M. P. Doyle. 1989. Fate and thermal inactivation of *Listeria monocytogenes* in beaker sausage and pepperoni. J. Food Prot. 52:226-231.

49. Golden, D. A., L. R. Beuchat, and R. E. Brackett. 1988. Evaluation of selective direct plating media for their suitability to recover uninjured, heat-injured, and freeze-injured *Listeria monocytogenes* from foods. Appl. Environ. Microbiol. 54:1451-1456.

50. Hansen, T. B. and S. Knochel. 1996. Thermal inactivation of *Listeria monocytogenes* during rapid and slow heating in sous vide cooked beef. Lett. Appl. Microbiol. 22:425-428.

51. Jay, J.M. 1996. Intrinsic and extrinsic parameters of foods that affect microbial growth, p.38-66. In Modern Food Microbiology. Chapman & Hall. New York, NY.

52. Jørgensen, F., T. B. Hansen, and S. Knochel. 1999. Heat shock-induced thermotolerance in *Listeria monocytogenes* 13-249 is dependent on growth phase, pH and lactic acid. Food Microbiol. 16:185-194.

53. Jørgensen, F., B. Panaretou, P. J. Stephens, and S. Knochel. 1996. Effect of pre and post heat shock temperature on the persistence of thermotolerance and heat shocked induced proteins in *Listeria monocytogenes*. J. Appl. Bacteriol. 80:216-224.

54. Jørgensen, F., P. J. Stephens, and S. Knochel. 1995. The effect of osmotic shock and cell morphology of *Listeria monocytogenes*. J. Appl. Bacteriol. 79:274-281.

55. Kroll, R.G. and R.A. Patchett. 1992. Induced acid tolerance in *Listeria monocytogenes*. Lett. Appl. Microbiol. 14:224-227.

56. Lou, Y. and A. E. Yousef. 1996. Resistance of *Listeria monocytogenes* to heat after adaption to environmental stresses. J. Food Prot. 59:465-471.

57. Mazzotta, A.S. 2001a. Thermal Inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. J. Food Prot. 64:315-320.

58. Mazzotta, A. S. 2001b. Thermal inactivation of stationary-phase and salt-adapted *Listeria monocytogenes* during postprocess pasteurization of Surimi-based imitation crab meat. J. Food Prot. 64:483-485.

59. McLauchlin, J. 1996. The relationship between *Listeria* and listeriosis. Food Control 7:187-193.

60. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food related illness and death in the United States. Emerg. Infect. Dis. 5:607-625.

61. Meng, J. and M. P. Doyle. 1998. Emerging and evolving microbial foodbourne pathogens. Institut Pasteur/Elsevier 96:151-164.

62. Montville, T. J. 1997. Principle which influence microbial growth, survival, and death in foods, p. 13-29. *In* T. J. Montville (ed.), Food Microbiology, Fundamentals and Frontiers. ASM Press, Washington, D.C.

63. Nair, S., I. Derré, T. Msadek, O. Gaillot, and P. Berche. 2000. CtsR controls class III heat shock gene expression in the human pathogen *Listeria monocytogenes*. Molecular Microbiol. 35:800-811.

64. Nichols, G., J. Mclauchlin, and J. DeLovois. 1998. The contamination of pâté with *Listeria monocytogenes*-results from the 1994 European community-coordinated food control program for England and Wales. J. Food Prot. 61:1299-1304.

65. O'Driscoll, B., C.G.M. Gahan, and C. Hill. 1996. Adaptive acid tolerance response in *Listeria monocytogenes* of an acid-tolerant mutant which demonstrates increased virulence. Appl. Environ. Microbiol. 62:1693-1698.

66. Pagan, R., S. Condon, and F. J. Sala. 1997. Effects of several factors on the heat-shock-induced thermotolerance of *Listeria monocytogenes*. Appl. Environ. Microbiol. 63:3225-3232.

67. Pagan, R., P. Manas, I. Alvarez, and F. J. Sala. 1998. Heat resistance in different heating media of *Listeria monocytogenes* ATCC 15313 grown at different temperatures. J. Food Safety 18:205-219.

68. Palumbo, S. A., J. L. Smith, B. S. Marmer, L. L. Zaika, S. Bhaduri, C. TurnerJones, and A. C. Williams. 1993. Thermal destruction of *Listeria monocytogenes* during liver sausage processing. Food Microbiol. 10:155-163.

69. Peleg, M. and M. B. Cole. 1998. Reinterpretation of microbial survival curves. Crit. Rev. Food Science 38:353-380.

70. Phan-Thanh, L. and F. Mahouin. 1999. A proteomic approach to study the acid response in Listeria monocytogenes. Electrophoresis 20:2214-2224.

