HEAT INACTIVATION OF *LISTERIA MONOCYTOGENES* IN BIOFILMS

by

REVIS A.N. CHMIELEWSKI

(Under the Direction of Joseph F. Frank)

ABSTRACT

Predictive models for the heat inactivation of *Listeria monocytogenes* in pure and mixed culture biofilms, formed on stainless steel and rubber surfaces and in the presence of food soil were developed using fraction negative data and logistics regression. The validation study indicated that at the 50% probability level of *L. monocytogenes* inactivation, the predictive model with strain 3990 on stainless steel surfaces was conservative in its estimate of *L. monocytogenes* biofilm inactivation while the Scott A model was not a reliable predictor of the heat inactivation of *L. monocytogenes* in a biofilm. The multispecies (*L. monocytogenes*, *Pseudomonas and P. agglomerans*) biofilm was an adequate predictor of *L. monocytogenes* biofilm inactivation and can be used in situations of low occurrence in a food product. A predictive model for the heat inactivation of *L. monocytogenes* on rubber surfaces was developed. The model provides for three prediction situations in the presence of soil, the fairly conservative assessment of risk using heat resistant Scott A strain and the less conservative predictions based on strain 3990 and *Listeria* in a mixed culture. For the low soil condition, the Scott A and 3990 strains showed adequate assessment of heat inactivation while the *L. monocytogenes* in a multi-species biofilm was conservative in its predictions. These predictive models could be used as a guide to apply hot water sanitation when chemical sanitation is ineffective for a process. Heat stress induced the increased production of biofilm for *L. monocytogenes* Scott A. There were changes in the proteins expression before and after heat stress.

INDEX WORDS: *Listeria monocytogenes*, predictive model, heat inactivation, stainless steel, buna-N rubber, glass wool, logistics regression

HEAT INACTIVATION OF *LISTERIA MONOCYTOGENES* IN BIOFILMS

by

REVIS A.N. CHMIELEWSKI

B.S., The University of Connecticut, Storrs, CT, 1984

M.S., The University of Georgia, Athens, GA, 1987

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2003

HEAT INACTIVATION OF *LISTERIA MONOCYTOGENES* IN BIOFILMS

by

Revis A.N. Chmielewski

Major Professor: Joseph F. Frank

Committee: Larry R. Beuchat
Milena Corredig
William L. Kerr
Jeffery Kornacki

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2003

DEDICATION

This work is dedicated to my daughter, Athena S. Chmielewski, my little champion.

ACKNOWLEDGEMENTS

I am most grateful to my major advisor Dr. Joseph Frank for his advice, patience and guidance during this challenging process. To the members of my committee, Dr. M. Corredig, Dr. L. Beuchat, Dr. W. Kerr and Dr. J. Kornacki, thanks for the guidance and help. To Isabel Blackman, Julie Ehlers, Kazue Takeuchi and James Folsom, I appreciate your patience, assistance, listening to my concerns and intellectual discussions. I am most grateful to my family, my Dad Winston and my sisters Marcia, and Wendy, and family members Karen, Aunt Rose, Sheila and Yolanda your prayers and support were immeasurable.

Research reported in this paper was supported by state and Hatch funds allocated to the Georgia Agricultural Experiment stations and by the University of Georgia Center for Food Safety. I would like to thank Nathanon Trachoo and James Folsom for the micrographs.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Listeria monocytogenes is common in nature, especially in food processing environments. Listeriosis is a public health concern especially to the immuno-compromised population. Although the incidence of listeriosis is low (5 cases per 1 million people per year), 95 % of the affected individuals are hospitalized. In 1999, for example, of the 2,500 incidences of listeriosis, 500 individuals died (54).

Some foods implicated in food-borne outbreaks include cheeses, milk, coleslaw and processed or ready-to eat- meats and poultry products (37, 53, 61, 99, 161, 170). The Food and Drug Administration has a zero tolerance for *L. monocytogenes* in ready-to-eat or minimally processed meat and poultry products. However, low occurrence in these products has raised concern of post-process contamination.

Microorganisms on wet surfaces have the ability to aggregate, grow into microcolonies and produce biofilm. Growth of biofilms in food processing environments leads to increased opportunity for microbial contamination of the processed product and disease transmission. Microorganisms within biofilms are protected from sanitizers, increasing the likelihood of survival and subsequent contamination of food. Extracellular polymeric substances associated with biofilm that are not removed by cleaning may provide attachment sites for newly arrived microorganisms to the cleaned system. Some of the methods used to control biofilm formation include mechanical and

manual cleaning, chemical cleaning and sanitation, and application of hot water. There are some studies showing the efficacy of sanitizers in controlling *L. monocytogenes* in a biofilm and in the presence of food products (10, 64, 121, 163, 202); however, in some cases, chemical sanitation is ineffective and hot water sanitation may be more applicable.

There are currently no studies evaluating the efficacy of hot water in controlling *L. monocytogenes* in a biofilm and in the presence of food. In this study, predictive models were developed to evaluate the required hot water treatment in inactivating *L. monocytogenes* in a biofilm on stainless steel and rubber surfaces in the presence of food. The protein expression by *L. monocytogenes* when subjected to preheat treatment was also determined.

key words: Listeria, biofilm, pathogen, food processing, sanitation, cleaning

CHAPTER 2

LITERATURE REVIEW [1]

General characteristics of *L. monocytogenes*

Listeria spp. are small (0.5 μm in diameter and 1-2 μm in length) gram positive rods, non spore-forming, non acid fast, catalase positive, facultative anaerobes. The bacterium is flagellated and motile at 20-25 ºC but not at 37 ºC (8, 37, 90, 164). On nutrient agar, colonies are smooth with a translucent blue-gray tint. Under a transmitted oblique light, colonies appear with blue-green iridescence .

Nutrient requirements

Listeria spp. primarily use glucose and glutamine as their carbon and nitrogen source respectively. It requires 4 B- vitamins: biotin, riboflavin, thiamine and thioctic and the following amino acids: arginine, cysteine, cystine, glutamine, isoleucine, leucine, methionine and valine (110, 164). *L. monocytogenes* ferments sugar to acid but does not produce gas. Glucose increases growth resulting in the production of lactic acid, acetic acid (197) as well as carboxylic and hydroxy acid and alcohols (48). It hydrolyzes erythrocytes, esculin and sodium hippurate.

Taxonomy

Genetically, *Listeria* consists of 7 species from 3 lines of descendants. The first descendants consist of *L. monocytogenes, L. innocua, L. invanoii, L. welshimeri* and *L. seeliger*i and the second line consists of *L. grayi* and the third are environmental isolates

closely related to *L. innocua* (127, 196). *Listeria* belongs to the clostridium sub-branch together with *Staphylococcus, Streptococcus, Lactobacillus and Brochothrix*. It transfers genetic material among *Streptococcus, Staphylococcus* and *Lactobacillus*. It has similar teichoic and lipoteichoic acid as do *Bacillus, Streptococcus, Staphylococcus and Lactobacillus* but has a blue green sheen when viewed under oblique light. There are at least 16 serovars but 95% of human isolates belong to 3 serovars: these isolates are 1/2a, 1/2b and 4b (127, 164).

Growth characteristics

This organism is a unique human pathogen because it is resistant to diverse environmental conditions such as low pH, and/or high salt, grows at low temperatures and survives in various environments such as water, soil and plants (90, 164). *L. monocytogenes* is psychrotolerant and its survival temperature is between 1 and 45ºC. The optimum growth temperature range is between 30 and 37ºC with its minimum growth temperature at 1.1 ºC ± 0.3ºC (90, 126, 164). It survives between pH 5 and 9.6, with optimum growth pH at 6 to 8 . It grows in 10% NaCl and survives 25.5% salt at 4ºC (90, 126). *Listeria monocytogenes* can grow at a_w as low as 0.90 (126). Studies (50, 155) show growth at 0.90 a_w in glycerol; 0.93 a_w in sucrose and 0.92 a_w in NaCl.

The growth at minimum pH and low NaCl is influenced by temperature, for example at pH 4.4 and 30ºC growth was observed up to 7 d; pH 4.4 and 20ºC growth occurred up to 14 d; and pH 5.3 and 4ºC growth occurred at 21d (72, 96). Other studies (39) demonstrate the interactive effect of temperature and sodium chloride where growth

was recorded in a medium of 4% NaCl at 30ºC for 8 dy and 6% NaCl at 30ºC for 13 d (37, 90).

Ecology

Listeria is common in natural environments; It's found in soil, animal feces, sewage, silage, vegetation and water. It occurs with microflora such as lactic acid bacteria, *Brochothrix,* and *corynebacterium* (96). In the food environment, *L. monocytogenes* occurs in raw milk (from infected cows), soft cheeses, fresh and frozen meats, poultry, seafood, fruits, and vegetables and further processed foods (37, 170). The lowest incidence is in ice cream and the highest in fresh meats (8). Some directly acidified soft cheeses having a pH 5.5 and processed without the presence of a starter culture provide an ideal environment for *Listeria's* growth. The most prevalent serotype in foods is 4b followed by ½ a and ½ b (8). When found in foods, *Listeria* usually occurs in low numbers, however, in smoked fish the incidence and levels are often high (ca.4 logs) (53, 61, 99). *Listeria* is particularly difficult to control in food processing facilities since the environment provides suitable conditions for its survival and growth. Food contact surfaces, shoes, clothing, floors, drains, condensate, stagnant water, hide, and soiled surface are vehicles and harborage for it to enter and exist within a food processing facility (69, 78).

Virulence factors and syndrome

An intracellular invasion by *L. monocytogenes* occurs when cells adhere to and colonize the human intestinal tract, invade the host tissues, multiply and spread from cell to cell until they reach the host blood stream (8, 127, 165). The invasion and

internalization of *Listeria* into the host tissue are accomplished when internalin proteins on the bacterial cell surface trigger the host's cell to engulf the bacterial cells forming a vacuole; *Listeria* then enters the somatic cells. Internalin-A (InlA) is one of the expressed surface bound proteins required to enter and invade epithelial cells. This protein is expressed at approximately 20ºC and best at 37ºC in the presence of high glucose. InI B is another surface bound protein expressed to enter hepatocytes while, p60 protein expression is required to invade fibroblast cells.

Once inside the somatic cells, *L. monocytogenes* escapes the vacuole by the lytic action of LLO toxin which forms pores in the vacuole's membranes. The listeriolysin (LLO) toxin is thiol activated. It is active at pH 5 and inactive at pH 7. This toxin also destroys phagocytic cells such as macrophages and hepatocytes that engulf the bacterial cells.

The surface protein Act A is involved in actin-based motility allowing the bacteria to spread from one somatic cell to another (8, 127, 165). *Listeria* cells then enter the cytoplasm where they become coated with the host actin filaments. The actin filament is used to propel these bacteria from one tissue cell to another by allowing protrusion into the neighboring cells. This spread is aided by phospholipases which cleave polar head groups of membrane phospholipids. A double membrane vacuole forms around the listeria cell. This vacuole is then lyzed and the listeria is released. Once *Listeria* enters the new cell, it replicates and continues to spread. In this way it avoids the immuno-defenses from the host's blood. It invades many types of cells such as

macrophages, hepatocytes, and epithelial cells. The principal site of infection is the liver. (8, 127, 165).

The virulence genes in *L. monocytogenes* clustered on the same operon are *prfA, plc A, inl A and B, hyl A*, *acta A* and *plc* C. The *prfA* is a global regulator of the virulence genes. *inl A and B* genes encode for surface bound proteins expressed upon a host cell invasion. *hyl A* encodes for the LLO toxin that causes phagosome's membrane lysis and hemolysis. *act A* gene encodes for Act A protein which promotes actin polymerization and intracellular movement of the bacterial cell. *plc A* and *plc C* encodes for the phospholipases needed for the cell to cell spread of *Listeria*. *iap* genes encodes for p60 protein which is associated with somatic cell invasion and a metabolic enzyme essential for cell division.

Listeria monocytogenes impair T-cells mediated immunity in the host by surviving within the macrophages and reproducing causing cascading reactions of T-cells and macrophage production. Listeria cells also contain lipid or LPS like components called mononucleosis producing agent (MPA) which stimulate the production of and kills mononuclear cells (monocytes) therefore the name monocytogenes. MAP also stimulates IgM antibodies. Lipoteichoc acid in the cell wall may also be responsible for immuno-suppression of the host. (8, 127, 165).

Mononucleosis type symptoms are meningitis sepsis, and encephalitis. Other symptoms include cervical and generalized lymph adenopathy; bacteremia, and febrile gastroenteritis: fever, vomiting, abdominal pain, diarrhea and abortion. Treatment is

coumermycin, rifampicin and ampicillin. The mortality is 38-40% in the immuno-compromised patient.

Infectious dose

In healthy individuals, a dose of 100 cells is inconsequential but not so for the unhealthy hosts. The consumption of 10^6cells/g food for healthy individuals can cause illness, but only 100 cells/g may be required for the immuno-compromised (8, 127, 165).

Detection and confirmation

Primary enrichment is done using half Fraser broth with the following selective agents: one volume of lithium chloride, and half volume of acriflavin and nalidixic acid. Full strength Fraser broth is used for the secondary enrichment. Colonies are isolated on selective agars such as Oxford, Palcam, and LSA containing selective agents such as esculin, polymyxin, acriflavin, mannitol, lithium chloride, ceftazidime. The agar plates are incubated for 18-24h at 30-37 ºC. The CAMP test is a definitive test for *Listeria monocytogenes* ; CAMP positive with *S. aureus* and CAMP negative for *R equi* are presumptive positive reaction of *L. monocytogenes* (2). Hemolysis and motility as well as biochemical method such as API- Listeria ™ could used as a confirmation test for *L. monocytogenes*. Other rapid methods such as DNA probes and real-time PCR are now available (2).

BIOFILM

Biofilms as they occur in nature consist primarily of viable and nonviable microorganisms embedded in polyanionic extracellular polymeric substances anchored to a surface (30, 199). Extracellular polymeric substances (EPS) may contain

polysaccharides, proteins, phosholipids, teichoic and nucleic acids, and other polymeric substances hydrated to 85-95% water (41, 188). EPS provide protection to the biofilm inhabitants by concentrating nutrients, preventing access of biocides, sequestering metals and toxins, and preventing dessication (30). Food industry biofilms may also have a high food residue and mineral content that originate with product and process water. These constituents also provide protection to microorganisms held within the biofilm.

Biofilms in nature can have a high level of organization, as they may exist in single or multiple species communities, form a single layer or three dimensional structure or take the form of aggregates such as flocs or granules (3, 4, 25). A natural biofilm community may function through collective behavior and coordinated activity such as cell to cell signaling (25, 137, 147, 154) which assists survival of constituent cells in stressful environments. The majority of microorganisms in natural habitats are attached to surfaces (49), indicating the extent of the selective advantage for biofilm growth. Environmental stresses such as low nutrient availability trigger phenotypic changes of planktonic (free living) cells to the sessile (attached) form (30, 40). Other factors that influence biofilm formation are substratum composition, surface chemistry and topography, and fluid flow (141). Biofilm formation can cause mechanical blockage in fluid handling systems, the impedance of heat transfer (173), and corrosion to metal surfaces (25), though these problems are not common in the food industry.

Poor sanitation of food contact surfaces, equipment and processing environments has been a contributing factor in food borne disease outbreaks, especially those involving *L. monocytogenes* and *Salmonella*. Improperly cleaned surfaces promote soil build-up,

and in the presence of water, contribute to the development of bacterial biofilms which may contain pathogenic microorganisms (18). Cross contamination occurs when food passes over contaminated surfaces, or via exposure to aerosols or condensate that originate from contaminated surfaces (7, 17, 20). Frank and Chmielewski (70) and Holah, et al. (89) demonstrated that the type of food contact surface and topography play a significant role in the inability to decontaminate a surface. Abraded surfaces accumulate soil and are more difficult to clean than smooth surfaces. Surface defects provide protection against the removal of soil and bacteria (17-20, 130), with the result that surviving bacteria can grow and produce a biofilm. Bacteria within a biofilm are more resistant to disinfectants which may assist the survival of *Listeria* spp. and other food borne pathogens in the food processing environment (20). Direct evidence that pathogen-containing biofilms play a role in the spread of food-borne illness is lacking, as identification and characterization of biofilms has not been included in food-borne illness investigations.

Biofilm formation

Biofilm formation consists of initial attachment, microcolony and EPS (extracellular polymeric substances) production, followed by maturation (49). This process is diagramed in Figure 1.1. Bacterial transition from planktonic to the sessile state is triggered by environmental signals. Natural ecosystems are generally low in available nutrients and biofilm formation is an important adaptation for survival under these conditions (141). Therefore, biofilm formation principles derived from natural ecosystem observations may not apply to nutrient rich food industry environments.

Attachment

Adhesion to a substratum can be active or passive depending on cell motility. Passive attachment is driven by gravity, diffusion and fluid dynamics. In active adhesion, the bacterial cell surface facilitates initial attachment. Cell surface properties such as flagella, pili, adhesin protein, capsules, and surface charge influence attachment (115). Flagella allow bacteria to move to a specific attachment site, while changes in cellular physiology that affect surface membrane chemistry, surface proteins such as pili and adhesins, synthesis of polysaccharides and cell aggregation all influence adhesion (49). Attachment often occurs within 5-30 seconds and occurs in two stages: reversible followed by irreversible adhesion (141).

Reversible attachment is an initial weak interaction of bacteria with a substratum. It involves van der Waals and electrostatic forces, and hydrophobic interactions. During reversible attachment bacteria still exhibit Brownian motion and are easily removed by application of mild shear force.

Irreversible attachment results from the anchoring of appendages and/or the production of extracellular polymers (188). Repulsive forces usually prevent direct bacterial contact with the substratum (often both the substratum and the bacterial cell are negatively charged). Bonding between bacterial appendages, (i.e. pili, flagella, adhesin protein) (160, 191) and the substratum involves short range forces such as dipole-dipole interaction, hydrogen bonds, hydrophobic, and ionic covalent bonding (17, 18, 20, 23, 76, 136, 181, 183). This bonding usually occurs within a few hours of contact (91). Several studies indicate that irreversible attachment takes from 20 min to a maximum of 4h at 4

to 20ºC (76, 128, 178, 179, 191). Removal of irreversibly attached cells is difficult, and requires application of strong shear force (scrubbing or scraping) or chemical breaking of the attachment forces through the application of enzymes, detergents, surfactants, sanitizers and/or heat (20, 71, 149, 163, 177).

Factors that affect microbial attachment to abiotic surfaces

Adhesion is affected by the chemical and physical properties of the cell and substratum surfaces and the composition of the surrounding medium. Abiotic substrata are modified by conditioning films that originate with the surrounding medium. Adherent properties of the cell are influenced by the cell envelope, whose chemistry changes in response to environmental stimuli and quorum sensing. Irreversible attachment is a physiological process under genetic regulation. Studies using *S. aureus*, *E.coli* and *S. epidermis* demonstrate that genes responsible for surface protein expression, attachment and EPS production are activated in response to external stimuli such as population density, stress or nutrient limitation (1, 43, 46, 76, 110, 137, 160).

Properties of food contact surfaces Bryers (25) and Boulange-Peterman et al. (18) observed that a critical level of interfacial tension promotes bacterial adhesion. The goal for bacterial adhesion is to minimize the interfacial free energy between the abiotic surface and the bacterial cell surface. For example, at a distance of 100 nm a cell surface can be affected by hydrophobic interaction. At 50nm, van der Waals forces can attract a cell towards a surface and at a closer distance, the strongest force, the electrostatic interaction (attraction or repulsion) can contribute to the adhesion process (20). Maximum attachment of bacterial cells depends upon high available free surface energy

(electrostatic and hydrophilic interactions) of an abiotic surface. Surfaces with high free surface energy, such as stainless steel and glass, are more hydrophilic. These surfaces generally allow greater bacterial attachment and biofilm formation than hydrophobic surfaces such as teflon, nylon, buna-N rubber and fluorinated polymers (13, 94, 130, 177). Smoot and Pierson (178) observed that initial attachment of *L. monocytogenes* to stainless steel was more rapid than to rubber, even though attachment to buna-N rubber was stronger. Additional evidence for the importance of free surface energy in attachment was reported by Bos et al. (16) who found that bacterial adhesion occurred mostly at the hydrophilic region of the hydrophilic - hydrophobic interface of a stainless steel surface. In addition, a study by Boulange-Peterman et al. (18) noted that the spreading pressure (πE) of bacteria as well as the balance of free energies (polar and van der Waals force) influenced adhesion, for instance, polar interaction of stainless steel and *Streptococcus thermophilus* resulted in decreased adherence. Boulange-Peterman et al. (18) and Sinde and Carballo (177) also demonstrated that cleaning stainless steel conditions the surface temporarily changing its properties. Cleaning with alkali or strong acid (4 M nitric acid) caused the surface to be hydrophilic while cleaning with weak acid produced a hydrophobic effect. Once stainless steel is exposed to air or water, it is passivated by forming a chromium oxide layer. Organic soil adheres to the oxide layer producing a conditioned substratum to which bacteria adhere (192). In most cases, bacteria attach more to hydrophilic than hydrophobic surfaces but the differences in attachment are not necessarily of practical significance (13, 45, 94, 177), since high growth rates on the

surface can make attachment differences a minor factor in the development of microbial load.

Substrate type also influences the attachment pattern. Bacteria tend to attach to glass (a hydrophilic surface) uniformly in a monolayer, while on hydrophobic surfaces such as nylon and tin, they tend to adhere in clumps (135).

Topography of food contact surfaces Stainless steel type 304, with either 2B (cold rolled), #4 (mechanically polished), or electropolished finish are usually used for fabricating equipment and utensils. Rubber, teflon, and nylon are used for gaskets, various utensils and equipment parts. These surfaces are abraded with repeated uses, increasing their ability to entrap bacteria and soil (89). This condition creates a harborage for bacterial growth and protection from cleaning and sanitation. Frank and Chmielewski (67) and Boulange-Peterman, et al. (19), observed that the average surface roughness (Ra) of stainless steel does not correlate to cell adherence, but, other measurements of surface roughness that indicate surface defects (Rdin and Rmax) correlate more closely with soil retention and removal (67). Studies by Jones et al. (100) and Holah and Thorne (89) also demonstrated that surface defects were associated with significant increases in bacterial attachment.

Contact time Contact time between the cell and the substratum is required for irreversible attachment. Lunden et al. (128) demonstrated that the most prevalent strain of *L. monocytogenes* (strain 1/2c) found in food processing plants had good adhesion ability and required only a short contact time for attachment.

Adhesive properties of the bacterial cell surface. Adhesion of bacterial cells is influenced by the physicochemical properties of the cells' surface which in turn are influenced by factors such as microbial growth rate, growth medium, and culture conditions (time and temperature). Bacteria have a net negative surface charge and usually behave as hydrophobic particles, but the degree of hydrophobicity can change with growth phase. Hydrophobicity generally decreases as growth rate increases (17, 19). Herald and Zottola (87), Hood and Zottola (91) and Smoot and Pierson (179) demonstrated that *Listeria* and *Yersinia* exhibited increased attachment when the microorganisms were at their highest metabolic activity. For *Listeria* the optimum metabolic activity and attachment conditions was at 30ºC and pH 7 and for *Yersinia* the optimum was at 21ºC and pH 8-9. Sorongon (181) observed that starvation of *Cytophaga* increases surface hydrophobicity. Other studies have correlated adhesion with surface charge and hydrophobicity. The adhesiveness of *Staphylococcus epidermidis* correlates directly with surface electronegativity and hydrophobicity while the adhesion of *Escherichia coli* is inversely proportional to the degree of negative surface charge but is not influenced by hydrophobicity (30, 76). Spores adhere better to food contact surfaces than vegetative cells due to the high hydrophobicity of their hairy surfaces (20). Growth media, pH and nutrients influence the surface charge of bacteria. Glucose and lactic acid in the growth medium decreased the electronegativity of *L. monocytogenes'* cell wall through the neutralization of the surface charge and the production of acid stress proteins (24). *L. monocytogenes* grown in tryptone exhibited less attachment ability than those grown with amino acids (110). Growth temperature also affects the surface properties of

Listeria. At high temperatures (37ºC), *Listeria* lose their flagella and their cell surfaces becomes less electronegative. When *Listeria* are grown at 15-20 ºC, the cell surface has a negative charge, suggesting that a negative charge results from the presence of flagella and glycolipids (23). Smoot and Pierson (23, 178) also found that *Listeria* grown at 30 ºC was more hydrophilic than when grown at 10 or 40 ºC. High growth temperature is also associated with increased attachment ability (179), possibly due to the production of heat stress proteins associated with the cell surface. Some studies (23, 159, 178) suggest that attachment ability is controlled by surface proteins other than flagella. The importance of flagella being primarily to bring the cells to attachment sites.

Structures that protrude from the cell membrane such as lipopolysaccharide (LPS), adhesins and other proteins, and lipoteichoic acids can play an important role in microbial attachment. *E. coli* and *L. monocytogenes* utilize flagella, pili and membrane proteins to initiate attachment (49, 191). The loss of these cell appendages changes surface properties, which may lead to decreased attachment ability on some abiotic surfaces (76, 83). LPS plays a role in initial attachment. *Pseudomonas* mutants deficient in the B-band of LPS exhibit reduced surface hydrophobicity and reduced ability to attach to hydrophilic surfaces. While in *E. coli*, the loss of LPS resulted in decreased ability of cells to attach to surfaces (49). Pili act like a strong adhesive to anchor bacteria to some surfaces (28, 145, 160) and also act as chemoreceptors, directing cells to move to specific attachment sites. Briandet et al. (23) and Smoot and Pierson (179) demonstrated that the greatest adhesion of bacteria on stainless steel occurred in a high ionic strength solution while the lowest attachment occurred under alkali conditions.

The low attachment indicates electrostatic repulsion between the cell and the attachment surface, and demonstrates the importance of the suspending solution in providing conditioning layer at the attachment surface to overcome this repulsion.

Substratum preconditioning Clean surfaces submerged in solution are rapidly changed by the adsorption of organic molecules and charged ions. This process is called preconditioning. Adsorption of an organic layer onto a substratum can occur within seconds of exposure to an aqueous environment. Numerous studies demonstrate that bacterial attachment occurs best on pre-conditioned surfaces in the presence of ions (7, 23, 183). Initiation of bacterial attachment is dependent on the surface properties of the preconditioned substrate (25). Bryers (25) and McEldowney and Fletcher (135) also pointed out that the presence of a surface layer of organic molecules can promote bacterial cell adhesion and that the maximum adsorption of organic molecules occurs on surfaces with high free energy. The importance of an organic preconditioned surface in bacterial adherence to stainless steel has also been demonstrated (192). Verran and Jones (192) concluded in their review that hydrophobic protein macromolecules adhered more to high free energy surfaces, and that fatty acids adhered better to hydrophobic polymeric surfaces and metals cleaned with solvents. McEldowney and Fletcher (135) and Criado (44) suggested that the adhesion of bacteria to an inert surface is greatly influenced by the compatibility of the preconditioning macromolecules with that of the surface properties of the bacteria. McEldowney and Fletcher (135) observed that hydrated layers of polymers and proteins that form on inert surfaces can either facilitate or reduce bacterial adhesion.

Milk and milk components will adsorb to surfaces within 5-10 seconds, forming a conditioning film that may encourage or inhibit bacterial attachment (141). Hood and Zottola (91) demonstrated the effect of dairy soil on attachment by using stainless steel exposed to whole, chocolate and diluted milk . Attachment of *L. monocytogenes* and *S. typhimurium* was inhibited by preconditioning with whole and chocolate milk, and was enhanced when using diluted milk. Data of Wong (202) and Barnes et al.(7) supported this finding by reporting that preconditioning with milk inhibits attachment of *Listeria* to stainless steel and buna-N rubber. Fletcher et. al (66), Bower, et al.(20) and Wong (202) found that various proteins such as bovine serum albumin (BSA), gelatin, fibrinogen and pepsin inhibited bacterial attachment to various surfaces. Although Fletcher (66) showed the inhibitory effects of BSA, this effect may not be entirely due to the properties of the conditioning layer, as serum albumin may also have modified the bacterial surface.

Sequence of attachment of multiple species influences the species composition of the resulting biofilm. The initial population that attaches can change the surface so that the following species can attach via cell to cell association. In some cases attachment of a second species can increase stability of the biofilm population (135). Hood and Zottola (91) demonstrated that *L. monocytogenes* was more likely to adhere to stainless steel in the presence of *Pseudomonas fragi*.

Microcolony formation

Microcolony formation will proceed after irreversible attachment given appropriate growth conditions. Microcolony formation results from simultaneous aggregation and growth of microorganisms and is accompanied by the production of

EPS. Images of microcolonies produced by water system bacteria on a polyvinyl chloride surface are presented in Figure 1.2. Studies of bacterial species in natural systems showed that aggregation may involve recruitment of planktonic cells from the surrounding medium as a result of cell to cell communication (quorum sensing)(137, 154). In *P. aeruginosa,* the *algC* gene is transcribed upon attachment; which results in down regulation of flagellum synthesis and up-regulation of *alg T* for the synthesis of alginate, the major component of EPS for this species (49). The production of acylhomoserine lactones (AHL) and other quorum sensing molecules (Lon protease) regulate the formation of typical biofilm structure of *P. aeruginosa* as well as various virulence factors (49, 154). In other microorganisms, adhesion and biofilm formation are under distinctly different genetic regulation (43, 83, 84).

P. aeroginosa, *E.coli* and *Vibrio cholerae* lose their flagella and increase their EPS production upon attachment to a surface (49). EPS is also produced in response to attachment and environmental stimuli such as osmotic pressure, pH, temperature, and starvation. Hood and Zottola (92) found that *P. fragi* only adhered to stainless steel under starvation conditions and produced EPS to anchor itself to the surface. The *crc* gene in *P. aeroginosa* codes for biofilm development as well as catabolite repression. This *crc* gene also regulates the *pilA & B* genes which encode for the main protein of type IV pili (49). The genetic control mechanism that links carbon metabolism *(crc* gene), and pilus assembly (*pil* gene) is unknown but data suggests a link between nutrient availability and biofilm formation (112). The composition of biofilm EPS is not known, but is likely a mixture of polymers. It cannot be assumed that EPS material produced in

broth culture is similar to that produced when attached to a surface. The EPS of pseudomonad biofilm attached to stainless steel contain galactose, glucose, rhamnose and uronic acid (123). Allison and Sutherland (3) provide evidence that EPS production does not always occur immediately after attachment. They demonstrated that polysaccharide production in Gram negative bacteria was initiated 5 to 6 hr after attachment. However, attachment EPS can also be produced by planktonic cells resulting in enhanced attachment (25).

Maturation of the biofilm

If conditions are suitable for sufficient growth and agglomeration biofilms in nature may develop an organized structure. This process is called maturation. The mature biofilm may consist of a single layer of cells in porous extracellular polymer or multilayered loosely packed microcolonies held together with EPS and interspersed with water channels. Examples of mature biofilms are presented in Figures 1.2 and 1.3. Lawrence et al. (117) observed the spatial redistribution of cells after microcolony formation to produce the mature biofilm structure by using confocal laser microscopy.

BIOFILM STRUCTURAL MODELS

Various models have been proposed to explain the development and properties of biofilms. These models are based on observations of biofilm structure and are therefore limited by the available visualization technology.

The monolayer biofilm theory

The earliest biofilm structure theory, the *continuum model* described biofilms as smooth, planar and homogeneous. This model was used in water engineering to predict

the rate of biofilm chemical activity based on diffusion, the physical effects of flow and pressure, and cell detachment rate (12, 198).

The multilayer- 3D structure theory

The continuum model was adequate for engineering predictions but observations using differential interference contrast (DIC) microscopy indicated that biofilms have a heterogeneous structure. The *Kreft* or swarm model described biofilms as a heterogenous mosaic with stacked microorganisms held together by EPS (198). This model was based on simple microbial physiology concepts. Cells metabolize substrates, excrete, maintain an energy level, divide or die depending on nutrient availability. If a cell divides it pushes other cells apart. If it dies the cell components are recycled as nutrients. The model assumed that biofilm cells had a specific affinity for each other, causing them to stick together. This model was able to explain the incorporation of diverse microbial communities with their own distinct characteristics into a biofilm.

The current theory

The *discrete model* is based primarily on evidence provided by confocal laser scanning microscopy. This instrumentation has allowed the visualization of a mushroom/ tulip structure of the biofilm with towers, pedestals and water channels. The upper portion is mushroom shaped with a narrow stalk penetrated by channels (198). The structural characteristics of this model are diagramed in Figure 1.1. This model proposes that cell growth in the periphery is rapid, and growth in the interior is slow. The towers or mushroom portion may have streamers which may break off and repopulate other sites. Zhang and Bishop (203) demonstrated that the porosity of mature biofilms range from

89% in the top layer to 5% in the bottom layer; evidence that confirms this model. In the discrete model, the effect of an individual cell or each microcolony is evaluated in relation to the entire group. For example, in a low concentration of substrate independent stacks of microorganisms form, but as solute concentration increases, microcolonies appear denser forming mushroom type structures with water channels interspersed within the structure. Davey and O'Toole (49) and Wimpenny et al. (198) described biofilm structures ranging from monolayers of single scattered cells to patches of cells which are interspersed throughout thick mucoid three dimensional layers (Figures 1.2 and 1.3). Organisms within the biofilm may compete, operate independently, cooperate or be predatory.

Factors influencing biofilm development

Some of the factors affecting biofilm development include surface and interface properties, nutrient availability, composition of microbial community, hydrodynamics, interspecies interaction and cellular transport. A study (100) of biofilm in mineral water bottles showed that the interfacial properties of a surface can create a micro-environment that selects for different microfloral communities. Although the smooth surfaces of the polyethylene terephthalate (PET) bottles were sparsely populated, they were colonized by rod shaped bacteria while the rougher and more hydrophobic surfaces of the high density polyethylene (HDPE) caps were populated with clumped coccoid bacteria.

Nutrient availability has a major influence on biofilm structure and the composition of the microbial community. Studies showing the effect of nutrients on pure culture *Listeria* biofilm development suggest (111) that low levels of phosphate initially

stimulate biofilm development, but after several days the effect was reduced. The type of sugar provided also influenced the development of biofilm, with trehalose and mannose allowing for only poor biofilm formation.

As the biofilm matures, it adapts to nutrient, oxygen and population changes and forms discrete microcolonies separated by water channels. The structural density of the matrix increases at the core while the top layers remain porous (12). Most metabolically active bacteria remain at the top layers of the biofilm matrix, near water channels (203). Water channels allow the dispersion and exchange of dissolved organics, metals cations and metabolites. Nutrients become trapped and concentrated in the biofilm matrix and move throughout the matrix by diffusion (25, 49) which results in a stratified habitat that selects for different microbial species (185). These species can then become involved in nutrient exchange with their neighbors. According to Wimpenny et al. (198) and Kumar and Anand (115), heterogeneous biofilms can possess different nutritional requirements, for example, when citrate was provided both *Pseudomonas* and *Burkholderia* grew as separate microcolonies but when an intermediate substrate was provided both organisms cooperated to create a metabolic symbiosis, and stayed together as a mixed culture biofilm (198).

Bishop (12) demonstrated that mature biofilms generate a dynamic redox potential gradient throughout the structure. At the biofilm core there was a 25 % diffusivity rate for oxygen, while biofilm/liquid interface exhibited 90% diffusivity. Such a structure creates a habitat for a heterogeneous and dynamic microbial population (12). At the liquid-biofilm interface there may be a high population of multiplying aerobic

cells. Towards the attachment surface where there are less available nutrients and oxygen, a niche is created for a population with various metabolic rates and processes that can recycle cell components and survive in low redox potential. The diverse communities or guilds form a food web creating symbiotic relationships. Other research indicates that the coexistence of microbial species in a biofilm depends on the ability of the microbial species to attach to a substratum and out-compete the competitors (185). Biofilms of mixed cultures are thicker and more stable to environmental stress than monospecies biofilms. The stabilization of mixed culture biofilms may be due to the production of a variety of EPS materials that result from the activity of different microorganisms (115).

Most of these investigations focused on biofilms in water and waste water systems. The implication of microbial diversity in food industry biofilms has not been determined. Some processing plant biofilms form in environments likely to have high microbial diversity (i.e. a floor drain), whereas other biofilms form in environments likely to be dominated by only one or a few microbial species (i.e., a plate heat exchanger). Jeong and Frank (97) grew multispecies biofilms containing *L. monocytogenes* and biofilm microflora from dairy and meat processing plants. This study demonstrated that *L. monocytogenes* is able to survive and grow at 10 and 21 ºC in the mixed microflora.

Hydrodynamics influence biofilm structure. After bacterial attachment, flow rate or shear force of the liquid environment affect biofilm structure and content. In the intracellular transport, molecular diffusion is dominant within laminar flow (25, 49).

Laminar flow causes patchy and rounded cells aggregates that are separated by cell free spaces , whereas turbulent flow produces patchy and elongated structures with streamers (49). High turbulence is also associated with increased EPS production (118) even though adhesion generally decreases with increased shear.

Detection methods

Biofilm development and structure has been analyzed using various methods. Biofilms have been grown in capillary tubes and flow through devices. Light, fluorescence (13, 101, 111), differential interference contrast, transmission electron (174), scanning electron (31), atomic force, and confocal laser scanning microscopy (CLSM) (12, 185, 198, 203) are used to analyze biofilm structure. Micro-electrodes can detect the presence of O_2 and observe molecular diffusion within the biofilm. Molecular biological methods including 16-23S rRNA hybridization and fluorescent in-situ hybridization (FISH) with CLSM (49) has been used to observe microstructure and metabolism of biofilms (198). The FISH method was used to confirm the decrease in viability of cells as the biofilm ages. Researchers were able to detect viable cells in the biofilm and determine that young biofilm had about 80% viable cells and about 50% in old biofilm (198).

The structure of biofilms found in the food industry has not been sufficiently studied to determine if the discrete model applies. This model was developed through observations on water and waste water biofilms that are probably unlike many biofilms found in food processing facilities. The presence of high levels of nutrients, macroscopic and microscopic deposits of food residues, and frequent stress from

cleaning, sanitizing or processing treatments will all influence biofilm structure. The influence of these factors is not accounted for in current biofilm theory.

Factors influencing detachment

Layers of biofilm can detach through erosion, sloughing and shear (35). As biofilm matures, it thickens creating an anaerobic environment on the interior. Bryers (25) suggests that the anaerobic condition results in an increase in acid and insoluble gas accumulation which weakens the biofilm structure causing sloughing of polymer layers from the supporting surface. Biofilm sloughing may also occur when there is an imbalance or fluctuation of nutrients. Low carbon availability can cause increased EPS production (111) which leads to detachment. High levels of available carbon can also trigger sloughing off. Shear force aids in sloughing off, especially for biofilm with the mushroom type structure and peripheral streamers (198). Bryers (25) states that after biofilm reaches a certain thickness, the rate of biofilm removal increases under constant laminar flow. Chae and Schraft (34) evaluated the growth and biofilm production of different strains of *L. monocytogenes* attached to glass slides in static conditions at 37ºC over a 10 day period. They noted that overall, there was a cycle of cell population and biofilm production. There was an initial increase in population within the first 3 days then the cell numbers and EPS production decreased. After 6 days, there was a resumption in population and biofilm production.

FOODBORNE PATHOGENS AND SPOILAGE MICROORGANISMS IN BIOFILMS

Listeria monocytogenes

L. monocytogenes is a hardy pathogen with ability to proliferate in cold wet environments that are ideal for biofilm formation. Listeria forms biofilms in pure culture (Figure 1.3), and can survive and grow in multispecies biofilms (13, 34, 36, 42, 64, 130). *L. monocytogenes* forms biofilms on stainless steel, plastic and polycarbonate surfaces and many other food contact surface materials. (52, 68, 85, 92, 98, 111, 130, 144). Therefore, *Listeria* species are well suited for growth and survival in various microniches found in food processing facilities. *L. monocytogenes* was isolated from the wooden shelves in a cheese ripening room (148) implicated in a listeriosis outbreak. *Listeria* has been isolated from environmental surfaces such as conveyor belts, floor drains, condensate, storage tanks and hand trucks (36, 42, 144). These are all surfaces on which biofilm is expected to have formed.

Nelson (144) and Charlton et al. (36) in 1990 isolated *Listeria* spp. throughout dairy processing plants, on processing and packing equipment and especially in wet difficult-to-clean environments such as conveyor belts and drains. A survey of milk processing plants in California in 1987 (24) showed that of the 156 plants sampled, 46 plants or 29.5% were positive for Listeria. Surveys of meat processing facilities produce similar findings with *Listeria* spp. found on various wet environments particularly in drains, conveyor belts, and ceilings that collect condensate. Meat processing equipment such as frankfurter casing strippers, conveyor belts and rollers, slicer blades and

packaging equipment are difficult to clean and are wet for extended periods of time, so provide ideal conditions for biofilm development and good harborage for *Listeria* spp. (146).

Listeria is found in vegetable processing facilities, as it is brought into the plant with soil and raw product. The potential hazard was demonstrated by outbreaks of listeriosis associated with broccoli and coleslaw (88, 116). As with meat and dairy processing, *Listeria* survives and grows in the wet, cold temperatures present in vegetable processing and storage environments (171).

Although *L. monocytogenes* has been isolated from suspected biofilm-forming growth niches in many food processing facilities, direct evidence that the presence of pathogen-containing biofilms leads to disease outbreaks is lacking. Most likely, the growth of *L. monocytogenes* in food plant biofilms increases the general contamination level in the food plant and is indicative of unsatisfactory cleaning/sanitizing procedures. Such conditions ultimately put exposed product at risk. Recent outbreaks of listeriosis, and salmonellosis have implicated post processing contamination of cheese, milk, hotdogs and ice cream as a contributing factor (24, 82).

***Pseudomonas* spp.**

Pseudomonads are ubiquitous spoilage organisms. They are found in food processing environments including drains and floors, on fruits, vegetables, meat surfaces and in low acid dairy products (24, 44, 91, 159). *Pseudomonas* spp. produce copious amounts of EPS and has been shown to attach and form biofilms on stainless steel

surfaces (7). They co-exist within biofilms with *Listeria, Salmonella* and other pathogens (4, 64, 97, 98).

Pantoea agglomerans

Pantoea agglomerans is ubiquitous in nature. It is found in the air of animal houses and in food processing plants especially in foods of plant origin (for example, edible seaweed) (142, 204). *P. agglomerans* are prolific biofilm producers and are often found in mixed microflora including Pseudomonads and other Enterobacteriaceae (29, 47). Del Campo et. al demonstrated that *P. agglomerans* co-exist in the presence of *L. monocytogenes* biofilm (29)

***Bacillus* spp**.

Bacillus is found throughout dairy processing plants (150). *Bacillus* survives heat processing and accumulates on pipelines and joints in the processing environment (98). If hot fluid continuously flows over a surface for over 16 h, *Bacillus* and other thermoduric bacteria may form a biofilm (69).

***Salmonella* spp.**

Salmonella can be isolated from poultry processing equipment especially in the slaughter and evisceration area (86, 104). The poultry processing operation is a wet environment and therefore ideal for biofilm formation. There is little information on the presence of *Salmonella* in biofilms in food processing environments. However, various studies (86, 101, 104) show that *Salmonella* can attach and form biofilms on surfaces found in food processing plants including plastic, cement and stainless steel.

BIOFILM REMOVAL AND CONTROL

Nutrient and water limitation, equipment design, and temperature control are important in biofilm control. Unfortunately, it is often not possible to reduce water availability, improve equipment design, or reduce operating temperatures, so biofilm control efforts most often focus on effective cleaning of potential growth sites (69). Biofilms will eventually form in wet areas even with minimal nutrients, but the presence of nutrients enhances growth. Once biofilms are allowed to form, cleaning the surface becomes more difficult because of the presence of adherent EPS.

Cleaning procedures should effectively remove food debris and other soils that may contain microorganisms or promote microbial growth. Most cleaning regimens include removal of loose soil with cold or warm water followed by the application of chemical cleaners, rinsing, and sanitation (69). Cleaning can be accomplished by using chemicals or a combination of chemical and physical force (water turbulence or scrubbing). High temperatures can reduce the need for physical force. Chemical cleaners suspend and dissolve food residues by decreasing surface tension, emulsifying fats, and peptizing proteins. The mechanism by which cleaning agents remove EPS associated with biofilms has not been determined.

Cleaning

Most chemical cleaning agents used in the food processing industry are alkali compounds that act as detergents for fat and protein. They can be used in combination with sequestrant or chelators and anionic wetting agents (compatible with acid or alkali cleaners). Many situations require the occasional use of acid cleaners to clean surfaces

soiled with precipitated minerals or having a high food residue/mineral content (i.e. milkstone). Nonionic wetting agents are used in some formulations since they are good emulsifiers and control foaming. Sequestrants, such as sodium phosphate derivatives, are often required to chelate minerals depending on water hardness. Chlorine compounds added to alkali help peptize protein. Cleaning compounds must be formulated with care, as many components are incompatible or most effective if applied separately (65, 122). Lewis (122) suggests that surfaces such as glass, ceramics and stainless steel should be cleaned with alkali or nonionic detergents; additionally, stainless steel should be cleaned with alkali or acid detergents. For plastics, alkali cleaners and nonionic detergents are recommended. Manufacturers recommend that detergent solutions be applied at temperatures between 40 and 90ºC depending on soil type and the risk of redeposition. Jackson (95) recommended that solution temperatures above 70ºC be used to clean milk pipelines and above 77ºC for cleaning pasteurizer and heat exchanger systems. Caution should be taken when using hot cleaning solutions, since soils high in carbohydrates and proteins may cook onto or attach more firmly to the surfaces. On the other hand if solutions are not sufficiently hot, soil can redeposit. The cleaning process can remove 90% or more of microorganisms associated with the surface, but cannot be relied upon to kill them. Bacteria can redeposit at other locations and given time, water and nutrients can form biofilm. Therefore, sanitation in addition to cleaning must be implemented (74).

In most food processing plants, food contact surfaces are cleaned and sanitized daily, however, many environmental surfaces such as storage tank and pump exteriors,

walls, and ceilings are cleaned infrequently. This infrequent cleaning provides the opportunity for biofilm formation if moisture is present. An effective cleaning procedure must break up or dissolve the EPS matrix associated with the biofilm so that sanitizing agents can gain access to the viable cells. Little is understood about the effectiveness of cleaning processes as currently used in the food industry to remove biofilm in this manner. Wirtanen et al. (200), Gibson, et al., (74) and Schwach and Zottola (176) provided evidence that mechanical and chemical treatments can destroy biofilms. The removal of *Bacillus* biofilm was influenced by flow rate, time and temperature of cleaning and the presence of chelators in the cleaning solution. Wirtanen et al.(200) found that alkali cleaning especially with chelators such as EDTA were more effective than acid cleaning in removing biofilm. Superheated water (125ºC, 30 min) was the most effective cleaning method even though it did not completely remove a 3-day old biofilm (200). However, Gibson et al. (74) found that alkali and acid cleaners were ineffective in removing *Pseudomonas* and *Staphylococcus aureus* biofilms on stainless steel, as they obtained only a 1 log reduction of microorganisms. In comparison, Dunsmore (56) observed a 3 log reduction of bacteria from a milk-soiled stainless steel surface on which biofilm had not formed. These studies suggest that prolonged cleaning with alkali cleaners containing chelators is necessary to remove biofilm. It can also be concluded that sanitizer application is essential to inactivate microorganisms remaining on the surface after cleaning (56, 57).

Sanitizing

The major types of sanitizers used in the food industry are halogens, peroxygens, acids and quaternary ammonium compounds. Effectiveness of chemical sanitizers is limited by the presence of soil, water hardness, temperature of applications and ability to physically contact the surviving microorganisms (74, 111).