71. Phan-Thanh, L., F. Mahouin, and S. Alige. 2000. Acid responses of *Listeria monocytogenes*. Int. J. Food Microbiol. 55:121-126.

72. Pine, L., G.B. Weaver, J.B. Malcolm, J.B. Brooks, and M.I. Daneshvar. 1989. Physiological studies on the growth and utilization of sugars by Listeria species. Canadian J. of Microbiology. 35:245-254.

73. Rocourt, J. 1996. Risk factors for listeriosis. Food Control 7:195-202.

74. Rocourt, J. and P. Cossart. 1997. *Listeria monocytogenes*, p. 337-352. *In* T. J. Montville (ed.), Food Microbiology, Fundamentals and Frontiers. ASM Press.

75. Rowan, N. J. and J. G. Anderson. 1998. Effects of above optimum growth temperature and cell morphology on thermotolerance of *Listeria monocytogenes* cells suspended in bovine milk. Appl. Environ. Microbiol. 64:2065-2071.

76. Ryser, E. T. and E. H. Marth. 1999. *Listeria*, Listeriosis, and Food Safety. Marcell Dekker, Inc., New York.

77. Schoeni, J. L., K. Brunner, and M. P. Doyle. 1991. Rates of thermal inactivation of *Listeria monocytogenes* in beef and fermented beaker sausage. J. Food Prot. 54:334-337.

78. Schuman, J. D. and B. W. Sheldon. 1997. Thermal resistance of *Salmonella* spp. and *Listeria monocytogenes* in liquid egg yolk and egg white. J. Food Protect. 60:634-638.

79. Seelinger, H.P.R. and Hohne, K. 1979. Serotyping of Listeria monocytogenes. In Methods in Microbiology, Vol 13. ed Bergan, T. & Norris, J.R. pp. 31-49. New York: Academic Press.

80. Seelinger, H.P.R., and Jones, D. 1986. Genus *Listeria* Pirie 1940. In: P.H.A Sneath, N.S. Mair, N.E. Sharpe, and J.G. Holt (eds.), Bergey's Manual of Systematic Bacteriology, Vol 2. pp. 788-795. Baltimore: Williams & Wilkins.

81. Smith, J. L. and R. L. Buchanan. 1990. Identification of supplements that enhance the recovery of *Listeria monocytogenes* on modified Vogel Johnson agar. J. Food Safety 10:155-163.

82. Smith, J. L., B. S. Marmer, and R. C. Benedict. 1991. Influence of growth temperature on injury and death of *Listeria monocytogenes* Scott A during a mild heat treatment. J. Food Protect. 54:166-169.

83. Southwick, F. S. and D. L. Parich. 1996. Intracellular pathogenesis of listeriosis. N. Engl. J. Med. 334:770-776.

84. Stephens, P. J., M. B. Cole, and M. V. Jones. 1994. Effect of heating rate on the thermal inactivation of *Listeria monocytogenes*. J. Appl. Bacteriol. 77:702-708.

85. Stryer, Lubert. 1995. Biochemistry. pp.484-517. New York: W. H. Freeman and Company.

86. Taormina, P.J, and L.R. Beuchat. 2001. Survival and heat resistance of *Listeria monocytogenes* after exposure to alkali and chlorine. Appl. Environ. Microbiol. 67:2555-2563.

87. U.S. Department of Agriculture/Food Safety Inspection Service. 1999. *Listeria* guideline for industry. Downloaded on 07/08/2003 from http://www/fsis.usda.gov/OA/topics/Imguide.htm

88. U.S. Department of Agriculture/Agricultural Research Service. 2001. Pathogen Modeling Program, Version 6.1. Downloaded on 07/31/2003 from http://www.arserrc.gov/mfs/PMP6_download.htm.

89. U.S. Department of Health and Human Services and United States Department of Agriculture (US DHHS/USDA). 2003. Risk assessment of the relative risk to pubic health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. Washington, D.C., FDA/USDA (unpublished data).

90. Wiedmann, M., T.J. Arvik, R.J. Hurley, and K.J. Boor. 1998. General stress transcription factor σ^B and its role in acid tolerance and virulence of *Listeria monocytogenes*. J. Bacteriol. 180:3560-3656.

91. Yen, L. C., J. N. Sofos, and G. R. Schmidt. 1991. Effects of meat curing ingredients on thermal destruction of *Listeria monocytogenes* in ground pork. J. Food Protect. 54:408-412.