Chlorine is commonly applied as a sanitizer due to its oxidizing and disinfecting power (51). Its most toxic form, hypochlorous acid (HOCL) is generated from hypochlorite ion at pH 4-7. Schwach and Zottola (176) found that sanitation with 150 ppm chlorine was not sufficient to remove a *Salmonella* biofilm matrix (EPS material) from stainless steel even though the cells were apparently killed. De Beer et al. (51) noted that chlorine could not fully penetrate a *Pseudomonas- Klebsiella* mixed biofilm (400 μm thick) after one hour of exposure. They suggested that the biofilm matrix itself inactivated the chlorine. LeChevallier et al. (120) reported that biofilms in drinking water distribution systems were not inactivated with a residual chlorine of 5 ppm. It took 15-20 ppm residual chlorine to control biofilm fouling of reverse osmosis membranes. Chlorine is less effective on older *Listeria* biofilms (121), and on abraded stainless steel and mineral resin surfaces with a high bacterial load (70), as well as, on ropy lactic acid bacteria biofilms (131). Ronner and Wong (166) found that chlorine and anionic sanitizers were better able to remove *Listeria* and *Salmonella* EPS material from stainless steel than quaternary ammonium compound (QAC) and iodine. Gelinas et al. (71) suggested that increasing the contact time for chlorine sanitizers from 5 to 30 min would greatly improve the efficacy of chlorine as demonstrated with *Pseudomonas* biofilm on

stainless steel. Chlorine is readily inactivated by organic material, so the presence of soil and biofilm may significantly reduce its effectiveness. Chlorine dioxide and chloramines are also used as sanitizers in the food industry. LeChevallier et al. (120) and Samrakandi et al. (172) found that monochloramine was better able to penetrate bacterial biofilm than chlorine, but chloramines require longer contact time for effectiveness.

QACs are cationic surfactant sanitizers and also have cleaning activity (135). They are often applied as a foam, which provides longer contact times on surfaces such as pipes, walls and ceilings than does water application. QAC is effective against gram positive and gram negative bacteria, molds, and yeast (32). It is non- corrosive, non-irritating and its activity is unaffected by organic load. QAC is not recommended for use in processing plants that use starter cultures because the residues inhibit these cultures. McCarthy (133) demonstrated that 400 ppm QAC, for 5 min contact time was required to inactivate *L. monocytogenes* biofilm on chitin while, work by Frank and Koffi (68) showed that *Listeria* biofilm treated with 800 ppm of QAC for 20 min was not completely inactivated. Quaternary ammonium compounds are often recommended for floors, walls and storage containers, surfaces which can be sanitized for long contact times, and for surfaces that do not require rinsing before production (nonfood contact surfaces) (75).

Peroxygen sanitizers include hydrogen peroxide and peracid compounds. Hydrogen peroxide is a broad spectrum sanitizer. It is both bactericidal and active against bacteria endospores (134). Peracetic acid (PAA) is the most widely used of the

peracid sanitizers. PAA is a more potent biocide than hydrogen peroxide and is often more effective than chlorine since it maintains activity with an organic load (134). Peracid sanitizers are often used for cold disinfection because of their activity at low temperatures. Peroxide based sanitizers were found to be more effective against *L. monocytogenes* and *Salmonella* spp. in a biofilm matrix than was hypochlorite (81). However, this study reported that *L. innocua* showed resistance to the peroxide sanitizer . Fatemi and Frank (64) determined the efficacy of various sanitizers on *Listeria/Pseudomonas* biofilms attached to stainless steel surfaces in the presence of milk soil. They found that peracetic acid was more effective than chlorine in inactivating *L. monocytogenes* in the milk-*Pseudomonas* biofilm. Richards (163) and Makela et al. (131) reported that PAA and QAC were more effective in inactivating *Listeria* biofilm and ropy lactic acid bacteria in dairy plants than hypochlorite. However, Rossoni and Gaylarde (168) showed that hypochlorite was more effective in inactivating a mixed culture of *E. coli, P. fluorescens and S. aureus* attached to stainless steel than peracetic acid. In general, peracetic acid has been found to be effective against biofilm bacteria, and is advantageous to use if the biofilm contains food residues.

Acid-anionic sanitizers such as phosphoric, sulfamic and acid blends are applied at a pH below 3 (75). They are fast acting on yeast and viruses but slower acting on bacteria. Anionic sanitizers have good wetting ability, are relatively unaffected by organic load or hard water, are non-corrosive, and can solubilize mineral films. They are often used in clean -in-place (CIP) systems, though not always on a daily basis. Gelinas et al. (71) showed that when an anionic sanitizer was applied at temperatures above 20º

C, its efficacy was greatly improved . Anionic sanitizers are neutralized by alkali cleaner residue and by cationic surfactants (32, 75). Frank and Koffi (68) found acid anionic santizers to be ineffective against *L. monocytogenes* biofilms.

Sanitizer selection should be based on whether or not a biofilm is likely to be present and the organic load likely to associated with the biofilm. All approved sanitizers work well in biofilm-free low organic load systems.

Equipment design

Ideally, equipment should be designed to prevent the accumulation of soil and allow for ease of cleaning, so that biofilms will not develop. Equipment must be fabricated using appropriate materials such as stainless steel and teflon. Proper layout of the processing equipment as well as process automation and installation of CIP system may minimize cleaning problems (75, 95). Unfortunately, such designs are sometimes either not practical or not implemented. Cleaning problems often occur at dead ends and where gaskets must be used, such as pumps and joints. Such locations may not receive sufficient exposure to cleaning and sanitizing chemicals to remove soil and kill microorganisms. Surviving microorganisms are then provided with sufficient nutrients to form a biofilm that resists subsequent cleaning and sanitation. Generally, food processing plants employing well designed equipment with effective cleaning programs will not have biofilm formation on food contact surfaces. In fact, a survey by Gibson et al. (74) found that biofilm formation within processing plants occurred only on environmental surfaces such as drains, walls and not on food contact surfaces even though there was bacterial attachment to those surfaces.

Formation of viable aerosols is often a by-product of cleaning. Aerosolization provides a means of dispersal of microorganisms present in biofilms. Aerosols are formed during the washing and spraying of surfaces and drains, or when biofilms dry and release dust particles (107). High pressure, low volume water is usually used to rinse surfaces, however flow above a pressure of 17.2 bars does not enhance biofilm removal (74). Flooding of a floor drain produced an aerosol that increased air-borne microflora for 40 minutes (106). Spurlock and Zottola (182)demonstrated that 210 min after aerosol generation, *L. monocytogenes* was still detectable in the air.

Biofilm detection

Various methods are used to detect and monitor the microbial load on surfaces in food processing plants. The conventional methods include plating of swabbing solution, contact plates and the dipstick technique. In general, these methods are easy to use and inexpensive. In the swab plating method, moistened swabs or sponges are used to remove microflora from the surfaces. The sample liquid is then plated onto plate count agar or a selective medium, incubated and colonies are enumerated and identified if desired. The advantage of this method is that with selective media, specific bacteria, yeast and mold can be isolated and identified. The major disadvantages are that the method is time consuming and, microorganisms may be selectively removed from the surface (34, 201). Contact plating directly samples a surface by pressing a plate of solidified agar against the surface. This method is simpler than swabbing, but it is not possible to sample irregular or rough surfaces, the very types of surfaces likely to harbor biofilms. The limitation of the method depends on how much pressure is applied to the

agar, contact time, presence of soil and if the agar picks up the bacterial contaminant (58). In addition, microorganisms do not quantitatively adhere to the agar surface upon application, again resulting in selection for a specific microflora or underestimating microbial numbers on the sampled surface.

ATP bioluminescence test is a rapid biochemical method for estimating total ATP collected by swabbing a surface. Total ATP is related to the amount of food residues and microorganisms collected by the swab. ATP from microbial cells and food residues reacts with the luciferin-luciferase resulting in emission of light, the intensity of which is related to the amount of ATP. A result can be obtained in 5 to 10 minutes. ATP bioluminescence is a good method for rapid determination of cleaning effectiveness, since both food residues and microorganisms are detected. Since the test is rapid, immediate corrective action can be taken. The ATP bioluminescence test cannot detect low levels of microorganisms, for example, more than 10^3 bacteria or 10 yeast cells must be collected by the swab to have positive results (192). There is no practical method for quantitative determination of biofilm microorganisms in the food industry environment. This is because swabs and sponges do not quantitatively detach firmly adherent microflora. However, swab and sponge sampling provide useful information on the extent of microbial growth on a surface and on the extent to which cleaning has been effective.

Consequences of biofilm development

Growth of biofilms in food processing environments leads to an increased opportunity for microbial contamination of the processed product. This increases the risk

of reduced shelf life and disease transmission. Microorganisms within biofilms are protected from disinfectants (68, 133, 166), increasing the likelihood of survival and subsequent contamination of food. EPS associated with biofilms that is not removed by cleaning provides attachment sites and nutrients for microorganisms newly arrived to the cleaned system (91). Wong (202) reported that undesirable microorganisms such as *Lactobacillus curvatus* and *Lactobacillus fermentum* persisted on milk residues in cheese processing plants even after repeated cleaning, subsequently contaminating products. Reduction in the efficiency of heat transfer (141) can occur if biofilms become sufficiently thick at locations such as plate heat exchangers. Some microorganisms in biofilms catalyze chemical and biological reactions causing corrosion of metal in pipelines and tanks.

Microorganisms on wet surfaces have been observed to aggregate and grow into microcolonies, form three dimensional structures and communal relationships, resulting in a complex biofilm. However, much of what we know about biofilms has been learned from studies of nutrient limited natural environments and simulations of these environments that are unlike what is often found in the food industry. The significance of biofilms in food processing is not well understood because of a lack of direct observation of biofilms in this environment and lack of research using model systems that closely simulate the food system environment. Food-borne pathogens and spoilage organisms can to attach to, and produce EPS on many food contact and environmental surfaces . Pathogenic bacteria can coexist within a biofilm with other environmental microflora; an example of this, is *L. monocytogenes* surviving in *Pseudomonas* biofilms.

Biofilms are difficult to remove from food processing surfaces and environments due to the production of EPS materials and the difficulties associated with cleaning complex processing equipment and processing environments. Therefore, biofilm control relies on the implementation of effective cleaning and sanitizing procedures, and on a design of processing equipment and the food processing environment that allows easy and thorough soil removal. Effectiveness of biofilm removal can be monitored using ATP-bioluminescence for rapid results or plate count procedures for sensitive results .

PREDICTIVE MICROBIOLOGY

Microbial risk assessment and predictive microbiology

Microbial risk assessment follows the same format as chemical risk assessment which includes the following steps: hazard identification and assessment, exposure assessment, dose-response assessment, risk characterization, and risk management (79). Predictive microbiology is a critical tool used in the risk assessment process of foods. It is used to evaluate and answer questions about the safety and shelf life aspects of food. Predictive microbiology is an essential means to assess food safety hazards before, during and after food processing by providing quantitative data to establish qualitative controls (critical control points and specific limits) in the HACCP management (6, 132, 139). It provides the scientific basis for studying the distribution, growth, survival or decline of spoilage microorganisms and pathogens in raw materials, changes in their population during food manufacturing, distribution, and consumer preparation. The quantitative data collected can be analyzed statistically and the generated models used to predict growth or survival of the microorganisms within evaluated parameters. The

models describe a mathematical relationship between the bacteria and their environment. Conventional methods for the detection of pathogens are slow and expensive and are usually performed at the end point of a process (195).

History of predictive microbiology

Predictive modeling is a technique to quantitatively analyze microbial behavior such as growth, survival or inactivation in response to time, temperature and other independent variables (139). According to McMeekin (139), the origins of predictive modeling started with Esty and Meyer (60) determining the thermal processes required to inactivate 10^{12} C. botulinum spores, which is known as the 12D process. The kinetic models were then introduced in the 1960's and 70's to address food spoilage issues which lead to the development of the probability models, growth models, growth/no growth interface models and the non-thermal death models (139, 140, 167).

Models are categorized as primary, secondary and tertiary (physiological) models. The primary model usually describe the changes in biomass over time and is represented as an microbial growth or inactivation curve or the probability of growth. Examples of the mathematical functions used in the primary growth models are the Gompertz and the Baranyi models (6, 197). In the primary thermal inactivation models, the rate of inactivation is usually described by first order kinetics. Examples are the log-linear thermal death calculations (D- value), exponential and exponential-tailing models, however these models usually ignore either the shoulder or tail effect of the inactivation curve or both(124).

Secondary growth models usually explain the growth or death kinetics due to limiting factors (temperature, NaCl, pH, a_w) and their interaction effect in the food environment (139). The square root, the Arrhenius type, modified Gompertz and the polynomial functions are some of the functions used (119, 140, 167, 180, 197). Secondary model ,for example, the modified Gompertz function accounts for the shoulder and most of the tailing effect.

The tertiary or physiological models are integral models that take into account the physiological response of microorganism to its growth or survival environment, for example, considering the effects of osmotic stress followed by acid stress preservative treatment or including the physical structure of the food in the model (73, 138, 139, 197).

Mathematics of predictive microbiology

Mathematical model development is based on the probability distribution of the population data. Normal or bell shaped distribution assumes that 68% of the data lies within one standard deviation of the mean and 95% of the data lies within two standard deviations of the mean. In non-normal distribution, the distribution deviates from the mean showing a positive or negative skew about the mean or demonstrates a discrete distribution (193) .

Some of the mathematical functions used to develop predictive models are as follows:

Linear functions describe a relationship between a response variable (Y) to one or more explanatory variables. This function assumes that the data distribution is normal that is the distribution occurs around the mean or center of the data. Some of the functions used

to analyze the normally distributed data are simple Arrhenius or first order kinetics which is used to calculate the inactivation of microorganisms (175). This model explains the relationship between the log reduction of microorganisms over time : dN/dt= -K (t) so, the $\log_{10}$ N= -kt + ln. N_o. When $\log_{10}$ N is plotted versus (t), a straight line with slope -k(t) is formed. The reciprocal of the slope (1/-k(t)) is the D- value or the thermal death time (153). The D- value is the time, at a specific temperature, for 1 log reduction in microbial load. D- values are calculated from the slope of the best fitting line using linear regression. This is the ideal thermal death model, but in reality there maybe shoulders or lag and tailing in the actual inactivation data (108).

Most microbial survival data in thermal inactivation studies show non-linear or skewed distribution. A non-linear model is used when the data is not normally distributed and there is more variance from the mean than in a normal distribution. Such continuous distributions include beta, gamma, exponential, log-normal, exponential, and Weibull distributions (114, 153). Microbial distribution can also be in the discrete form such as binomial distribution, meaning that the outcome or response may have two possible values, failure or success. So the conditional mean is bound between 0 and 1. This distribution is used to obtain the probability *p* of success in *N* trials and assumes that *p* is independent in each trial (93) .

Features of discrete logistics regression

Logistic regression analysis can be used to evaluate and predict microbial growth, survival, dose response or sporatic population outburst (26). Continuous logistic equations are used to explain microbial growth while discrete logistics are used to

explain random outbursts of population (152). Discrete logistics regression follows the same principle as linear regression where there is a relationship between the response variable (*Y*) and the explanatory variable (x). The difference is that the response variable (*Y*) is binomial or dichotomous, that is, there is either growth or no growth. Therefore *Y* is a binary response variable and lies between 1 and 0 depending on what events occurred. So, logistic regression describes the relationship between the explanatory variables (x) and the binary response variable *(Y)*. The binary response variable *(Y)* is estimated by transforming the maximum likelihood estimator *(Z)*. Z yield values for the unknown predictor variables β and combines the x's. Z is expressed as, $z= \beta o + \beta 1 * x$. This likelihood function expresses the maximum probability of the observed data being a function of the unknown predictor variable (x). In other words the maximum likelihood estimator closest to the observed values (77). The logistic regression model is the transformed maximum likelihood estimator with the binary response (Y) as the estimator of risk. The logistics regression is expressed as :

$$Pr(Y) = (\exp(\beta o + \beta 1 * x)/ (1+ \exp(\beta o + \beta 1 * x). \text{ or } Ln(P/1-P)= (\beta o + \beta 1 * x)$$

The general assumption in this logistics function is that the distribution of errors is binomial (93, 162) and the conditional mean is bound between 0 and 1.

Requirements and limitations of predictive modeling

When selecting a predictive model certain criteria must be followed. A goodness of fit test must be performed to determine the adequacy of the model or how well the model fits the data; The model must be simple as simplicity of a model is essential for practical usage. Also, there must be parsimony in the number of parameters in the model;

and the model must be able to predict over the range of the data and models should explain the confidence limits of the prediction (195).

The accuracy of the predictive models can be determined or validated with new observations or by splitting the original data. Validation models can be compared with the prediction model through graphical comparisons, by bias factor and accuracy factor calculations. The bias factor is calculated using the average deviation of predicted and observed data points and is expressed as the antilog of the average log of the ratio between predicted and observed data $\{Bf= 10^{(\Sigma \log(\text{observed}/ \text{predicted}/n}\}$(140).

Limitations of modeling

Models are limited by the quality of data collected as well as the statistical analysis method. It must be noted that outbreaks may occur from outlier situations where a pathogen has adapted to a new environmental condition. Some limitations to microbial presence and survival prediction are that many models cannot account for variation among strains and physiological state of cells not incorporated into the model (184, 195).

Application of predictive microbiology

The application of predictive modeling is important for food safety, product development, developing HACCP plans, and educating non-microbiologists since it provides a more accurate prediction in complex situations (195).

In terms of food safety, predictive models determine the risk of microorganisms surviving in a food under predicted conditions. In other words, the assessing of the likelihood, nature and magnitude of microbial survival after food processing (38). Models can also project how to reduce the level of that risk (124, 138, 139).

In the canning industry, thermal process calculation is determined from prediction models based on the inactivation of critical microbial spores found in the food product (5, 186). Ball and Olson (5) and others (186) developed process lethality tables for the canning process. Thermal death time or decimal reduction time (D-values) are first calculated from a semi-logarithmic plot. The D-value is the time required (min) to reduce the microbial load by 1 log at a specific temperature. In canning, the12D thermal process is the minimum process lethality to inactivate *C. botulinum*, that is, allow the probability of survival of 10^{-12} spores (189). The sterilization process is calculated with a z value. Z is the number of degrees (ºF) in temperature required to get one log reduction or the temperature necessary to get a 10 fold change in D-value. A z- value of 18º F is used to calculate a sterilization process (F_o) for a particular product (156, 157).

In New Zealand, the meat industry developed predictive modeling as a scientific basis to set regulatory criteria in processing. They determined, through predictive modeling, that the cooling of hot boned boxed meat to 7ºC allowed only a 2-2.5 log increase in E. coli growth and that was acceptable risk. They also used a predictive model to determine the safety of warming meat to 10ºC after extended chilling period (187).

Today with the consumer demand for minimally processed, safe food products, the development of predictive models to optimize parameters and determine the growth/no growth interface in a food product for a variety of pathogenic microorganisms is critical (15, 138, 139). Bolton and Frank (15) demonstrated the behavior of *L.*

monocytogenes in Mexican style cheese under various conditions, such quantitative information can be used to verify processes or develop new safe processes.

THERMAL INACTIVATION AND RESISTANCE

Thermal inactivation

Thermal processing is one of the most important methods for controlling *L. monocytogenes* in further processed foods since this organism grows and survives refrigeration temperatures and, is more heat resistant than most food borne pathogens (63, 129). It is therefore important to review its heat resistance in different food products. Lou and Yosef (126) summarized a sequence of thermal inactivation studies in the 1950's and '60's of *Listeria* in different food products which conflicted with the results of more recent studies. Those studies (9) showed thermal resistance of *Listeria* at milk pasteurization temperatures (71.7 ºC, for 15 s and 63 ºC for 30 min). Later research with improved methodology provided reassuring evidence that the dairy product pasteurization process specifically high temperature short time HTST (71.1 for 15 sec) process was adequate for food safety (22). In dairy products, studies (21, 22, 33, 55) using heat exchangers, glass tubes, open vessels and sealed capillary tubes show $D_{71.7\,^{\circ}C}$ of 0.7s in raw milk, 2.0s in autoclaved skimmed milk, and 2.7s in whole milk for inoculum size of 10^5 cfu/ml of *Listeria* while the $D_{63.3\,^{\circ}C}$ was 19.9 s. In general, the HTST pasteurization process of dairy products reduces cells to below detectable levels showing ca. 5-10 log reduction (D) whereas, the vat pasteurization method had a 39D (129). The only concern raised from those studies was with re-pasteurized milk which had a $D_{71.7\,^{\circ}C}$ of 1.7s which was higher than that of raw inoculated milk although it fell

within the range of process safety (22). The heat inactivation rates of *L. monocytogenes* in beef and poultry products were significantly higher than in milk.. The heat inactivation in meat was $D_{60\,^{\circ}C}$ between 6.6-8.4 min for two strains of *L. monocytogenes*, NCTC 11994 and ScottA; for frankfurters $D_{70\,^{\circ}C}$ = 2 min; in sausage meat $D_{61\,^{\circ}C}$ = 6.1 min but when cure is added at $D_{62\,^{\circ}C}$ = 7.1 min indicating that protective effects of cure compounds (nitrite, dextrose, lactose corn syrup and 3% NaCl) (63, 129). In a sous-vide study of vacuum packaged minced and whole muscle meat with a heat treatment between 50ºC to 60 ºC, *L. monocytogenes* inactivation rate in the minced meat was $D_{52ºC}$=21.3 min and $D_{56ºC}$ =3.0 min, while for the whole muscle $D_{52ºC}$ was 29 min and $D_{56ºC}$ = 3.3 min (80). In chicken breast meat study, $D_{65ºC}$= 1.71 min and $D_{70ºC}$ = 0.99 (143). The sous -vide study of salmon fillet showed that the heat inactivation of *Listeria* (58-80ºC) had a heat inactivation of $D_{60ºC}$ = 4.23-4.48 min with a z= 6.4 (59, 167). In liquid eggs, the D values at temperatures between 51.5 ºC with hydrogen peroxide, and 57.7ºC were 37.6 and 8.3 min respectively (151). The pasteurization temperature of liquid eggs is important since there is a loss in functional properties at 49ºC at holding time of 30 min . In cabbage juice the resistance was considerably less ($D_{56ºC}$ = 2.04 and 3.64, at ph 4-6) compared to other foods (11). All these studies show considerable heat resistance of *L. monocytogenes* in heat processed foods.

Thermal resistance

Growth conditions of *L. monocytogenes* have significant influence on its thermal resistance. Some of the factors affecting thermal resistance of pathogens include intrinsic factors such as genetic resistance, physiological state of the cell ,as well as, extrinsic

factors such as environmental stresses including sublethal heating, pretreatment of inoculum prior to heating (acid and osmotic shock), growth phase, growth temperature, recovery and heating medium (103)(52).

The analysis of fatty acid profile of *L. monocytogenes* showed that cells grown at high temperatures (> 37ºC) had higher percentage of long chain fatty acid accompanied by increase in heat resistance (105). Other studies (14, 27, 62, 103, 158) demonstrated that the exposure of *Listeria* to temperatures above optimal growth conditions, known as heat shock (ca.45-50ºC) triggers the synthesis of heat shock protein (hsp) resulting in increased thermotolerance. This heat shock condition is transient and is known as adaptive thermotolerance (27). The magnitude of time and temperature of pre-shock exposure seems to have significant effect on degree of thermotolerance and is supported by reports of slow heating rates producing increased thermal resistance (14, 109, 169). Thermal resistance is also affected by the phase of bacterial growth. Cells in the log phase of growth that were heat shocked (48ºC, for 10-20 min) prior to treatment had twice the $D_{55ºC}$ compared to cells in stationary phase (113, 125). Jorgensen (102) demonstrated that heat shocked induced thermotolerance of log phase *Listeria* was dependent on the pH of the heating medium. Cross protection can be inferred from one environmental stress to another. Acid exposure as well as osmotic stress can induce other stress responses including thermotolerance (102, 105, 190). The composition of heating medium have a significant influence on the thermal resistance of bacteria. *Listeria* has high thermal resistance in substrates with high fat and protein content. When grown in dairy products, particularly butter, the D-values were 7 times higher than in

broth (33, 143). The D-value in chicken breast meat was $D_{55^{o}C}$=50.8min and $D_{70^{o}C}$=0.99min; versus in peptone $D_{55^{o}C}$ =20.3min; $D_{70^{o}C}$= 0.103min. The Food and Drug Administration has recommended that if the fat content of a dairy product is greater than 10% that the specified pasteurization temperature must be increased by 2.9º C. For example, ice cream mix must be heated for 79.4ºC for 25 s. Thermotolerance has also been demonstrated in other substrates. *Listeria* survives better after heat treatment on potato substrate than in meat (194). The recovery medium is critical for the detection and enumeration of thermally injured *L. monocytogenes*. Knabel (113) suggests that growth at 43ºC in anaerobic conditions recovers six times more cells than at 37ºC.

MICROBIAL ENUMERATION AND DATA COLLECTION

There are several methods for the collection and enumeration of data. There is direct enumeration, end point testing, fraction negative or quantal enumeration. Direct enumeration involves recording colony forming units serially diluted on agar plates. Endpoint testing or thermal death time curve involve heating a test unit until the point where the inoculated unit is sterile and fraction negative or quantal data as the recording of percent positive growth in tubes or quantal data analysis using MPN (157) .

Removing listeria biofilm from the food and non food contact surfaces is a difficult task due to the nature of biofilm development and confounding effect of food soil. Most biofilm inactivation studies use agar overlay for enumeration (70). This method has its limitation in recovering injured cells, therefore in this study we studied the heat inactivation of *Listeria* biofilm and mixed culture biofilm using the fraction negative method employing enrichment culture. This method is more sensitive to low counts.

Most inactivation studies ignore the fact that thermal death is not a log-linear process, knowing this we propose to develop a predictive model using non-linear logistic statistical analysis to evaluate the survival of *Listeria monocytogenes* in a biofilm after heat exposure.

---

[1] Chmielewski, R.A.N. and J.F. Frank 2003. Portions published in the *Comprehensive Reviews in Food Science and Food Safety* 2:22-32.

References

1. **Adams, J., and R. Mclean.** 1999. Impact of rpoS deletion on *Escherichia coli* biofilms. Appl. Environ. Microbiol. **65:**4285- 4287.

2. **Allerberger, F.** 2002. *Listeria*: growth, phenotypic differentiation and molecular microbiology. FEMS Immunology and Medical Microbiology **1469:**1-7.

3. **Allison, D., and I. Sutherland.** 1987. The role of exopolysaccharide in adhesion of freshwater bacteria. J. Gen. Microbiol. **133:**1319-1327.

4. **Bagge, D., M. Hjelm, C. Johansen, I. Huber, and L. Gram.** 2001. *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. Appl. Environ Microbiol. **67:**2319-2325.

5. **Ball, C. O., and F. C. W. Olson.** 1957. Sterilization in Food Technology. McGraw-Hill, New York.

6. **Baranyi, J., and T. A. Roberts.** 1995. Mathematics of predictive food microbiology. Int. J. Food Microbiol **26:**199-218.

7. **Barnes, L. M., M. F. Lo, M. R. Adams, and A. H. Chamberlain.** 1999. Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. Appl. and Environ. Microbiol. **65:**4543- 4548.

8. **Batt, C. A.** 1999, posting date. Listeria. Academic Press. [Online.]

9. **Bearns, R. E., and K. F. Girard.** 1958. The effect of pasteurization on *Listeria monocytogenes*. Can. J. Microbiol. **4:**55-61.

10. **Best, M., M. E. Kennedy, and F. Coates.** 1990. Efficacy of a variety of disinfectants against *Listeria* spp. Appl. Environ. Microbiol. **56:**377-380.

11. **Beuchat, L. R., R. E. Brackett, D. 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.

12. **Bishop, P. L.** 1997. Biofilm structure and kinetics. Wat. Sci . Tech **36:**287-294.

13. **Blackman, I. C., and J. Frank.** 1996. Growth of *Listeria Monocytogenes* as a biofilm on various food processing surfaces. J. Food Prot. **59:**827-839.

14. **Bolton, D. J., C. M. McMahon, A. M. Doherty, J. J. Sheridan, D. A. McDowell, I. S. Blair, and D.Harrington.** 2000. Thermal inactivation of *Listeria*

monocytogenes and *Yersinia enterocolitica* in minced beef under laboratory conditions and in sous -vide prepared minced and solid beef cooked in a commercial retort. J. Appl. Microbiol. **88:**626-632.

15. **Bolton, L., and J. F. Frank.** 1999. Defining the growth/no-growth interface for Listeria monocytogenes in mexican-style cheese based on salt, pH and moisture content. J. Food Prot. **62:**601-609.

16. **Bos, R., H. C. V. d. Mei, J. Gold, and H. J. Busscher.** 2000. Retention of bacteria on a substratum surface with micro-patterned hydrophobicity. FEMS Microbiol. Lett. **189:**311-315.

17. **Boulange-Peterman, L.** 1996. Processes of bioadhesion on stainless steel surfaces and cleanability: a review with special reference to the food industry. Biofouling **10:**275-300.

18. **Boulange-Peterman, L., B. Barroux, and M. N. Bellon-Fontaine.** 1993. The influence of metallic wettability on bacterial adhesion. J. Adhesion Sci. Technol. **7:**221-230.

19. **Boulange-Petermann, L., J. Rault, and M.-N. Bellon-Fontaine.** 1997. Adhesion of *streptococcus thermophilus* to stainless steel with different surface topography and roughness. Biofouling **11:**201-216.

20. **Bower, C. K., J. McGuire, and M. A. Daeschel.** 1996. The adhesion and detachment of bacteria and spores on food contact surfaces. Trends Food Sci. Tech. **7**.

21. **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.

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

23. **Briandet, R., T. Meylheue, C. Maher, and M. N. Bellon-Fontaine.** 1999. *Listeria monocytogenes* Scott A: cell surface charge, hydrophobicity , and electron donor and acceptor characteristics under different environmental growth conditions. Appl. Environ. Microbiol. **65:**5328-5333.

24. **Brocklehurst, T. F., C. M. Zaman-Wong, and B. M. Lund.** 1987. A note on the microbiology of retail packs of prepared salad vegetables. J. Appl. Bacteriol. **63:**409-415.

25. **Bryers, J. D.** 1987. Biologically active surfaces: processes governing the formation and persistence of biofilms. Biotechnol. Prog. **3:**57-68.

26. **Buchanan, R. L., M. H. Golden, R. C. Whiting, J. G. Phillips, and J.L.Smith.** 1994. Non-thermal inactivation models for *Listeria monocytogenes*. J. Food Sci. **59:**179-188.

27. **Bunning, V. K., R. G. Crawford, J. T. Tierney, and J. T. Peeler.** 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella typhimurium* after sublethal heat shock. Appl. Environ. Microbiol. **56:**3216- 3219.

28. **Butler, J. L., J. C. Stewart, C. Vanderzant, Z. L. Carpenter, and G. C. Smith.** 1979. Attachment of microorganisms to pork skin and surfaces of beef and lamb carcasses. J. Food Prot. **42:**401-406.

29. **Campo, J. D., F. Carlin, and C.Nguyen-The.** 2001. Effects of epiphytic Enterobacteriaceace and Pseudomonads on the growth of *Listeria monocytogenes* in model media. J. Food Prot. **64:**721-724.

30. **Carpentier, B., and O. Cerf.** 1993. Biofilm and their consequences with particular reference to hygiene in the food industry. J. Appl. Bacteriol. **75:**499-511.

31. **Carr, J. H., R. L. Anderson, and M. S. Favero.** 1996. Comparison of chemical dehydration and critical point drying for the stabilization and visualization of aging biofilm present on interior surfaces of PVC distributuion pipe. J. Appl. Bacteriol. **80:**225- 232.

32. **Carsberg, H.** 1996. Selecting your sanitizers. Food Qual. **2:**35-36,61-62.

33. **Casadei, M. A., R. E. 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.

34. **Chae, M. S., and H. Schraft.** 2001. Cell viability of *Listeria monocytogenes* biofilms. Food Microbiol. **18:**103-112.

35. **Characklis, W. G.** 1990. Biofilm processes, p. 195-231. *In* W. G. Characklis and K. C. Marshall (ed.), Biofilms. John Wiley & Sons, New York.

36. **Charlton, B. R., H. Kinde, and L. H. Jensen.** 1990. Environmental Survey for Listeria species in California Milk Processing Plants. J. Food Prot. **53:**198-201.

37. **Cliver, D. O.** 1990. Listeria Monocytogenes, p. 395. *In* D. O. Cliver (ed.), Foodborne Diseases. Academic press, San Diego.

38. **Cohrssen, J. J., and V. T. Covelle.** 1989. Risk Analysis: A guide to principles and methods for analyzing health and environmental risks. The National Technical Information Service, Springfield.

39. **Cole, M. B., M. V. Jones, and C. Holyoak.** 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. J. Appl. Bacteriol. **69:**63-72.

40. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. Ladd, J. C. Nickel, M. Dagupta, and T. Marrie.** 1987. Bacterial biofilm in nature and disease. Ann. Rev. Microbiol. **41:**435-484.

41. **Costerton, J. W., and R. T. Irvin.** 1981. The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. **83:**299-324.

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

43. **Cramton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Gotz.** 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infec. Immun. **67:**5427-5433.

44. **Criado, M. T., B. Suarez, and C. M. Ferreiros.** 1994. The Importance of bacterial adhesion in the dairy industry. Food Tech. **48:**123-126.

45. **Cunliffe, D., C. A. Smart, J. Tsibouklis, S.Young, C.Alexander, and E. N. Vulfuson.** 2000. Bacterial absorption to thermoresponsive polymer surfaces. Biotech Lett. **22:**141-145.

46. **Dalton, H. M., and P. E. March.** 1998. Molecular genetics of bacterial attachment and biofouling. Environmental Biotechnology **9:**252-255.

47. **Daly, B., W. B. Betts, A. P. Brown, and J. G. O'Neill.** 1998. Bacterial loss from biofilms exposed to free chlorine. Microbios **96:**7-21.

48. **Daneshvar, M. I., J. B. Brooks, G. B. Malcolm, and L. Pine.** 1989. Analyses of fermentation products of Listeria species by frequency-pulsed electron-capture gas liquid chromatography. Can. J. Microbiol. **35:**786-793.

49. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847-867.

50. **Daza, M. S. T. d., Y. Villegas, and A. Martinez.** 1991. Minimal water activity for growth of *Listeria monocytogenes* as affected by solute and temperature. Int. J. Food Microbiol **14:**333-337.

51. **DeBeer, D., R. Srinivasan, and P. S. Stewart.** 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. **60:**4339-4344.

52. **Dhir, V., and C. E. R. Dodd.** 1995. Susceptibility of suspended and surface attached *Salmonella enteritidis* to biocide and elevated temperatures. Appl. Environ. Microbiol. **61:**1731-1738.

53. **Dillon, R., T. Patel, and S. Ratnam.** 1992. Prevalence of *Listeria* in smoked fish. J. Food Prot. **55:**866-870.

54. **Donnelly, C. W.** 2003. Getting a handle on Listeria. Food Safety Magazine **8:**18-27.

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

56. **Dunsmore, D. G.** 1981. Bacteriology Control of Food Equipment Surfaces by Cleaning Systems. I. Detergent effects. J. Food Prot. **44:**15-20.

57. **Dunsmore, D. G., and M. A. Thomson.** 1981. Bacteriological Control of Food Equipment Surfaces by Cleaning Systems. II. Sanitizer Effects. J. Food Prot. **44:**21-27.

58. **Elliot, R. P.** 1980. The microbiology of sanitation, p. 51-55. *In* A. M. Katsuyama and J. P. Strachan (ed.), Principles of Food Processing Sanitation. Food Processors Institute, Washington, D.C.

59. **Embarek, P. K. B., and H. H. Huss.** 1993. Heat resistance of *Listeria monocytogenes* in vacuum packaged pasteurized fish fillets. International Journal of Food Microbiology **46:**251-261.

60. **Esty, J. R., and K. F. Meyer.** 1922. The heat resistance of the spore of B. botulinus and allied anaerobes XI. J. Infec. Dis. **31:**650-663.

61. **Farber, J. M.** 1991. *Listeria monocytogenes* in fish products. J. Food Prot. **54:**922-924.

62. **Farber, J. M., and B. E. Brown.** 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. Appl. Environ. Microbiol. **56:**1584-1587.

63. **Farber, J. M., and P. I. Peterkin.** 1999. *Listeria monocytogenes* in meat products, p. 505-564. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria*, Listeriosis, and Food Safety. Marcel Dekker, Inc, New York.

64. **Fatemi, P., and J. F. Frank.** 1999. Inactivation of *Listeria monocytogenes*/*Pseudomonas* biofilms by peracid sanitizers. J. Food Prot. **62:**761-765.

65. **Flemming, H.-C., and T. Griebe.** 2000. Control of biofilms in industrial waters and processes, p. 125-141. *In* J. Walker, S. Surman, and J. Jass (ed.), Industrial Biofouling. Wiley, Chicester.

66. **Fletcher, M.** 1976. The effects of proteins on bacterial attachment to polystyrene. J. Gen. Microbiol. **94:**400-404.

67. **Frank, J., and R. Chmielewski.** 2001. Influence of surface finish on the cleanability on Stainless Steel. J. Food Prot. **68:**1178-1182.

68. **Frank, J., and R. Koffi.** 1990. Surface-adherence growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J. Food Prot. **53:**550-554.

69. **Frank, J. F.** 2000. Control of biofilm in the food and beverage industry, p. 239. *In* J. Walker, S. Surman, and J. Jass (ed.), Industrial Biofouling. John Wiley & Sons Ltd., Chicester, New York.

70. **Frank, J. F., and R. A. Chmielewski.** 1997. Effectiveness of sanitation with quaternary ammonium compound or chlorine on stainless steel and other domestic food-preparation surfaces. J. Food Prot. **60:**1-6.

71. **Gelinas, P., J. Goulet, G. M. Tastayre, and G. A. Picard.** 1984. Effect of temperature and contact time on the activity of eight disinfectants- a classification. J. Food Prot. **47:**841- 847.

72. **George, S. M., B. M. Lund, and T. F. Brocklehurst.** 1988. The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. Lett. Appl. Microbiol. **6:**153-156.

73. **Gerwin, S. J. C. v., and M. H. Zwietering.** 1998. Growth and inactivation models to be used in quantitative risk assessments. J. Food Prot. **1:**1541-1549.

74. **Gibson, H., J. H. Taylor, K. E. Hall, and J. T. Holah.** 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J. Appl. Microbiol. **87:**41-48.

75. **Giese, J.** 1991. Sanitation: the key to food safety and public health. Food Tech. **45:**74-80.

76. **Gilbert, P., D. J. Evans, E. Evans, I. G. Duguid, and M. R. W. Brown.** 1991. Surface characteristics and adhesion of *Escherichia coli* and *Staphylococcus epidermidis*. J. Appl. Bacteriol. **71:**72-77.

77. **Gonzalez-Martinez, C., M. G. Corradini, and M. Peleg.** 2002. Probablistic models of microbial safety and nutritional quality. Journal of Food Engineering.

78. **Gravani, R.** 1999. Incidence and control of *Listeria* in food processing facilities. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria,* Listeriosis and Food Safety, 2nd ed. Marcel Dekker, New York.

79. **Haas, C. N., J. B. Rose, C. P. Gerba, and C. S. Crobett.** 1997. What predictive food microbiology can learn from water microbiology. Food Technol. **51:**91-94.

80. **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.

81. **Harkonen, P., S. Salo, T. Mattila-Sanholm, G. Wirtanen, D. G. Allison, and P. Gilbert.** 1999. Development of a simple *in vitro* test system for the disinfection of bacterial biofilm. Wat. Sci. Tech. **39:**219-225.

82. **Hedberg, C. W., J. A. Korlath, J.-Y. D'Aoust, K. E. White, W. L. Schell, M. R. Miller, D. N. Cameron, K. l. MacDonald, and M. T. Osterholm.** 1992. A multistate outbreak of *Salmonella javiana* and *Salmonella oranienburg* infections due to consumption of contaminated cheese. Jama **268:**3203-3207.

83. **Heilmann, C.** 1996. Molecular basis of intracellular adhesion in the biofilm forming *Staphylococcus epiderrmidis*. Mol. Microbiol. **20:**1083-1091.

84. **Heilmann, C., C. Gerke, F. Perdreau-Remington, and F. Gotz.** 1996. Characterization of *TN917* insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. Infec. Immun. **64:**277-282.

85. **Helke, D. M., E. B. Somers, and A. C. L. Wong.** 1993. Attachment of *Listeria monocytogenes*and *Salmonella typhimurium* to stainless steel and buna-N in the presence of milk and individual milk components. J. Food Prot. **56:**479-484.

86. **Helke, D. M., and A. C. L. Wong.** 1994. Survival and growth characteristics of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and buna-N rubber. J. Food Prot. **57:**963- 968, 974.

87. **Herald, P. J., and E. A. Zottola.** 1988. Scanning electron microscopic examination of *Yersinia enterocolitica* attached to stainless steel at selected temperatures and pH values. J. Food Prot. **51:**445-448.

88. **Hines, E.** 1999. *Listeria* control strategies: a brief history of *Listeria monocytogenes*. Food Qual. **5:**32-34.

89. **Holah, J. T., and R. H. Thorne.** 1990. Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. J. Appl. Bacteriol. **69:**599-608.

90. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. Bergey's Manual of Determinative Bacteriology, 9th ed. Williams and Wilkins, Baltimore.

91. **Hood, S., and E. Zottola.** 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. Int. J. Microbiol. **37:**145-153.

92. **Hood, S., and E. Zottola.** 1997. Growth media and surface conditioning influence the adherence of *Pseudomonas fragi, Salmonella typhimurium*, and *Listeria monocytogenes* cells to stainless steel. J Food Prot. **60:**1034-1037.

93. **Hosmer, D. W., and S. Lemeshow.** 2000. Applied Logistic Regression, 2nd ed. Wiley, New York.

94. **Hyde, F. W., M. Alberg, and K. Smith.** 1997. Comparison of fluorinated polymers against stainless steel, glass and polypropylene in microbial biofilm adherence and removal. J. Ind. Microbiol. Biotech **19:**142 -149.

95. **Jackson, A. T.** 1985. Cleaning of Food Processing Plant, p. 95- 125. *In* S.Thorne (ed.), Developments in Food Preservation-3, vol. 3. Elsevier Science Publ. Co., New York.

96. **Jay, J.** 1996. Modern Food Microbiology, 5th ed. Chapman &Hall, New York.

97. **Jeong, D. K., and J. F. Frank.** 1994. Growth of *Listeria monocytogenes* at 10ºC in biofilms with microorganism isolated from meat and dairy processing environments. J. Food Prot. **57:**576-586.

98. **Jeong, D. K., and J. F. Frank.** 1994. Growth of *Listeria monocytogenes* at 21ºC in biofilms with microorganisms isolated from meat and dairy processing environments. Lebensm-wiss. u. technol. **27:**415-424.

99. **Jinneman, K. C., M. M. Wekell, and M. W. Eklund.** 1999. Incidence and behavior of *Listeria monocytogenes* in fish and seafood products. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria,* Listeriosis and Food Safety. Marcel Dekker, New York.

100. **Jones, C. R., M. R. Adams, P. A. Zhdan, and A. H. L. Chamberlain.** 1999. The role of surface physiochemical properties in determining the distribution of the autochthonous microflora in mineral water bottles. J. Appl. Microbiol. **86:**917-927.

101. **Jones, K., and S. B. Bradshaw.** 1997. Synergism in biofilm formation between *Salmonella enteritidis* and a nitrogen-fixing strain of *Klebsiella pneumoniae.* J. Appl. Microbiol. **82:**663-668.

102. **Jorgensen, 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.

103. **Jorgensen, 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 shock-induced protein in *Listeria monocytogenes*. J. Appl. Bacteriol. **80:**216-224.

104. **Joseph, B., S. K. Otta, I. Karunasagar, and I. Karunasagar.** 2001. Biofilm formation by Salmonella spp. on food contact surfaces and their sensitivity to sanitizers. Int. J. Food Microbiol. **64:**367-372.

105. **Juneja, V., T. Foglia, and B. Marmer.** 1998. Heat resistance and fatty acid composition of *Listeria monocytogenes*: Effect of pH , acidulants and growth temperature. J. Food Prot. **61:**683-687.

106. **Kang, Y.-J., and J. F. Frank.** 1990. Characteristics of biological aerosols in dairy processing plants. J. Dairy Sci. **73:**621-626.

107. **Kang, Y.-J., and J.F.Frank.** 1989. Biological aerosols: a review of airborne contamination and its measurement in dairy processing plants. J. Food Prot. **52:**512-524.

108. **Kilsby, D., K. W. Davies, P. J. McClure, C. Adair, and W. A. Anderson.** 2000. Bacterial thermal death kinetics based on probability distributions: The heat destruction of *Clostridium botulinum* and *Salmonella Bedford*. J. Food Prot. **63:**1179-1203.

109. **Kim, K.-T., E. A. Murano, and D. G. Olson.** 1994. Heating and storage conditions affect survival and recovery of *Listeria monocytogenes* in ground pork. J. Food Sci. **59:**30-32, 59.

110. **Kim, K. Y., and J. F. Frank.** 1994. Effect of growth nutrients on attachment of *Listeria monocytogenes* to stainless steel. J. Food Prot. **57:**720- 726.

111. **Kim, K. Y., and J. F. Frank.** 1995. Effect of nutrients on biofilm formation by *Listeria monocytogenes* on stainless steel. J. Food Prot. **58:**24-28.

112. **Kjelleberg, S., B. Humphrey, and K. Marshall.** 1983. Initial phases of starvation and activity of bacteria at surfaces. Appl. Environ. Microbiol. **46:**978-984.

113. **Knabel, S. J., H. W. Walker, P. A. Hartman, and A. F. Mendonca.** 1990. Effect of growth temperature and strictly anaerobic recovery on survival of *Listeria monocytogenes* during pasteurization. Appl. Environ. Microbiol. **56:**370-376.

114. **Kormendy, I., and L. Kormendy.** 1997. Consideration for calculating heat inactivation processes when semilogarithmic thermal inactivation models are non-linear. Journal of Food Engineering **34:**33-40.

115. **Kumar, C. G., and S. K. Anand.** 1998. Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol. **42:**9 - 27.

116. **Kuntz, L. A.** 1995. New bugs on the block. Food Prod. Design **5:**113-121.

117. **Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell.** 1991. Optical sectioning of microbial biofilms. J. Bacteriol. **173:**6558-6567.

118. **Lazarova, V., V.Pierzo, D. Fontvielle, and J. Manem.** 1994. Integrated approach for biofilm characterisation and biomass activity control. Wat. Sci. Tech. **29:**345-354.

119. **Lebert, I., V. Robles-Olvera, and A. Lebert.** 2000. Application of polynomial models to predict growth of mixed cultures of *Pseudomonas spp.* and *Listeria* in meat. Int. J. Food Microbiol **61:**27-39.

120. **LeChevallier, M. W., C. D. Cawthon, and R. G. Ramon.** 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. **54:**2492-2499.

121. **Lee, S.-H., and J. Frank.** 1991. Inactivation of surface adherent *Listeria monocytogenes* hypochlorite and heat. J. Food Prot. **54:**4-6.

122. **Lewis, K. H.** 1980. Cleaning, disinfection and hygiene, p. 232- 258. *In* J. H. Silliker, R. P. Elliot, A. C. Baird-Parker, F. L. Bryan, J. H. B. Christain, D. S. Clark, J. C. Olson, and T. A. Roberts (ed.), Microbial Ecology of Foods, vol. 1. Academic Press, New York.

123. **Lindberg, L. E., B. R. Holmbom, O. M. Vaisanen, A. M.-L. Weber, and Salkinoja.** 2001. Sugar composition of biofilms produced by paper mill bacteria. Appl. Microbiol. Biotech. **55:**638-643.

124. **Linton, R. H., W. H. Carter, M. D. Pierson, C. R. Hackney, and J. D. Eirert.** 1995. Use of a modified gompertz equation to predict the effects of temperature, pH and NaCl on the inactivation of Listeria monocytogenes Scott A heated in infant formula. J. Food Prot. **59:**16-23.

125. **Linton, R. H., M. D. Pierson, and J. R. Bishop.** 1990. Increase in heat resistance of Listeria monocytogenes Scott A by sublethal heat shock. J Food Prot. **53:**924-927.

126. **Lou, Y., and A. Yousef.** 1999. Characteristics of *Listeria monocytogenes* important to food processors, p. 131-224. *In* E. T. Ryser and E. H. Marth (ed.), Listeria, Listeriosis and Food Safety, 2nd ed. Marcel Dekker, New York.

127. **Low, J. C., and W. Donachie.** 1997. A review of *Listeria monocytogenes* and listeriosis. The Veterinary Journal **153:**9-29.

128. **Lunden, J. M., M. K. Miettinen, T. J. Autio, and H. J. Korkeala.** 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact time. J. Food Prot. **63:**1204-1207.

129. **Mackey, B. M., and N. Bratchell.** 1989. The heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. **9:**89-94.

130. **Mafu, A. A., D. Roy, J. Goulet, and P. Magny.** 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces after short contact time. J. Food Prot. **53:**742- 746.

131. **Makela, P. M., H. J. Korkeala, and E. K. Sand.** 1991. Effectiveness of commercial germicide products against the ropy slime-producing lactic acid bacteria. J. Food Prot. **54:**632-636.

132. **Marks, H., and M.Coleman.** 1998. Estimating distribution of numbers of organisms in food products. J. Food Prot. **61:**1535-1540.

133. **McCarthy, S. A.** 1992. Attachment of *Listeria monocytogenes* to chitin and resistance to biocides. Food Tech. **46:**84-88.

134. **McDonnell, G., and A. D. Russell.** 1999. Antiseptics and disinfectants: activity, action and resistance. Clin. Microbiol. Rev. **12:**147- 179.

135. **McEldowney, S., and M. Fletcher.** 1987. Adhesion of bacteria from mixed cell suspension to solid surfaces. Arch. Microbiol. **148:**57-62.

136. **McGuire, J., and V. Krisdhasima.** 1991. Surface chemical influences on protein adsorption kinetics. Food Tech. **45:**92-96.

137. **McLean, R. J. C., M. Whiteley, D. Stickler, and W. C. Fuqua.** 1997. Evidence of autoinducer activity in nauturally occurring biofilms. FEMS Microbiol. Lett. **154:**259-263.

138. **McMeekin, T. A., J. Olley, D. A. Ratkowsky, and T. Ross.** 2002. Predictive microbiology: towards the interface and beyond. Int. J. Food Microbiol **73:**395-407.

139. **McMeekin, T. A., and T. Ross.** 2002. Predictive Microbiology: providing knowledge-based framework for change management. Int. J. Food Microbiol **78:**133-153.

140. **Mellefont, L. A., T. A. McMeekin, and T. Ross.** 2002. Performance evaluation of a model describibg the effects of temperature, water activity, pH and lactic acid concentration on the growth of *Escherichia coli*. Int. J. Food Microbiol **In press**.

141. **Mittelman, M. W.** 1998. Structure and functional characteristics of bacterial biofilms in fluid processing operations. J. Dairy Sci. **81:**2760- 2764.

142. **Moore, J. E., J. Xu, and B. C. Millar.** 2002. Diversity of the microflora of edible macoalga (Palmaria palmata). Food Microbiol. **19:**249-258.

143. **Murphy, R. Y., B. P. Marks, E. R. Johnson, and M. G. Johnson.** 2000. Thermal inactivation kinetics of *Salmonella* and *Listeria* in ground chicken breast meat and liquid medium. J. Food Sci. **65:**706- 710.

144. **Nelson, J.** 1990. Where are *Listeria* likely to be found in dairy plants? Dairy, Food and Environ. San **10:**344- 345.

145. **Netting, J.** 2001. Sticky situation. Sci. News **160:**28-30.

146. **Nickelson, N., and C. Schmidt.** 1999. Taking the hysteria out of *Listeria*: the mechanics of *Listeria* and the strategies to find it. Food Qual. **5:**28-35.

147. **Nilsson, P., A. Olofsson, M. Fagerlind, T. Fargerstrom, S. Rice, S. Kjelleberg, and P. Steinberg.** 2001. Kinetics of the AHL regulatory system in a model biofilm system: How many bacteria constitute a "quorum"? J. Molecular Biology **309:**631-640.

148. **Noterman, S.** 1994. The significance of biofouling to the food industry. J. Food Tech. **48:**13-14.

149. **Oh, D.-H., and D. L. Marshall.** 1995. Destruction of *Listeria monocytogenes* biofilms on stainless steel using monolaurin and heat. J. Food Prot. **57:**251-255.

150. **Oosthuizen, M. C., B. Steyn, D. Lindsay, V. S. Brozel, and A. v. Holy.** 2001. Novel method for the proteomic investigation of dairy-associated *Bacillus cereus* biofilm. FEMS Microbiol. Lett. **194:**47-51.

151. **Palumbo, M. S., S. M. Beers, S. Bhaduri, and S. A. Palumbo.** 1996. Thermal resistance of *Listeria monocytogenes* and *Salmonella* spp. in liquid egg white. J. Food Prot. **59:**1182-1186.

152. **Peleg, M.** 1997. Modeling microbial populations with the original and modified versions of the continuous and discrete logistic equations. Critical Reviews in Food Science and Nutrition **37:**471-490.

153. **Peleg, M., C. M. Penchina, and M. B. Cole.** 2001. Estimation of the survival curve of *Listeria monocytogenes* during non-thermal heat treatments. Food Research International **34:**383-388.

154. **Pesci, E. C., J. B. J. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg, and B. H. Iglewski.** 1999. Quinolone signaling in the cell-cell communication system of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. **96:**11229-11234.

155. **Petran, R. L., and E. A. Zottola.** 1989. A study of factors of affecting growth and recovery of *Listeria monocytogenes* Scott A. J. Food Sci. **54:**458-460.

156. **Pflug, I. J., and R. Christensen.** 1980. Converting an F-value determined on the basis of one z-value to an F-value determined on the basis of a second z-value. J. Food Sci. **45:**35-40.

157. **Pflug, I. J., R. G. Holcomb, and M. M. Gomez.** 2000. Principle of the thermal destruction of microorganisms. *In* S. S. Block (ed.), Disinfection, Sterilization and Preservation, 5th ed. Lippincott, Williams and Wilkins, Philadelphia.

158. **Phan-thanh, L., and T. Gormon.** 1995. Analysis of heat and cold shock proteins in *Listeria* by 2-dimensional electrophoresis. Electrophoresis **16:**444-450.

159. **Piette, J. P. G., and E. S. Idziak.** 1991. Role of flagella in adhesion of *Pseudomonas Fluorescens* to tendon slices. Appl. Environ. Microbiol. **57:**1635-1639.

160. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of Escherica coli biofilm formation:roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285-293.

161. **Ranga, P. J., W.-J. Lin, and E. A. Johnson.** 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. **57:**3046-3048.

162. **Ratkowsky, D. A.** 2002. Some examples of, and some problems with, the use of nonlinear logistic regression in predictive food microbiology. Int. J. Food Microbiol **73:**119-125.

163. **Richards, R.** 1999. Efficacy of sanitizers on *Listeria monocytogenes* biofilms. Food Austral. **51:**624-625.

164. **Rocourt, J.** 1999. The genus Listeria and Listeria monocytogenes: Phylogenic position, taxonomy, and identification. *In* E. T. Ryser and E. H. Marth (ed.), Listeria, Listeriosis and Food Safety, 2nd ed. Marcel Dekker, Inc, New York.

165. **Rocourt, J., and P. Cossart.** 1997. Listeria monocytogenes, p. 337-352. *In* M. P. Doyle, L. P. Beuchat, and T. J. Thomas (ed.), Food Microbiology Fundamentals and Frontiers, 1st ed. ASM Press, Washington, D.C.

166. **Ronner, A., and A. Wong.** 1993. Biofilm development and sanitizer inactivation of *Listeria Monocytogenes* and *Salmonella Typhimurium* on stainless steel and buna-n rubber. J. Food Prot. **56:**750-758.

167. **Ross, T., P. Dalgaard, and S. Tienungoon.** 2000. Predictive modelling of the growth and survival of *Listeria* in fishery products. Int. J. Food Microbiol **62:**231-245.

168. **Rossoni, E. M., and C. C. Gaylarde.** 2000. Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy. J. Food Microbiol. **61:**81-85.

169. **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:**2065-2075.

170. **Ryser, E. T., and E. H. Marth.** 1999. *Listeria*, Listeriosis and Food Safety. Marcel Dekker, New York.

171. **Saguy, I.** 1992. Simulated growth of *Listeria monocytogenes* in refrigerated foods stored at variable temperatures. Food Tech. **46:**69-71.

172. **Samrakandi, M. M., C. Roques, and M. Georges.** 1997. Influence of tropic conditions on expolysaccharide production: bacterial biofilm susceptibility to chlorine and monochloramine. Can. J. Microbiol. **43:**751-758.

173. **Sandu, C., and R. K. Singh.** 1991. Energy increase in operation and cleaning due to heat-exchanger fouling in milk pasteurization. Food Tech. **45:**84-91.

174. **Sanford, B. A., V. L. Thomas, S. J. Mattingly, M. A. Ramsay, and M. M. Miller.** 1995. Lectin-biotin assay to slime present in *in-situ* biofilm produced by *Staphylococcus epidermis* using transmission electron microscopy. J. Ind. Microbiol. **15**.

175. **Schaffner, D. W., and T. P. Labuza.** 1997. Predictive microbiology: Where are we and where are we going? Food Technol. **51:**95-99.

176. **Schwach, T. S., and E. Zottola.** 1984. Scanning electron microscopic study on some effects of sodium hypochlorite on attachment of bacteria to stainless steel. J. Food Prot. **47:**756-759.

177. **Sinde, E., and J. Carballo.** 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. Food Microb. **17:**439-447.

178. **Smoot, L. M., and M. D. Pierson.** 1998. Effect of environmental stress on the ability of *Listeria monocytogenes* Scott A to attach to food contact surfaces. J. Food Prot. **61:**1293-1298.

179. **Smoot, L. M., and M. D. Pierson.** 1998. Influence of environmental stress on the kinetics and strength of attachment of *Listeria monocytogenes* Scott A to buna-n rubber and stainless steel. J. Food Prot. **61:**1286-1292.

180. **Soboleva, T. K., A. B. Pleasants, and G. L. Roux.** 2000. Predictive microbiology and food safety. Int. J. Food Microbiol **57:**183-192.

181. **Sorongon, M., R. Bloodgood, and R. Burchard.** 1991. Hydrophobicity, adhesion and surface-exposed proteins of gliding bacteria. Appl. Environ. Microbiol. **57:**3193- 3199.

182. **Spurlock, A. T., and E. A. Zottola.** 1991. The survival of *Listeria monocytogenes* in aerosols. J. Food Prot. **54:**910-912, 916.

183. **Stanley, P.** 1983. Factors affecting the irreversible attachment of *Pseudomonas aeruginosa* to stainless steel. Can. J. Microbiol. **29:**1493-9.

184. **Steeg, P. F., and J. E. Ueckert.** 2002. Debating the biological reality of modelling preservation. Int. J. Food Microbiol **73:**409-414.

185. **Stewart, P. S., A. K. Camper, S. D. Handran, C. T. Huang, and M. Warnecke.** 1997. Spatial distribution and coexistence of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in biofilms. Microb. Ecol. **33:**2-10.

186. **Stoforos, N. G., J. Noronha, M. Hendrickx, and P. Tobback.** 1997. A critical analysis of mathematical procedures for the evaluation and design of in-container thermal processes for foods. Critical Reviews in Food Science and Nutrition **37:**411-441.

187. **Sumner, J., and K. Krist.** 2002. The use of predictive microbiology by the Australian meat industry. Int. J. Food Microbiol **73**.

188. **Sutherland, I. W.** 1983. Microbial exopolysaccharides- Their role in microbial adhesion in aqueous systems. Crit. Rev. Microbiol. **10**.

189. **Toledo, R.** 1991. Fundamentals of Food Process Engineering, 2nd ed. Chapman & Hall, New York.

190. **Vasseur, C., L. Baverel, M. Hebraud, and J. Labadie.** 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. J. Appl. Microbiol. **86:**469-476.

191. **Vatanyoopaisarn, S., A. Nazli, C. E. R. Dodd, C. E. D. Rees, and W. M. Waites.** 2000. Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Appl. Environ. Microbiol. **66:**860-863.

192. **Verran, J., and M. Jones.** 2000. Problems of biofilm in the food and beverage industry, p. 145- 173. *In* J. Walker, S. Suramn, and J. Jass (ed.), Industrial Biofouling. John Wiley, Cichester.

193. **Vose, D.** 2000. Risk analysis: a quantitative guide, 2nd ed. John Wiley & Sons, Inc, New York.

194. **Walsh, D., J. J. Sheridan, G. Duffy, I. S. Blair, D. A. McDowell, and D. Harrington.** 2001. Thermal resistance of wild-type and antibiotic resistant *Listeria monocytogenes* in meat and potato substrates. J. Appl. Microbiol. **90:**555-560.

195. **Whiting, R.** 1997. Microbial Database Building: What Have We Learned? Food Technol. **51:**1997.

196. **Wiedman, M., J. L. Bruce, C. Keating, A. E. Johnson, P. L. M. Donough, and C. A. Batt.** 1997. Ribotype and virulence gene polymorphisms suggest three distinct *Listeria monocyotogenes* lineages with differences in pathogenic potential. Infect. Immun. **65:**2707-2716.

197. **Wilson, P. D. G., T. F. Brocklehurst, S. Arino, D. Thuault, M. Jacobsen, M. Lange, and J. F. V. Impe.** 2002. Modelling microbial growth in structured foods: towards a unified approach. Int. J. Food Microbiol **73:**275-289.

198. **Wimpenny, J., W. Manz, and U. Szewzyk.** 2000. Heterogeneity in biofilms. FEMS Microbiol. Rev. **24:**661-671.

199. **Wimpenny, J. W. T., S. L. Kinniment, and M. A. Scourfield.** 1993. The physiology and biochemistry of biofilm, p. 51-94. *In* S. P. Denyer, S. P. Gorman, and M. Sussman (ed.), Microbial biofilms: formation and control. Blackwell Scientific Publ., London.

200. **Wirtanen, G., U. Husmark, and T. Matilla-Sandholm.** 1996. Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems. J. Food Prot. **59:**727-733.

201. **Wirtanen, G., and T. Matilla-Sandholm.** 1993. Epifluorescence image analysis and cultivation of foodborne biofilm bacteria grown on stainless steel surfaces. J. Food Prot. **56:**678-683.

202. **Wong, A. C. L.** 1998. Biofilm in food processing environments. J. Dairy Sci. **81:**2765-2770.

203. **Zhang, T. C., and P. L. Bishop.** 1994. Structure, activity and composition of biofilm. Wat. Sci. Tech. **29:**335-344.

204. **Zucker, B. A., S. Trojan, and W. Muller.** 2000. Airborne gram-negative bacterial flora in animal houses. J. Vet. Med. B **47:**37-46.

Figure. 1.1. The process of biofilm formation. Planktonic cells attach to a surface, biofilm genes are turned on and other cells are recruited through quorum sensing. Cells grow on the surface forming microcolonies. Microcolonies continue to grow producing a mature biofilm structure with channels (blue). The green represents bacterial cells and the white and light blue opaque areas represent EPS.

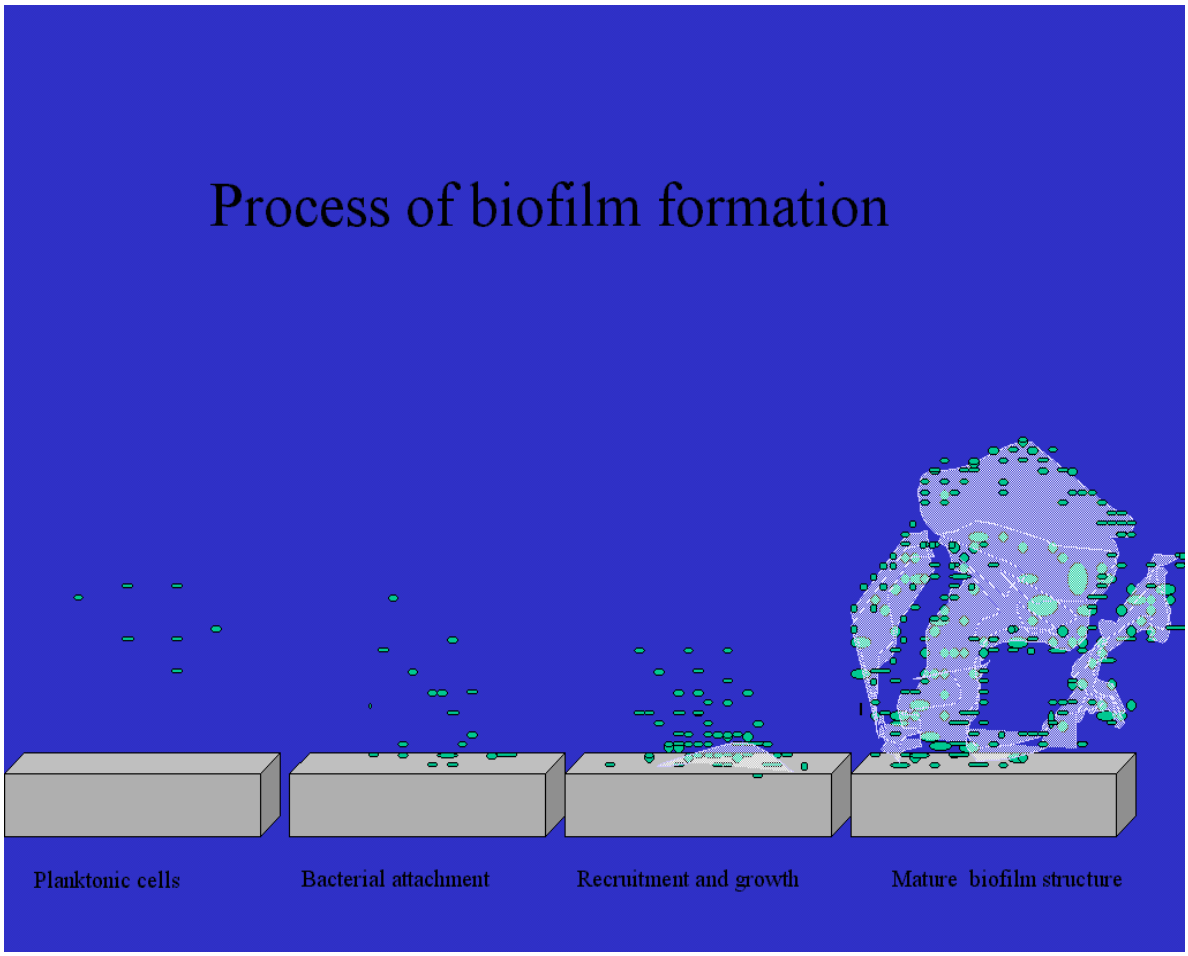
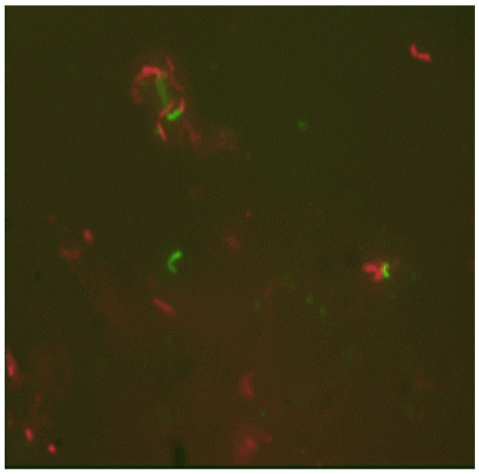
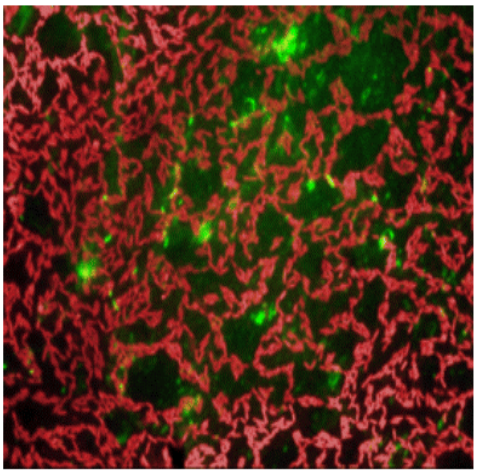

Process of biofilm formation
Planktonic cells
Bacterial attachment
Recruitment and growth
Mature biofilm structure

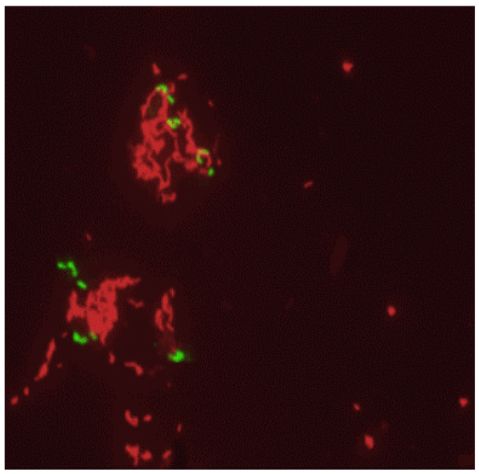
Figure 1.2. Epifluorescent micrographs of four unidentified water biofilm isolates grown on polyvinyl chloride surface with *Campylobacter jejuni*. All biofilms were produced in 7days at 21ºC. Red represents the water biofilm isolates stained with Syto™ red and green represents *Campylobacter* labeled with fluorescent antibody. Images illustrate (a) biofilm at the beginning stage of production (b) a typical mature biofilm structure (c) and (d) different degrees of microcolony formation. Images courtesy of Nathanon Trachoo.



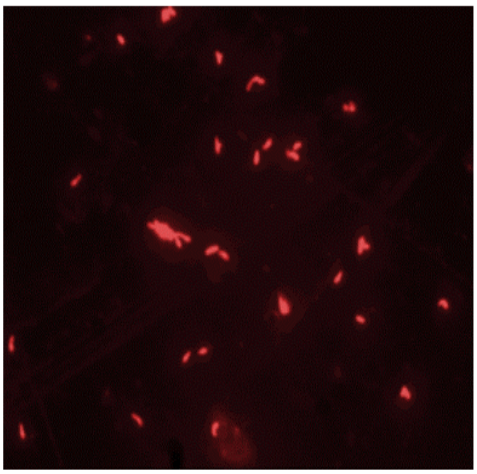
a



b

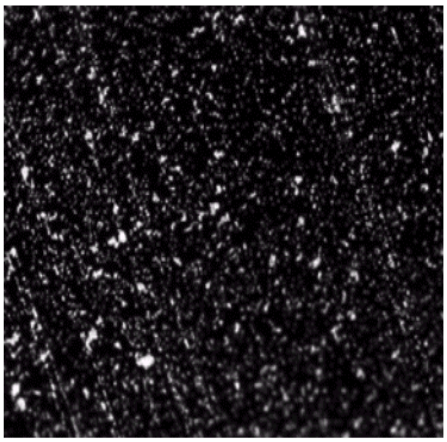


c

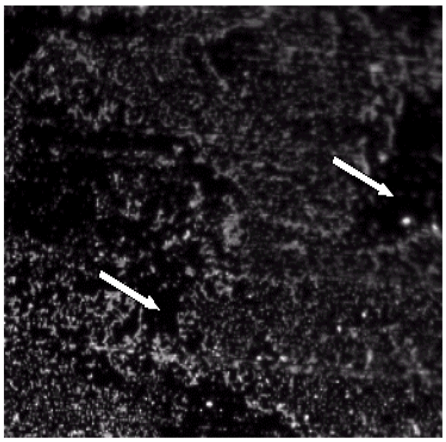


d

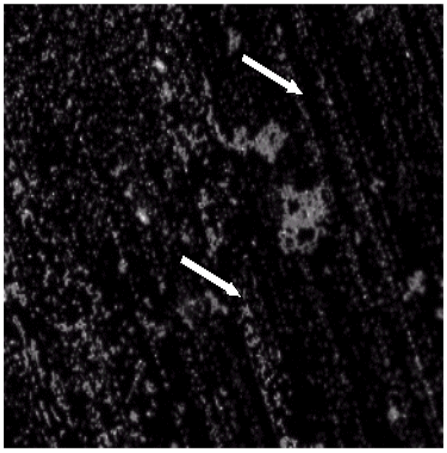
Figure 1.3 Epifluorescent micrographs of biofilm produced on stainless steel by 4 strains of *L. monocytogenes*. Images illustrate (a) confluent growth of biofilm. (b) channels within the biofilm. (c) growth along the striations of the stainless steel. (d) multilayered microcolonies. Images courtesy of James Folsom.



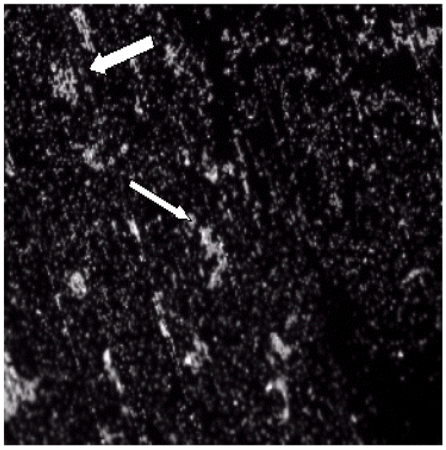
a



b



c



d

CHAPTER 3

A PREDICTIVE MODEL FOR HEAT INACTIVATION OF *LISTERIA MONOCYTOGENES* BIOFILM ON STAINLESS STEEL [1]

[1] Chmielewski, R.A.N. and J.F. Frank 2003. To be submitted to the *Journal of Food Protection*

ABSTRACT

Heat inactivation of bacteria is calculated based on log-linear decimal reduction times. However, inactivation of *Listeria* in a biofilm cannot be assumed as log linear. The purpose of this study is to develop a heat inactivation predictive model for *Listeria monocytogenes* (Scott A and 3990) in monoculture and in multispecies (*Pseudomonas* and *P. agglomerans*) biofilms formed on stainless steel surfaces and in the presence of soil. Biofilms were produced by immersing stainless steel surfaces in diluted Tryptic Soy broth (TSB) inoculated with the appropriate cultures, followed by incubation for 4 h at 25ºC, rinsing with sterile phosphate buffer (0.01M), transferring to diluted TSB and incubation for 48 h at 25 ºC. Duplicate biofilm samples were heat treated for 1, 3, 5, 15 min at 70, 72, 75, 77 and 80 ºC and tested for survivors using enrichment media. Controls were shaken with glass beads and enumerated using Plate count agar and Listeria Selective agar. The experiment was repeated six times. A predictive model was developed using fraction negative data and logistic regression analysis. Probability plots showing the percent probability of *L. monocytogenes* inactivation in biofilms after heat treatment were generated from the predictive equation. The predictive model shows that the hot water sanitation of stainless steel can be effective in inactivating the *L. monocytogenes* in a biofilm on stainless steel if time and temperature are controlled within an enclosed system. This model provides processors with a risk management tool that provides predicted levels of *L. monocytogenes* inactivation and three heat resistance assumptions. The predictive model was validated using a five *L. monocytogenes* strain cocktail in the presence of soil.

INTRODUCTION

The sporatic outbreaks of listeriosis due to the consumption of contaminated ready-to-eat meats have raised concern that recontamination may occur after processing. One possible cause of recontamination in food processing plants maybe due to ineffective cleaning (6). Food contact surfaces and areas in processing lines such as joints, butterfly valves, pitted surfaces, equipment gaskets, dead ends and non-food contact surfaces such as drains, walls, ceiling condensate, equipment surfaces and hand trucks as possible sources of *Listeria* contamination (3, 5, 11, 16) .

Listeria attached to or growing on equipment surfaces is difficult to remove and has increased resistance to biocides (14). Microorganisms are more difficult to remove from abraded surfaces compared to smooth surfaces (12) and rubber surfaces compared to stainless steel (9, 19). Older biofilms are more difficult to remove than younger biofilms and planktonic cells. Biofilms grown for seven days are more resistant to biocide compared to those grown for two days (14). Biofilm in wastewater consists of heterogeneous populations coexisting in different niches so, previous biofilm formation by one specie may be a harborage by another specie, this can be especially significant in areas such as drains where a non-pathogenic species can take residence, develop a biofilm and a pathogen such as *Listeria* can establish residence (1). The difficulty of biofilm removal from surfaces may be compounded by the presence of food residue. This is especially true for oily surfaces where high temperature cleaning operations may be necessary; *Listeria* survival in such an environment may be of concern in regards to recontamination in food processing plants.

Removing listeria biofilm from the food and non-food contact surfaces is a difficult task due to the extracellular polymeric nature of biofilm and the protection of food soil. In this study, we propose use of fraction negative data analyzed using non-linear logistic statistical analysis to predict the survival of *Listeria monocytogenes* in biofilms after heat exposure.

The objective of this research is to develop a predictive model for the inactivation of *Listeria monocytogenes* in monoculture and multispecies of biofilms on stainless steel surfaces following heat treatment. The effect of adding soil to the biofilm was also determined.

MATERIALS AND METHODS

Surface preparation.

Stainless steel coupons (type 304, No.4 finish) cut to 10 cm^2 (5x 2 cm) were degreased in acetone, sonicated (Aquasonic, model 550HT, VWR Scientific, Atlanta, GA) for 60 min at 55ºC in 2g/l alkali detergent (Micro™,International products corp., Burlington, NJ), rinsed with deionized water, and sonicated for 20 min in 30g/L of phosphoric acid- based cleaner (Formula 3586, Zep manufacturing, Atlanta, GA) then rinsed three times in deionized water. Coupons were autoclaved in deionized water.

Culture source

Cultures used in this study are listed in Table 3.1. Strain selection of *Listeria monocytogenes* was based on the degree of biofilm production and/or heat tolerance according to preliminary data (data not shown). *L. monocytogenes* Scott A and 3990 were high biofilm producers and heat tolerant, and therefore were selected for the

prediction model development. *L. monocytogenes* strains YM96 and 303 were moderate biofilm producers and *L. monocytogenes* 17 which produced minimal biofilms were used in the validation study along with the *L. monocytogenes* Scott A and 3990 strains.

Biofilm preparation

In the predictive study, cultures were activated from frozen beads (Microbank™, Prolab diagnostics, Austin, TX) in Tryptic Soy Broth (Difco Brand, Becton Dickson, Sparks, MD) for 18 h at 32ºC. Biofilms were produced by immersing stainless steel coupons in diluted TSB(1:10) for 4 h at 25ºC in a 0.1% (v/v) of 10^6 cells/ ml inoculum . The monoculture inoculum of *L. monocytogenes* are strains Scott A and 3990, respectfully. Other monoculture species are *Pseudomonas spp*. and *P. agglomerans* . There were two multispecies culture inocula, the first consisted of a mixture of 4 parts *L. monocytogenes*, Scott A broth culture to 1 part *Pseudomonas* spp broth culture and, the second consisting of 8 parts *L. monocytogenes*, 3990 broth culture to one part *Pseudomonas* spp. broth culture and one part *P. agglomerans* broth culture (Tb 3.1). Following the 4 h attachment, coupons were rinsed with phosphate buffer (0.01M, pH 7.0), transferred to diluted TSB (1:10) and incubated for 48 h at 25 ºC. Biofilms formed according to this procedure were considered as a‘low-soil’ condition.

Soiling of biofilm

The soil formulation consisted of an emulsion of rendered chicken fat (137.7 mg /ml), sterile chicken serum (211-338 mg protein/ml)(Sigma C-5405, St. Louis, MO) and lecithin (0.05mg/ml)(Sigma P-5638, St. Louis, MO). The soil mixture was blended for 1 min with an electric hand blender (Braun, Lynnfield, MA). The hand blender was

disinfected (70% ethanol v/v) prior to use. Stainless steel coupons containing biofilm were dipped in the soil then placed in disposable centrifuged tubes and incubated at 10º C overnight (18h). This was considered as 'soiled' biofilm (15mg soil /cm^2). This soil mixture were analyzed for initial microbial load which was determine to be less than 10 cfu /cm^2.

Biofilm preparation for the validation study

Sterile stainless steel coupons were submerged into broth containing a five strain cocktail of *L. monocytogenes,* Scott A; 3990, 17, YM 96 and 303, used in equal parts. Following a 4 h attachment as previously described, coupons were rinsed with phosphate buffer (0.01M, pH 7.0), transferred to diluted TSB (1:10) and incubated for 48 h at 25 ºC. Biofilm were soiled according to the biofilm 'soil' procedure.

Experimental control

For the inoculated (positive) and uninoculated (negative) controls, coupons containing biofilm were rinsed with phosphate buffer(0.01M, pH 7.0) then placed in 0.1% (wt/v) peptone with glass beads (3 g/10ml, 450-600 , Sigma-Aldrich, St Louis MO), and shaken on a wrist action shaker (model 75,Wrist action shaker ™, Burrell Co., Pittsburgh, PA) for 3 min, serially diluted then plated and enumerated using Plate count agar (PCA), Pseudomonas Isolation agar (PIA) and LSA and incubated at 32ºC for 24-48h. *L. monocytogenes* was isolated on LSA, *Pseudomonas* on PCA or Pseudomonas isolation agar (PIA)(Difco Brand, Becton Dickson, Sparks, MD) with confirmation by observing the morphology microscopically and *P. agglomerans* were isolated using Violet red bile agar (VRB)(Difco Brand, Becton Dickson, Sparks, MD) . Mixed culture

biofilm was analyzed only for surviving *Listeria*. Counts were recorded as colony forming units (cfu)/ cm^2.

Heat treatment

Duplicate samples containing either 'low' soil or 'soiled' biofilm of *L. monocytogenes* in monoculture or multispecies were submerged in preheated test-tubes with 25 ml phosphate buffer heated in a circulating water bath (Precision Scientific, Winchester, VA). Samples were heated for 1, 3, 5, 15 min at 70, 72, 75, 77, 80 ºC. Temperatures were confirmed using a two-channel thermocouple (Traceable®, Houston, TX). One thermocouple was suspended in the test-tube with buffer and the other was attached to the coupon surface. Treated coupons were submerged in 25 ml Tryptic Soy broth (30g/L) with yeast extract (6g/L) (TSB-YE) at 35 ºC for 24 h then 1 ml aliquot was transferred to Fraser broth and incubated at 35 ºC for 48 h. Samples were streaked on LSA and incubated at 35ºC for 24h for presumptive identification. 10% of positive samples were confirmed using API-Listeria ™. The analysis of the heating data were based on the fraction negative enumeration method, that is the data was recorded as the fraction of the duplicate samples that shows no growth of *L. monocytogenes*. The response would be one of three possibilities, no survivors (0 samples positive out of 2); 50% survival (1 positive sample /2 samples)or 100% (2 positive sample /2 samples) survival with observation values of 0, 0.5 and 1. The purpose of using fraction negative data is to overcome the tailing effect demonstrated by clumped cells during heat inactivation. The fraction negative method allows for the recovery of low numbers of survivors that is cannot be detected using conventional enumeration methods. This

method also shows if the target population was completely inactivated. This experiment was replicated six times.

Heat treatment for the validation study

New data were collected using a five strain cocktail of *L. monocytogenes* (Scott A, 3990, YM 96, 303 and 17) that was subjected to the same growth, and soiling conditions previously described. Samples were heated for 1, 8, and 15 min at 70, 76 and 80ºC. The experiment was replicated six times with duplicate samples for each experiment. Data collection and analysis followed the previously described procedure.

Experimental design

Four monocultures and two multispecies biofilms were grown on stainless steel surfaces and exposed to two soil conditions. The surfaces were subjected to five heating temperatures and four heating times. Data for the experiments were recorded as either positive or negative for the presence of *L. monocytogenes* and as positive or negative for the presence of *Pseudomonas* and *P. agglomerans*. The mixed culture biofilms were analyzed for *Listeria* only. The experiment was replicated six times with duplicate samples for each experiment. The resulting data from each observation had one of three possibilities, no survivors, 50% survival or 100% survival with values of 0/2, 1/2 or 2/2 positives. After data removal and pooling a total of 480 observations were analyzed for the final model.

Table 3.1 Microbial cultures used in biofilm formation on stainless steel

Name of microorganisms	serovars	Origin/Source*
Listeria monocytogenes Scott A	4b	Human isolate
Listeria monocytogenes 3990	4b	Vecherin Mont'd or cheese isolate
Listeria monocytogenes YM 96	1/2a	Monkey environment
Listeria monocytogenes 303	1/2a	Monkey liver
Listeria monocytogenes 17	4b	Food processing plant environment
Pseudomonas spp M21		Food processing plant environment
Pantoea agglomerans		Food processing plant environment

Name of microorganisms	Codes
L. monocytogenes, Scott A	Scott A
L. monocytogenes, 3990	LM 3990 or 3990
L. *monocytogenes* Scott A + *Pseudomonas* spp	LMPs
L. monocytogenes, 3990 +*Pseudomonas* spp. + *P. agglomerans*	LMPsP

*Center for Food Safety, Griffin, GA.

.

Statistical analysis

A statistical model was developed to estimate the probability of inactivation of *L. monocytogenes* in a biofilm treated with various time, temperature combinations. Logistics analysis using SAS/STAT software (SAS®, Duncan, SC) was used to estimate the probability of complete inactivation of *L. monocytogenes* in a biofilm afer heat treatment. This equation shows, Ln $[P/(1-P)]= \beta_0 + \beta_1 (C) + \beta_2 (C) + \beta_3 (TEMP) + \beta_4$ (Time) the probability of binomial response (survival or inactivation) is dependent upon the parameters culture, soil, temperature and time. Culture and soil are classified variables, while time and temperature are continuous variables. The subset within the classified parameters were tested for if they could be combined or kept as independent subset. For model development, each parameter in the model was tested for adequacy of as a main effect and for interactive effect with the other main parameters. The selected equation was evaluated using a score goodness-of -fit statistic, Schwartz-Bayes Criterion (SC). The SC test is the most conservative and reliable in evaluating a good fit of the observed data with the predicted data. The predictive power of the model was evaluated using plots of the predicted points. The predictive power of the selected models would be limited by the range of the time (1-15 min) and temperature (70ºC- 80ºC) parameters employed in this experiment. Predictive power was considered good if the predicted points, at or above 75% probability of total inactivation, fell within the range of the heating parameters tested.

PREDICTIVE MODEL DEVELOPMENT

Monoculture and multispecies biofilm models without soil

Individual models were developed to predict the heat inactivation of *L. monocytogenes, Pseudomonas* and *P. agglomerans* in monoculture and *L. monocytogenes* in multispecies biofilms. The prediction equations were derived from the logistic regression analysis of the fraction negative data. The prediction equation (1) Ln(P/1-P)= 11.3506 -0.1477* TEMP -0.0826* TIME was used to estimate the probability of total inactivation of *Pseudomonas* biofilm after heat treatment for different time- temperature combinations**.** The odds ratio of the logistic model (0.912) indicated that time was the most important factor contributing to this model. The data suggested that after heating at 80ºC, for 15 min, there was an 85% probability that *Pseudomonas* would be inactivated. This prediction equation (2) Ln(P/1-P)= 23.3581 -0.3243* TEMP -0.0620* TIME estimated that the probability of total inactivation Scott A, a heat treatment at 80ºC for 15 min was about 97%. The odds ratio 0.93 for the time variable means that time was the factor that contributed most to this model. The prediction equation (3) Ln(P/1-P)= 53.5295 -0.7438*TEMP -0.1908* TIME for LMPs biofilm inactivation estimated that the probability of total inactivation of Listeria in this LMPs mixed culture biofilm heated at 80ºC for 15 min was 99.985 %. The odds ratio (.81) showed that time contributed most to this predictive model. This prediction equation (4) Ln(P/1-P)= 10.7211-0.1303*TEMP -4.0129* TIME for *P. agglomerans* biofilm estimated that the probability of total inactivation of *P. agglomerans* after heating at 80ºC for 1 min was 99.8% and no chance for survival after 5 min. The prediction

equation (5) Ln(P/1-P)= 6.2671 -0.1016*TEMP -0.1026* TIME for the survival of strain 3990 biofilm after heat inactivation estimated that the probability of total inactivation of strain 3990 after heating at 80ºC for 15 min was 97%. While the prediction equation (6) for LMPsP biofilm Ln(P/1-P)= 8.0866 -0.1197*TEMP -0.1557* TIME estimated that the probability of total inactivation of *Listeria* in a mixed culture biofilm heat treated at 80ºC for 15 min was 97.9%.

These individual heat inactivation models demonstrated that a monoculture of *Pseudomona*s biofilm was the most heat resistant of those tested. The *Pseudomonas* model predicted an 84% chance of total inactivation after heating at 80º C for 15 min while the monocultures of *Listeria* strains (Scott A and 3990) showed a 97 % probability of total inactivation at a heat treatment of 80º C for 15 min. Monocultures (Scott A and 3990) also showed greater heat resistance than *Listeria* within a multispecies (LMPs and LMPsP) biofilm. Combining the data to create one model creates parsimony, since temperature and time variables were the same in each model and culture types could be included as a classified variable.

The integrated monoculture and mixed culture model without soil

The monoculture biofilms of *Pseudomonas* sp. and *P. agglomerans* were controls used to determine if their biofilm production influenced heat inactivation of *Listeria*. Although *Pseudomonas* monoculture biofilm showed significant heat resistance, when *Pseudomonas* was included in the multispecies biofilm, it did not influence the heat inactivation of *L. monocytogenes*. *P. agglomerans* did not survive heat inactivation in biofilm and had no influence on the survival of *L. monocytogenes* in a mixed culture biofilm. Therefore, the data for the non-*Listeria* monoculture biofilms were not considered in further model development. Additional analysis was implemented to determine the influence of *Listeria* alone or within a mixed culture biofilm. Stepwise selection to evaluate the statistical contribution of the parameters in the model, indicated that cultures, time and temperature contributed to the significance of the model ($p<0.0001$). The Pearson correlation coefficient test to evaluate correlation between cultures, showed that the response of monoculture and multispecies biofilms were significantly independent ($p<0.0001$) so culture types were analyzed as a classified variable. Therefore, the monoculture and multispecies integrated model without soil was developed. It included time and temperature as the continuous variables and culture type (Scott A; LMPs; LM 3990; LMPsP) as the classified variable and the interactive effect of culture and temperature. This model (Eq. 7) is expressed as:

$$\text{Ln}\,[P/(1-P)] = 22.2515 + \begin{bmatrix} 2.2342 * S\cot tA \\ 128.1520 * LMPs \\ -15.9024 * LM3990 \\ -14.4838 * LMPsP \end{bmatrix} + \begin{bmatrix} 0.3359 * S\cot tA \\ -0.7052 * LMPs \\ -0.1020 * LM3990 \\ -0.3152 * LMPsP \end{bmatrix} * TEMP - 0.1148 * TIME$$

(Eq. 7)

This integrated monoculture and multispecies model without soil (7) indicated, for example, that Scott A showed a lower probability of total inactivation than Strain 3990. Scott A heated at 80ºC for 15 min would have 20% probability of inactivation while Strain 3990 had 97% probability of inactivation. The goodness of fit test (SC value) indicated that the reduction of this model from the full ranked factorial model was acceptable (Tb.3.2). However, this model gave predictive values out of the range of the heating parameters tested (temperature of 70-80 ºC and time 1-15min), and therefore was rejected.

Table 3.2 P -values of the full ranked and reduced monoculture and multispecies integrated model without soil

Parameters	Full model P> Chi-sq	Reduced model P> Chi-sq
C	<0.0001	<0.0001
TEMP	<0.0001	<0.0001
TIME	0.89	<0.0001
TEMP*C	<0.0001	
TIME*C	0.13	
TEMP*TIME	0.71	
SC values	839.3	755.9

Analysis of the integrated model with soil

The soil data was included in the previously described integrated model without soil (Eq. 7) resulting in an integrated model with soil. This predictive model included time and, temperature as the continuous variables and soil (low soil, soiled) and culture type (Scott A; LMPs; LM 3990; LMPsP) as the classified variables and the interactive effects of culture and temperature. The resulting model (Eq. 8) is as follows:

$$\text{Ln}\,[P/(1-P)] = 17.9118 - 0.9012 * soil + \begin{bmatrix} 6.8334 * S\cot tA \\ 1.9748 * LMPs \\ -5.1345 * LM3990 \\ -3.6737 * LMPsP \end{bmatrix} - 0.2420 * TEMP - 0.1085 * TIME + \begin{bmatrix} -0.0947 * TEMP * S\cot tA \\ -0.0264 * TEMP * LMPs \\ 0.0722 * TEMP * LM3990 \\ -0.0489 * TEMP * LMPsP \end{bmatrix}$$

(Eq. 8)

The goodness-of-fit test (SC value) for the integrated model with soil (Eq. 8) indicated that reduction of this model from the full ranked model was acceptable (Tb3.3). However, the reduced model produced predictive values out of the range of the tested parameters (temperature of 70-80 ºC and time 1-15min), and the predictive equation was too complex for calculations and therefore was rejected.

Table 3.3 P-values of full ranked and reduced integrated model with soil.

Parameters	Full model	Reduced model	
	P> Chi sq	Estimate	P> Chi-sq
Intercept	<0.0001	17.91	<0.0001
Soil	0.049	0.90	<0.0001
Culture	0.008	-	<0.0028
Scott A	-	6.83	-
LMPs	-	1.97	-
LM3990	-	-5.13	-
LMPsP	-	-3.67	-
TEMP	<0.0001	-0.24	<0.0001
TIME	0.46	-0.11	<0.0001
TEMP*C	0.52		<0.0021
TEMP*Scott A	-	-0.09	-
TEMP*LMPs	-	-0.03	-
	-	0.07	-
TEMP*LM3990	-	-0.05	-
TEMP*LMPsP			
SC values	2079.8	2017.7	

Final predictive model

The data of the two multispecies biofilms (LMPs and LMPsP) were merged as one multispecies culture type. The resulting culture variable contains three culture types instead of four. The three culture types include monocultures of *L. monocytogenes* Scott A and 3990 strains and *L. monocytogenes* within a multispecies biofilm. Separately the two multispecies biofilms had similar survival patterns, so merging the data improved predictive power. Also, in evaluating the soil parameter, the low-soil data did not significantly contribute to the final model, so the final predictive model (Eq. 9) was developed by removing the soil variable from the model.

Model validation

The validation model was developed to assess the quality of the final prediction model. The same statistical approach as used for the prediction model was used to generate the model (Eq.10). The validation model was graphically compared with the predicted model (Eq. 9). The accuracy factor of the predictive and validated data was calculated and is expressed as the antilog of the average log of the ratio between the predicted and validated data ($AF = 10^{(\sum \log(\text{predicted}/\text{observed})/n)}$)

RESULTS

Conventional predictive models for the inactivation of bacteria in suspension and in food matrices use log-linear calculations. Microorganisms within biofilms exhibit increased heat resistance and demonstrate a tailing effect in heat inactivation. Direct enumeration is not sufficiently sensitive to detect low levels of survivors after heat inactivation so, a positive/negative enumeration method with enrichment was media used so that survival estimate is based on the out-growth of at least one cell. The calculations in this study are based on non-linear inactivation rates. The survival data had a binomial distribution and were analyzed using logistic regression.

The final predictive heat inactivation model for *Listeria monocytogenes* in a biofilm is shown in Eq. 9 and the statistical parameters are presented in Table 3.4. This model indicates that culture, temperature and time had statistical significance in the prediction of *Listeria's* survival. Goodness of fit test (SC values) comparing the full ranked factorial model with the final reduced model (Tb. 3.4) indicate that the final reduced model had a good fit and predicts within the range of the parameters tested.

Final model

$$\text{Ln}\,[P/(1-P)] = 18.0527 + \begin{bmatrix} -0.4706 * S\cot tA \\ -0.5316 * LM3990 \\ -0.0616 * multispecies \end{bmatrix} 0.2299 * TEMP - 0.1108 * TIME$$

(Eq. 9)

Validation model

$$\text{Ln}(P/1-P) = 35.9399 - 0.4655 * \text{TEMP} - 0.1892 * \text{TIME} \qquad \text{(Eq. 10)}$$

Table 3.4. Parameter estimates and Chi sq. values of full ranked and final reduced prediction model

Parameters	Full model	Reduced model		
	P> Chi sq	Estimate	S.E	P> Chi sq
Intercept	<0.0001	18.05	1.65	<0.0001
C	0.67			<0.0001
Scott A	-	-0.47	0.09	
LM3990	-	-0.53	0.11	
Multispecies	-	-0.06	0.00	
TEMP	<0.0001	-0.22	0.02	<0.0001
TIME	0.08	-0.11	0.01	<0.0001
TEMP*C	0.68	-	-	-
TIME*C	0.69	-	-	-
TEMP*TIME	0.04	-	-	-
TEMP*TIME*C	0.66	-	-	-

[A] Mixed culture was set as the baseline for comparison

The prediction equation (Eq. 9) estimates the probability of survival of *L. monocytogenes* in monoculture and multispecies biofilms after heat treatment at different time/temperature conditions in the presence of soil. Plots (Figs. 3.1-3.3) were developed from the predictive equation showing the probability of complete heat inactivation at 90, 75 and 50% in the biofilms in the presence of soil. The data indicate that to obtain a 90 and 75% probability of total inactivation of Scott A, heating at 80ºC for 12.5 and 2.5 min, respectively, is required. For a 50% probability of inactivation of Scott A, a heating regiment of 76ºC for less than a minute is needed (Fig. 3.1). The heat inactivation requirement of strain 3900 with 90, 75 and 50% probability of total inactivation, at 80ºC is 21.6, 11.7 and 1.7 min respectively (Fig. 3.2). While the prediction estimates at 90, 75 and 50% probability of total inactivation of *Listeria* in a multispecies biofilm indicate that heat treatment at 80ºC for 16.2, 6.3 min and less than a minute, respectively, is required (Fig. 3.3). The predictive model demonstrates that there are differences in heat resistance between monoculture *L. monocytogenes* biofilms and *Listeria* in the multispecies biofilm in the presence of soil. Scott A in monoculture biofilm exhibited the lowest heat resistance followed by the *Listeria* in multispecies biofilm with the strain 3990 monoculture biofilm exhibiting the most heat resistance. This predictive model can therefore employ three underlying heat resistance assumptions, the least survival prediction with the Scott A strain, a moderate prediction of survival with the mixed culture and a conservative prediction using the strain 3990 strain.

The prediction model (Eq. 9) was verified by heating biofilms containing a five-strain cocktail of *L. monocytogenes* in the presence of soil. The verification plot

generated from the equation (10) is graphically compared with the prediction plots of each biofilm type. The 50% probability plots indicated that Scott A strain (Fig. 3.1) had higher inactivation probabilities than the verification cocktail, for example heat treatment at 75ºC requires 3 min for total inactivation of Scott A biofilm versus 5 min for the cocktail strains. This suggests that Scott A biofilm on soiled stainless steel may not be a representative strain of *L. monocytogenes* for heat inactivation studies of biofilm. *L. monocytogenes* within multispecies biofilms (Fig. 3.2) exhibited inactivation levels similar to the validation cocktail while *L. monocytogenes* 3990 biofilm demonstrated the greatest heat resistance. The final model that included strain 3990 (Fig. 3.3) as the representative culture is a conservative predictor of *L. monocytogenes* inactivation in a biofilm after heat treatment and therefore provides a safe guide for development of hot water sanitation processes. The accuracy factor of the predicted data versus the validated data was 0.85 suggesting that validated data closely follows the prediction.

DISCUSSION

Sanitation of food and non-food contact surfaces with hot water is a common practice in the food industry. Chemical sanitizers are not always effective in inactivating microorganisms within a biofilm since organic matter may reduce activity or sanitizers may not be able to penetrate the polymeric matrix. Surfaces that are not sufficiently cleaned to be free of biofilm especially pitted surfaces, elbows, gaskets and joints are typical areas that would facilitate microbial growth. When chemical cleaning methods prove to be ineffective and cooking on of organic residue is not a concern, hot water sanitation becomes a viable alternative. To date, little data is available on which to base

hot water sanitation requirements in food processing facilities. Therefore, a predictive model to estimate inactivation of *L. monocytogenes* in a biofilm will be useful. The static heating employed in this study simulates conditions in protected areas in a CIP system such as joints with gaskets.

Although *L. monocytogenes* is often found in the processing environment there is insufficient information to select a physiologically representative strain. The predictive model developed in this study include three heat resistant assumptions using two *L. monocytogenes* strains in mono-culture and one in multispecies biofilms. The probability plots generated from the predicted model also give three probability levels (90, 75 and 50%) for *L. monocytogenes* inactivation in biofilms.

The results of the heat inactivation study of *L. monocytogenes* on stainless steel indicate that strain 3990 biofilm is more heat resistant in the presence of soil than Scott A and *L. monocytogenes* with the multispecies biofilm on stainless steel. The validation study shows that at the 50% probability level of *L. monocytogenes* inactivation, the predictive model with 3990 strain is conservative in its estimate of *L. monocytogenes* biofilm inactivation. The model based on this strain should be used in situations where there is high risk of *L. monocytogenes* occurrence in ready to eat product. The five-strain cocktail shows higher survival than the Scott A strain and similar inactivation predictions as *L .monocytogenes* within a multispecies biofilm. Therefore the Scott A model is not a conservative predictor of *L. monocytogenes* inactivation in a biofilm after heat treatment on stainless steel, so, this model would have limited use. *L. monocytogenes* within the multispecies biofilm showed similar inactivation as the

cocktail cultures with a slightly more conservative prediction at the higher levels of heat treatment. *L. monocytogenes* within a multispecies biofilm is therefore an adequate representation of *L. monocytogenes* biofilm survival and can be used in situations of low risk of *L. monocytogenes* exposure to product.

The five-strain cocktail contained strain 3990 which was expected to be heat resistant and predominate however, the higher than expected inactivation of the cocktail biofilm suggests that within the five-strain cocktail there was an imbalance of growth among the strains during biofilm formation. We were unable to determine the predominant culture in the validated biofilm because of the genetic similarity of the *L. monocytogenes* strains. Some studies (13) show imbalance in *L. monocytogenes* growth in biofilm, for example, *L. monocytogenes* grew well in the presence of *Pseudomonas* but in the presence of other microflora, grew only after *Pseudomonas* reached stationary phase.

The presence of soil could have a protective effect on the heat inactivation of microorganisms in suspension or attached to a surface. The heat resistance of *L. monocytogenes 3990* biofilm dramatically increased in the presence of soil (chicken fat and serum)(Eq. 9) compared to the "low" soil (diluted TSB) conditions (Eq. 7) however this was not true of Scott A. Casadei (2) and Chhabra (4) showed that high fat substrates increased heat resistance of *L. monocytogenes* 1151 and Scott A in a suspension, with strain 1151 showing higher heat resistance the Scott A. While Flint (7) noted that *S. thermophilus* attached to stainless steel in the presence of skim milk then heated in water was more heat resistant than similarly treated planktonic cells. The presence of soil could

promote bacterial growth thereby changing the pH of the biofilm environment . This can subsequently influence the inactivation as well as survival of cells (10).

Nutrient depletion, slow growth rate and surface attachment of bacteria are some of the growth conditions that lead to biofilm formation of *L. monocytogenes* (8). Cells within a biofilm may be protected from stress if they are located in the biofilm strata where nutrients are depleted, cell growth is slow and stress response is induced. Dense clustering of cells and production of extracellular polymer effectively change the heating menstrum. Frank and Koffi (8) and Lee and Frank (15) showed biofilm that remained attached to a surface had significantly higher survival after exposure to heat than detached biofilm and planktonic cells. For example, adherent cells survived 70ºC heat for 15 min while planktonic cells were killed in 30 s. Lee (15) demonstrated that the greater the cell density within a biofilm the higher the survival of *L. monocytogenes* after heating.

When developing a predictive model consideration should be given to selecting a representative bacterial strain or serovar, since not all strains or serovars have the same level of heat resistance or biofilm production. In our model the calculated odds ratio indicated that time of heating and culture type (in particular, *L. monocytogenes* 3990) were the predominant factors contributing to the prediction of *L. monocytogenes* survival in biofilm after heat treatment. Our study showed that Scott A responded differently than strain 3990, with strain 3990 being more heat resistant in the presence of soil. Stress response in biofilm growth can be different for different strains. Sorqvist (17) studied the heat resistance of 30+ strains and serovars of *L. monocytogenes* and noted greater heat resistance among ½ and 3b serovars but, within the 3b group, not all had equal heat

resistance. Vasseur (18) studied the effect of osmotic, alkali, acid or thermal stresses on *L. monocytogenes* and observed varied stress response between strains.

The results of this study indicate that there is need to verify the efficiency of hot water sanitation processes. CIP systems may not be able to exceed 77ºC and temperatures may drop lower than this. Therefore, the risk of *L. monocytogenes* surviving hot water sanitation process is significant if adequate time/temperature controls are not maintained. The predictive model, based on total inactivation of *L. monocytogenes*, provides three heat resistance assumptions to predict *L. monocytogenes*'s inactivation on stainless steel, with Scott A strain showing the highest probability of inactivation followed by *L. monocytogenes* within a multispecies biofilm and strain 3990 showing a conservative heat inactivation prediction. The validation data supports the strengths and confirms the limitations of the predictive model. The accuracy factor shows that the validation data closely follows the predictive data. This study shows that *L. monocytogenes* within a biofilm on stainless steel may be effectively inactivated if heat treatment is controlled. However, it may be a challenge to control sanitation temperatures in the processing environment especially in systems where cold spots have not been identified. The times and temperatures selected for this study were based on common practice in the food industry. Our predictive model could be used in risk assessment planning allowing processors to select a heat treatment based on the likelihood of product contamination and on the level of risk acceptable.

References

1. **Bishop, P. L.** 1997. Biofilm structure and kinetics. Wat. Sci . Tech **36:**287-294.

2. **Casadei, M. A., R. E. 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.

3. **Charlton, B. R., H. Kinde, and L. H. Jensen.** 1990. Environmental Survey for Listeria species in California Milk Processing Plants. J. Food Prot. **53:**198-201.

4. **Chhabra, A. T., W. H. Carter, R. H. Linton, and M. A. Cousin.** 2002. A predictive model that evaluates the effect of growth conditions on the thermal resistance of *Listeria monocytogenes*. International Journal of Food Microbiology **78:**235-243.

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

6. **Dunsmore, D. G.** 1981. Bacteriology Control of Food Equipment Surfaces by Cleaning Systems. I. Detergent effects. J. Food Prot. **44:**15-20.

7. **Flint, S., J. Brooks, P. Bremer, K. Walker, and E. Hausman.** 2002. The resistance to heat of thermo-resistant streptococci attached to stainless steel in the presence of milk. Journal of Industrial Microbiology and Biotechnology **28:**134-136.

8. **Frank, J., and R. Koffi.** 1990. Surface-adherence growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J. Food Prot. **53:**550-554.

9. **Frank, J. F., and R. A. Chmielewski.** 1997. Effectiveness of sanitation with quaternary ammonium compound or chlorine on stainless steel and other domestic food-preparation surfaces. J. Food Prot. **60:**1-6.

10. **George, S. M., B. M. Lund, and T. F. Brocklehurst.** 1988. The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. Lett. Appl. Microbiol. **6:**153-156.

11. **Greene, A. K., T. G. Reynolds, and E. M. Southerland.** 1991. Sanitary evaluation of target flowmeter used in a dairy processing plant. J. Food Prot. **54:**966-968.

12. **Holah, J. T., and R. H. Thorne.** 1990. Cleanability in relation to bacterial retention
on unused and abraded domestic sink materials. J. Appl. Bacteriol. **69:**599-608.

13. **Lebert, I., V. Robles-Olvera, and A. Lebert.** 2000. Application of polynomial models to predict growth of mixed cultures of *Pseudomonas spp.* and *Listeria* in meat. Int. J. Food Microbiol **61:**27-39.

14. **LeChevallier, M. W., C. D. Cawthon, and R. G. Ramon.** 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. **54:**2492-2499.

15. **Lee, S.-H., and J. Frank.** 1991. Inactivation of surface adherent *Listeria monocytogenes* hypochlorite and heat. J. Food Prot. **54:**4-6.

16. **Lindsay, D., I. Geornaras, and A. v. Holly.** 1996. Biofilm associated with poultry processing equipment. Microbios **86:**105-116.

17. **Sorqvist, S.** 1994. Heat resistance of different serovars of *Listeria monocytogenes*. J. Appl. Bacteriol. **76:**383-388.

18. **Vasseur, C., L. Baverel, M. Hebraud, and J. Labadie.** 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. J. Appl. Microbiol. **86:**469-476.

19. **Wong, A. C. L.** 1998. Biofilm in food processing environments. J. Dairy Sci. **81:**2765-2770.

Figures 3.1 and 3.2. The probability of heat inactivation of *Listeria monocytogenes* Scott A (Fig. 3.1) and, 3990 (Fig. 3.2) after heat treatment on stainless steel with soil. Heating temperatures range from 70 to 80 ºC and heating times of 1, 3, 5,and 15 min. Heat inactivation probability plots were represented at 90% (P=0.90), and 75% (P=0.75) levels and validated probability level at 50% (VP=0.50)

Figure 3.1

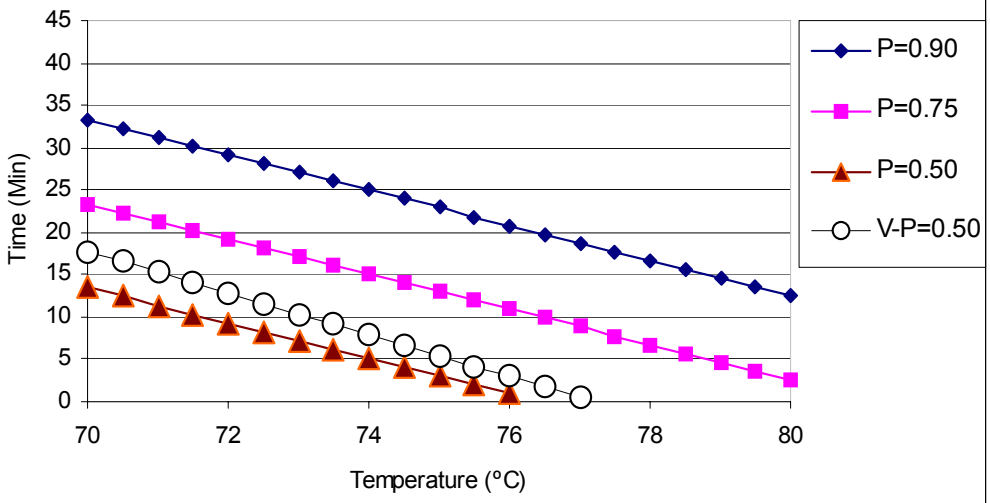


Figure 3. 2

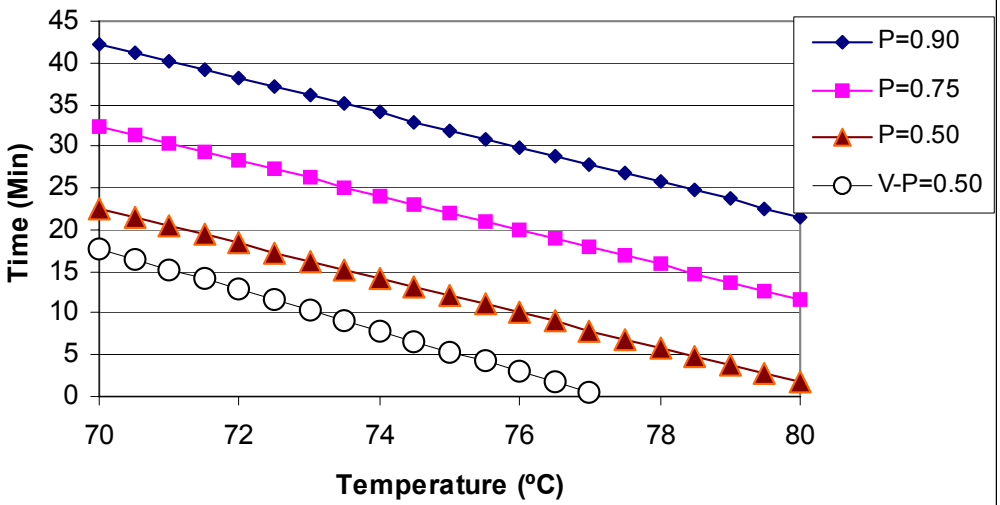
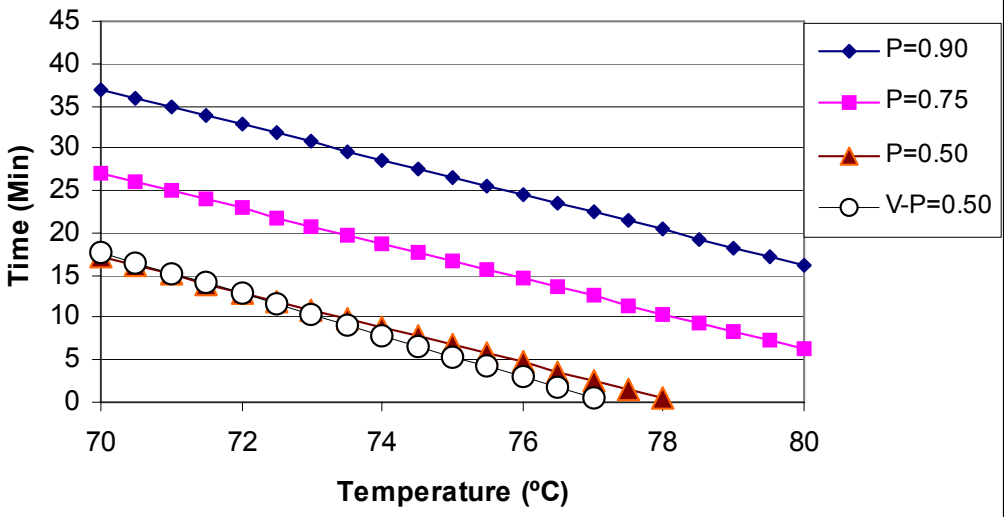


Figure 3.3. The probability of heat inactivation of *Listeria monocytogenes* in multispecies biofilm after heat treatment on stainless steel with soil. Heating temperatures range from 70 to 80 ºC and heating times of 1, 3, 5,and 15 min. Heat inactivation probability plots were represented at 90% (P=.90), and 75% (P=.75) levels and validated probability level at 50% (VP=.50)

Figure 3.3


45
40
35
30
25
20
15
10
5
0
Time (Min)
70
72
74
76
78
80
Temperature (ºC)
P=0.90
P=0.75
P=0.50
V-P=0.50

CHAPTER 4

A PREDICTIVE MODEL FOR HEAT INACTIVATION OF *LISTERIA MONOCYTOGENES* BIOFILM ON BUNA-N RUBBER [1]

---

[1] Chmielewski, R.A.N. and J.F. Frank 2003. To be submitted to the *Journal of Food Protection*

ABSTRACT

Buna -N rubber is used to make gaskets for the food processing industry. Exposure with alkali cleaners, sanitizers, high temperatures and high pressure can cause rubber gaskets to deteriorate and provide harborage for microbial growth resulting in biofilm formation. The purpose of this study is to develop a predictive model for the heat inactivation of *Listeria monocytogenes* in pure and multispecies biofilms formed on buna-N rubber in the presence of soil. Biofilms were produced by immersing rubber disks in a 1:10 dilute Tryptic Soy broth (TSB) inoculated with the monocultures of *L. monocytogenes*, strains Scott A or 3990 or with *L. monocytogenes* in multispecies (*Pseudomonas spp.* and *Pantoea agglomerans*) biofilms followed by incubation for 4 h at 25ºC, rinsing with phosphate buffer (0.01M), transferring to diluted TSB and incubation for 48 h at 25 ºC. Duplicate biofilm samples were heat treated for 1, 3, 5, 15 min at 70, 72, 75, 77 and 80ºC and tested for survivors using enrichment media. Controls were vortexed with glass beads and enumerated using plate count agar and Listeria Selective agar. The experiment was repeated six times. A predictive model was developed using fraction negative data and logistic regression. Probability plots showing the percent probability of *L. monocytogenes* survival in biofilm after heat treatment were generated from the predictive equation. The predicted model was validated using a five strain cocktail of *L. monocytogenes*. The validated prediction model indicates that with proper maintenance of the time /temperature controls *L. monocytogenes* in biofilms on rubber surfaces will be inactivated. This model can be used as a tool in the selection of hot water sanitation processes for rubber surfaces.

INTRODUCTION

Gaskets in food processing equipment are often made from Buna-N rubber, also known as Nitrile butyl rubber (10, 11). Repeated cleaning and sanitation of buna-N rubber ages the surface leading to the development of cracks. These deteriorated surfaces are difficult to clean creating a favorable environment for food residue accumulation, and biofilm formation (10, 11).

The attachment and biofilm formation of *Listeria* and other microorganisms are influenced by the physico-chemical properties of the surface such as surface charge, hydrophobicity, pH, temperature and nutrient composition of the preconditioning menstrum (4, 6, 9, 12). Surface materials used in food processing allows differing degrees of biofilm formation (1, 5, 11) for example, *Listeria* and *Salmonella* were found to adhere more to hydrophobic surfaces than to hydrophilic surfaces although the attachment may be weak (8). The presence of food residue on buna-N rubber can affect microbial attachment and biofilm formation. Milk and milk components inhibited attachment of *L. monocytogenes* and *S. typhimurium* on buna-N rubber (4), while cream and fat increased biofilm formation of some *Bacillus* and *Pseudomonas* species (10). Buna-N rubber exhibited inhibitory effects on the growth and attachment of microorganisms including *L. monocytogenes* and *P. agglomerans* (7, 10, 11). Much information is available on the attachment, growth and biofilm formation of environmental and foodborne bacteria and the efficacy of chemical cleaning and sanitation used for their control. However, there are limited data available on the effect of

hot water sanitation on the inactivation of bacterial biofilm in the food processing environment.

The objective of this research was to develop a predictive model for the heat inactivation of monoculture and multispecies of *Listeria monocytogenes* biofilms on rubber surfaces and in the presence of soil

MATERIALS AND METHODS

Surface preparation

Buna-N rubber disks (1 cm^2)(McMaster-Carr, Atlanta, GA) were heated (100ºC) in a flask with 2g/L alkali detergent (Micro™, International products corp., Burlington, NJ) then washed for ten cycles in an automatic dishwasher using alkali detergent (Jet Clean, Fisher Scientific, Pittsburgh, PA) to age the surface of the disks and to remove chemical residues. Disks were then sonicated (Aquasonic, model 550HT, VWR scientific, Atlanta, GA) for 40 min in an alkali detergent (Micro™) at 50º C rinsed three times in deionized water, and then autoclaved in deionized water.

Culture source

Microorganisms used in this experiment are listed in Table 4.1. Strains of *L. monocytogenes* isolated from different sources were selected based on their heat resistance and the degree of the biofilm production determined in preliminary studies. *L. monocytogenes* Scott A and 3990 were selected for use in the predictive model because they were the most heat resistant and were high biofilm producers. *L. monocytogenes* strainsYM96 and 303 the moderate biofilm producers and *L. monocytogenes* 17 which

produced minimal biofilm were used in the validation study along with the Scott A and 3990 strains.

Biofilm preparation

In the predictive study, cultures were activated from frozen beads (Microbank™, Prolab diagnostics, Austin, TX) in Tryptic Soy Broth (Difco brand, Becton Dickson, Sparks, MD) with incubation for 18 h at 32ºC. Biofilms were produced by immersing rubber surfaces in diluted TSB(1:10) inoculated with the 0.1%(v/v) inoculum (10^6 cells/ ml) appropriate cultures. The monoculture inoculum of *L. monocytogenes* are strains Scott A and 3990 respectively. The other monoculture inocula are *Pseudomonas* spp. and *P. agglomerans* respectively. The two multispecies culture inocula first consisted of four parts *L. monocytogenes,* Scott A and one part *Pseudomonas* spp and, the next consisted of 8 parts *L. monocytogenes*, 3990, one part *Pseudomonas* spp. and one part *P. agglomerans*. The cultures used in the validation study consisted of a five strain cocktail of *L. monocytogenes,* Scott A; 3990, 17, YM 96 and 303, used in equal parts. Following the 4 h attachment of cells to the rubber surface at 25ºC, the surface was rinsed with phosphate buffer (0.01M, pH 7.0), transferred to diluted TSB and incubated for 48 h at 25 ºC. Biofilms from this protocol were considered as a 'low-soil' condition.

Soiling of biofilm

The soil formulation consisted of an emulsion of rendered chicken fat (137.7 mg /ml), chicken serum (211-338 mg protein/ml)(Sigma C-5405, St. Louis, MO) and lecithin (0.05mg/ml)(Sigma P-5638, St. Louis, MO). The mixture was blended for 1 min with a disinfected electric hand blender (Braun, Lynnfield, MA). Rubber disks containing

biofilm were dipped in the soil mixture then placed in disposable petri dishes and incubated at 10º C overnight (18h). These were considered as 'soiled' biofilm (Disks contained 2mg soil/cm^2).

Surface preparation for validation study

Sterile stainless rubber disks were submerged into broth containing a five strain cocktail of *L. monocytogenes,* Scott A; 3990, 17, YM 96 and 303, used in equal parts. Following a 4 h attachment as previously described, coupons were rinsed with phosphate buffer (0.01M, pH 7.0), transferred to diluted TSB (1:10) and incubated for 48 h at 25 ºC. Biofilms were soiled according to the previously described procedure.

Experimental Controls

Inoculated (positive) and uninoculated (negative) control disks were rinsed in phosphate buffer (0.01M, pH 7,0) and placed in a 0.1% (w/v) peptone with glass beads (3g/10ml, 450-600 μm Sigma, St. Louis, MO), vortexed for 1 min, serially diluted and then plated on Plate count agar (PCA), Listeria selective agar (LSA), or Pseudomonas isolation agar (PIA) and incubated at 32ºC for 24-48h. *L. monocytogenes* was isolated on LSA, *Pseudomonas* spp. on PCA or Pseudomonas isolation agar (PIA) with confirmation by observing the morphology microscopically and *P. agglomerans* were isolated using violet red bile agar (VRB). Multispecies biofilm was analyzed only for surviving *Listeria*. Counts were recorded as colony forming unit (cfu)/ cm^2.

Table 4.1. Microbial cultures used in biofilm formation on rubber surfaces.

Name of microorganisms	serovars	Origin/Source*
Listeria monocytogenes Scott A	4b	Human isolate
Listeria monocytogenes 3990	4b	Vecherin Mont'd or cheese isolate
Listeria monocytogenes YM 96	1/2a	Monkey environment
Listeria monocytogenes 303	1/2a	Monkey liver
Listeria monocytogenes 17	4b	Food processing plant environment
Pseudomonas spp M21		Food processing plant environment
Pantoea agglomerans		Food processing plant environment

Name of microorganisms	Codes
L. monocytogenes, Scott A	Scott A
L. monocytogenes, 3990	LM 3990 or 3990
L. *monocytogenes* Scott A + *Pseudomonas* spp	LMPs
L. monocytogenes, 3990 +*Pseudomonas* spp. + *P. agglomerans*	LMPsP

*Center for Food Safety, Griffin, GA.

Heat inactivation treatment

Duplicate rubber disks containing either low soil or soiled biofilm of *L. monocytogenes* spp., *Pseudomonas* spp., and *P. agglomerans* in monoculture and *L. monocytogenes* within multispecies biofilms (LMPs and LMPsP)(Tb. 4.1) were submerged in a wide mouth dilution bottle containing 100ml of phosphate buffer (0.01M, pH 7.0) preheated to the appropriate temperatures. Disks were heat treated for 1, 3, 5, 15 min at 70, 72, 75, 77 and 80 ºC. A circulating water bath (Precision Scientific, Winchester, VA) was used for heating the phosphate buffer and temperatures were confirmed using a two-channel thermocouple (Traceable®, Houston, TX). One thermocouple was suspended in the heated buffer and the other was attached to the disk surface. Heat treated disks were transferred to 24 well microtiter plates (Corning, Inc., Corning, NY) containing TSB-YE (30g/L Tryptic soy broth and 6g /L yeast extract) at room temperature then incubated at 35 ºC for 24 h . 100 µl aliquot of TSB-YE broth was transferred to Fraser broth and incubated at 35 ºC for 48 h. Samples were streaked for presumptive identification of *Listeria* on LSA and incubated at 35ºC for 24h. Ten percent of positive samples were confirmed on API-Listeria ™ for *L. monocytogenes*. This experiment was replicated 6 times.

Heat treatment for the validation study

New data was collected using a five strain cocktail of *L. monocytogenes* t(Scott A, 3990, YM 96, 303 and 17) that was subjected to the same growth and soiling as previously described. Samples were heated for 1, 8 and15 min at 70, 76 and 80ºC . The experiment was replicated six times with duplicate samples for each experiment.

Test for inhibitory substances in rubber

Cleaned disks were tested for microbial inhibitors using the zone of inhibition method (5) using *L. monocytogenes* Scott A and 3990 as indicator strains. In addition, an extract was prepared from twenty rubber disks by autoclaving them in type I quality water for 45 min with additional storage for 3 days at 25 ºC. 2.5 ml aliquots each of Scott A and strain 3990 were added into 2.5 ml the extract and incubated at room temperature. Samples were collected every hour for 4h for plate counts. Positive and negative controls and treated samples were spiral plated (250ul) on LSA and incubated at 35 ºC for 48 h. Colonies were enumerated as cfu /cm^2.

Experimental design

Four monocultures and two multispecies biofilms were grown on rubber surfaces and exposed to one of two soil conditions. The surfaces were subjected to five heating temperatures and four heating times. Data for the monoculture experiments were recorded as either positive or negative for the presence of *L. monocytogenes* and as positive or negative for the presence of *Pseudomonas* and *P. agglomerans*. The multispecies biofilms were analyzed for *Listeria* only. The experiment was replicated six times with duplicate samples for each experiment. The data from each replication had one of three possibilities, no survivors (0/2), 50% survival (1/2) or 100% survival(2/2). There were 960 experimental observations used in the final model.

Statistical analysis

A statistical model was developed to estimate the probability of *Listeria* in a biofilm survival treated with various time, temperature combinations. The logistics

analysis using SAS/STAT software (SAS®, Duncan, SC) was used to estimate the probability of inactivation Ln [P/(1-P)]= $\beta_0 + \beta_1$ (C)+ β_2 (TEMP) +β_3 (Time) afer heat treatment. For model development, stepwise selection analysis was performed to determine which main effects contributed to the prediction of the data. Factorial combination of the main effects were also tested. After the parameters were selected, a score goodness-of -fit statistic, Schwartz-Bayes Criterion (SC-value) was calculated. The SC score statistic test for the statistical significance of the combined effects of independent variables in the model. It is the most conservative and reliable in evaluating a good fit. The predictive power of the model was evaluated using plots of the predicted points. The initial load was determined to be an insignificant parameter and was not included as a parameter in the model. The predictive power of the selected models was limited by the time (1- 15 min) and the temperature range (70ºC- 80ºC) parameters employed in this experiment. Predictive power was considered as reasonable if the data points fell within the range of these parameters. The predictive efficiency was expressed as an accuracy factor (AF) $AF=10^{(\sum \log(\text{predicted/observed})/n)}$ for comparing the final prediction model and validation model.

MODEL DEVELOPMENT

Pure and multispecies models without soil

Six individual models were developed to predict the heat inactivation of *L. monocytogenes, Pseudomonas* and *P. agglomerans* respectively in monoculture and *L. monocytogenes* in multispecies biofilms. The prediction equations were derived from the logistic regression analysis of the binomial data. The predictive model equation for the

inactivation of *Pseudomonas* in a biofilm indicated that time and temperature were equally important factors in estimating the inactivation of *Pseudomonas* after heat treatment. The prediction equation Ln(P/P-1)= 37.1366 -0.5146* Temp -0.3630* Time (Eq. 1) estimated that the probability of total inactivation of *Pseudomonas* in a biofilm on buna-N rubber after heat treatment of 80 ºC for 1 min was about 99.8 % . For Scott A biofilm the prediction equation Ln(P/P-1)= 24.6210-0.3507*Temp-0.3239*Time (Eq. 2) estimated that there was a 98.8% probability of total inactivation of *Listeria* surviving in a biofilm after heat treatment at 80ºC for 1 min. In the multispecies biofilm (LPs), after a heat treatment at 80ºC, for 15 min there was a 94.6% probability of total inactivation of *Listeria* [Ln(P/P-1)= 10.7749-0.1680* Temp -0.2071*Time (Eq 3). The probability of *P. agglomerans* inactivation in a biofilm on buna-N rubber after heat treatment of 80 ºC for 1 min was about 99.99%, Ln(P/P-1)= 30.4214-0.4917* Temp +0.0627*Time (Eq. 4). For strain 3990 biofilm the prediction equation estimated that *Listeria* did not survive heat treatment of 70ºC or higher. In the multispecies, LPsP biofilm, after a heat treatment at 80ºC, for 15 min there was a 98.6% of total inactivation Ln(P/P-1)= 10.7211-0.1303* Temp -4.0129*Time (Eq 6). The odds ratio demonstrated that time was the predominant factor contributing to each of the predictive models for bacterial survival in a biofilm on rubber.

The integrated model without soil

In developing a model, a balance must be maintained between having parsimony (minimum number of variables in a model) and reducing of experimental error (Σij). By pooling the data of the cultures and including a new explanatory variable (culture) in the

model a balance was maintained and the predictive power of the model was improved. The culture variable was classified with 4 classes of biofilm, Scott A, 3990 strains and *Listeria* in multispecies LPs and LPsP. The integrated model without soil is expressed in (Eq. 7)

$$\text{Ln}(P / P - 1) = 12.8196 + \begin{bmatrix} 4.0045 * \text{ ScottA} \\ 3.7602 * \text{ LPs} \\ -9.5734 * \text{LM 3990} \\ -1.8087 * \text{LPsP} \end{bmatrix} - 0.2446 * \text{ Temp} - 0.2837 * \text{Time}$$

(Eq. 7)

This model predicted that strain 3990 would be inactivated instantly at 70ºC but for a 90 % probability of total inactivation of Scott A, a heat treatment of 1.5 min for 76 ºC min was required. This model was an adequate predictor of *L. monocytogenes* survival in biofilms in a 'low soil' condition. The model predicted within the range of the data, the explanatory variables were statistically significant and the Score statistic SC indicated a good fit.

The integrated model including soil

The addition of soil data yielded the integrated model (Eq 8) with the addition of the classified soil variable. This variable allows the selection of the soil /no soil option. This integrated model (Eq 8) with soil is expressed as:

$$\text{Ln}(P / P - 1) = 20.8366 + 0.6853 * \text{ soil} + \begin{bmatrix} 0.7296 * \text{ ScottA} \\ 0.0118 * \text{ LPs} \\ 0.0422 * \text{LM 3990} \\ -0.7836 * \text{LPsP} \end{bmatrix} - 0.3109 * \text{ Temp} - 0.1173 * \text{Time}$$

(Eq. 8)

This equation predicts that to achieve a 90% probability of total inactivation of Scott A, a heat treatment at 76ºC for 1.7 min was required and, for total inactivation of strain 3990, heating for less than a minute was adequate. Although this integrated model with soil had good fit to the data and good predictive power, the data for the multispecies biofilms (LPs and LPs P) gave similar predictions and could be pooled. This resulted in the final predictive model (Eq. 9).

Final predictive model

$$\mathrm{Ln}(P/P-1) = 19.8811 + \begin{bmatrix} 0.3718 * \mathrm{Soil} \\ 0.000 * \mathrm{Soil} \end{bmatrix} + \begin{bmatrix} 0.4092 * \mathrm{Scott\ A} \\ 0.1245 * \mathrm{LM\ 3990} \\ -0.5337 * \mathrm{Mixed} \end{bmatrix} - 0.2981 * \mathrm{Temp} - 0.1122 * \mathrm{Time}$$

(Eq. 9)

Validation model

The validation model was developed to assess the quality of the final prediction model. The same statistical approach was used to generate the model expressed as:

$$\mathrm{Ln}(P/P-1) = 21.1155 - 0.3175 * \mathrm{TEMP} - 0.1317 * \mathrm{TIME} \qquad \text{(Eq.10)}$$

The validated model was graphically compared with the predicted model (Eq. 9). The accuracy factor of the predictive and validated data was calculated and expressed as the antilog of the average log of the ratio between the predicted and validated data [$AF = 10^{(\sum \log(\text{predicted/observed})/n)}$].

RESULTS

The final heat inactivation predictive model for *L. monocytogenes* in a biofilm is shown in Eq. 9 and the statistical data and parameters are presented in Table. 4.2. The final model indicates that soil, culture, temperature and time parameters were the main effects that had statistical significance in the prediction of *Listeria's* survival. Soil and culture are the classified variables and time and temperature are the continuous variables. The soil variable contains the soil/ low soil options. The culture variable has four classes of biofilm, Scott A, strain 3990 and *L. monocytogenes* in two different multispeciess. Goodness of fit test (SC) comparing the full ranked model with the final model (Table 4.2) indicates that the final model had a good fit. The evaluation of the predictive power shows that this model predicts within the range of the parameters tested.

Final Predictive model

$$\mathrm{Ln}(P/P-1) = 19.8811 + \begin{bmatrix} 0.3718 * \mathrm{Soil} \\ 0.000 * \mathrm{No\ Soil} \end{bmatrix} + \begin{bmatrix} 0.4092 * \mathrm{Scott\ A} \\ 0.1245 * \mathrm{LM\ 3990} \\ -0.5337 * \mathrm{Mixed} \end{bmatrix} - 0.2981 * \mathrm{Temp} - 0.1122 * \mathrm{Time}$$

(Eq. 9)

This study shows that *L. monocytogenes* in biofilms grown on rubber in the presence of soil has a low probability of survival at a heat treatment of 78 ºC for 15 min. However, Scott A in a monoculture was the most heat resistant biofilm on buna-N. The prediction for Scott A strain (Fig. 4.1) in a biofilm showed that to achieve a 95% and 90% probability of total inactivation with hot water, a heat treatment of 15 and 9 min respectively at 80ºC is required. For a 75% probability of inactivation, a heat treatment of

less than 1 minute at 80ºC is required. The prediction for heat inactivation of *L. monocytogenes* 3990 (Fig. 4.2) in a biofilm showed that to achieve 95%, and 90% probability level of inactivation required heat treatment of 76 ºC for less than 6 min, and less than one minute respectively. The heat inactivation prediction for *L. monocytogenes* in multispecies biofilm (Fig. 4.3) showed that to achieve a 95% and 90% probability of total inactivation, heat treatments at 76 ºC for less than one minute are required. The predictive model in the presence of soil indicates three heat resistance conditions for which this model can be used. The Scott A model is the most conservative in its prediction and as it advises a more intense heat treatment for inactivation while models based on *L. monocytogenes* 3990 and *L. monocytogenes* in multispecies biofilms are less conservative in their recommended heat treatments.

In the low soil prediction model with Scott A biofilm (Fig. 4.4), a heat treatment at 76ºC for a 95% and 90% probability of total inactivation, requires heating for 5.3 min and less than a minute respectively. While for strain 3990 (Fig. 4.5), to achieve a 95 % and 90% probability of total inactivation, heat treatment at 76º C for 2.6 min and less than 1 minute respectively is needed. For *L. monocytogenes* in multispecies biofilm in low soil conditions (Fig. 4.6), for a 95% and 90% probability inactivation, heat treatment at 76º C for 6.3 min and less than 1 minute respectively is required. Overall, strain 3990 biofilms on rubber surfaces were readily inactivated following heat treatment. The Scott A biofilm in both low and high soil conditions (Fig. 4.1 and 4.4) and *Listeria* in the multispecies biofilm under low soil (Fig. 4.6) conditions showed more heat resistance than the strain 3990 biofilm in both soil conditions (Fig. 4.2 and 4.5). This observation is

contrary to the previous study using stainless steel surfaces (unpublished data) in which strain 3990 biofilm showed greater heat resistance than both Scott A and *Listeria* in the multispecies biofilm under soiled conditions (Fig. 4.1, 4.2, 4.4 and 4.5). This observation illustrates the variable response of bacterial strains in the presence of soil and surface materials and, their influence on biofilm persistence and heat resistance.

Comparison of the validation model

$$Ln(P/P-1)= 21.1155-0.3175*TEMP-0.1317*TIME \qquad (Eq.\ 10)$$

with the predictive model indicates that the predictive model was conservative in its assessment of risk. The accuracy factor for predicted versus the validated data was 0.8. this means that predicted heat treatment requirements are greater for the predictive model than the validation model. The heat inactivation prediction for Scott A in a biofilm in soiled conditions was highly conservative showing substantially higher heat inactivation requirements than the validation model indicated. The 3990 strain in a biofilm (Fig. 4.2) was less conservative in its prediction than Scott A strain (Fig. 4.1) but the predicted heat treatments requirements were higher than the validation model. *L. monocytogenes* in a multispecies biofilm showed similar probabilities of inactivation as the validation model predictions. Under low soil conditions, Scott A and 3990 (Fig. 4.4 and 4.5) closely paralleled the validation model estimate of inactivation. While the inactivation prediction of *Listeria* in multispecies (Fig. 4.6) biofilms were slightly conservative compared to the validation model estimate.

DISCUSSION

This study showed that *L. monocytogenes* in biofilms grown on rubber were adequately inactivate by heat treatment ranging from 78-80º C for 10-15 min. This suggests that hot water sanitation during intercycle cleaning will inactivate *Listeria* in biofilms formed on rubber so long as adequate time/ temperature controls are maintained. This study produced a predictive model estimating the probability of *L. monocytogenes* inactivation in monoculture and in multispecies biofilms formed on buna-N rubber and in the presence of low-soil and soiled conditions. The model provides for three prediction situations in the presence of soil, the fairly conservative prediction using heat resistant Scott A strain and the less conservative predictions based on strain 3990 and *Listeria* in a multispecies. For the low soil condition, the Scott A and 3990 strains paralleled the validated model while the *L. monocytogenes* in a multispecies biofilm was conservative in its assessment of risk. This prediction model when compared with the five strain *L. monocytogenes* model indicated higher probabilities of inactivation at equivalent heat treatments. The accuracy factor (.81) confirms the conservative predictions of the predictive model with soil in required heat treatment for the inactivation of *L. monocytogenes* in biofilms.

Buna-N rubber is one of the materials used for gaskets in the food processing industry. Continuous exposure with alkali cleaners, sanitizers, high temperatures and high pressure from sealing the coupling can cause rubber to deteriorate leading to development of cracks (11). As rubber ages it accumulates more soil and allows more biofilm formation that is difficult to clean (11). Our study indicates that hot water is a

reasonable sanitation option to inactivate *Listeria* in a biofilm on used buna-N surfaces. However, long term inadequate cleaning may lead to

biofilm that are more heat resistant than those tested in this study. In addition, processors may not have good knowledge of the actual heat treatment applied to a gasketed joint as this heat treatment could be less than indicated by water flowing through the system.

Various factors influence bacterial attachment to a surface. *Listeria* has a net negative surface charge and relatively low hydrophobic cell surface. However under stressed conditions *Listeria*'s cell hydrophobicity increases (3) and interacts with hydrophobic surfaces through hydrophobic-hydrophobic interactions. Rubber becomes less hydrophobic as it ages and this may lower the hydrophobic interaction between bacterial cells and the rubber surface. However, preconditioning of the solid surface with food residue may negate the effect of the surface charge and facilitate or inhibit bacterial attachment (4, 9) while surface charge and bacterial attachment to a surface and increase their thermal resistance (2, 4). The effect of soil on the heat resistance of *L. monocytogenes* biofilm in this study was inconclusive. The prediction of *Listeria* inactivation on soiled buna-N surface indicates substantially higher heat resistance of Scott A biofilm, slightly higher resistance of the strain 3990 but significantly less resistance in the multispecies biofilm with soil as compared to low soil conditions.

Previous research indicate that new and heated rubber may release bacteriostatic substances that inhibit microbial colonization (5, 11). However the inhibition test (data not shown) on the precleaned and aged rubber disks did indicate the presence of

listericidal agents. The diluent of heated rubber did not detect the release of Listeriocidal compound during heating. In conclusion, our validated prediction model indicates that with proper maintenance of the time /temperature controls *Listeria* in biofilms will be inactivated on rubber surfaces. This predictive model therefore allows food processors to manage hot water sanitation processes for rubber surfaces to achieve an acceptable level of risk.

References

1. **Blackman, I. C., and J. Frank.** 1996. Growth of *Listeria Monocytogenes* as a biofilm on various food processing surfaces. J. Food Prot. **59:**827-839.

2. **Driessen, F. M., J. D. Vries, and F. Kingma.** 1984. Adhesion and growth of thermoresistant *Streptococci* on stainless steel during heat treatment of milk. J. Food Prot. **47:**848-852.

3. **Dykes, G., J. Mills, and R. G. Bell.** 2001. The effect of chilled storage on *Listeria monocytogenes* cell surface hydrophobicity and attachment to beef muscle. Int. J. Food Sci. Technol. **36:**783-788.

4. **Helke, D. M., E. B. Somers, and A. C. L. Wong.** 1993. Attachment of *Listeria monocytogenes*and *Salmonella typhimurium* to stainless steel and buna-N in the presence of milk and individual milk components. J. Food Prot. **56:**479-484.

5. **Helke, D. M., and A. C. L. Wong.** 1994. Survival and growth characteristics of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and buna-N rubber. J. Food Prot. **57:**963- 968, 974.

6. **McGuire, J., and V. Krisdhasima.** 1991. Surface chemical influences on protein adsorption kinetics. Food Tech. **45:**92-96.

7. **Ronner, A., and A. Wong.** 1993. Biofilm development and sanitizer inactivation of *Listeria Monocytogenes* and *Salmonella Typhimurium* on stainless steel and buna-n rubber. J. Food Prot. **56:**750-758.

8. **Sinde, E., and J. Carballo.** 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. Food Microb. **17:**439-447.

9. **Smoot, L. M., and M. D. Pierson.** 1998. Effect of environmental stress on the ability of *Listeria monocytogenes* Scott A to attach to food contact surfaces. J. Food Prot. **61:**1293-1298.

10. **Storgards, E., H. Simola, A.-M. Sjoberg, and G. Wirtanen.** 1999. Hygiene of gasket material used in food processing equipment part 1: new materials. Trans IChemE **77:**137-145.

11. **Storgards, E., H. Simola, A.-M. Sjoberg, and G. Wirtanen.** 1999. Hygiene of gasket material used in food processing equipment part 2: aged materials. Trans IChemE **77:**146-155.

12. **Wong, A. C. L.** 1998. Biofilm in food processing environments. J. Dairy Sci. **81:**2765-2770.

Table 4.2. The results of logistic regression analysis showing the parameters, parameter estimates and Chi square values of the full ranked and final prediction model for rubber surfaces.

Parameters	Full model	Final model	
	P> Chi sq	Estimate	P> Chi sq
Intercept	<0.0001	19.8811	<0.0001
Soil	0.99	0.3718	0.0001
Culture	0.99		<0.0001
Scott A		0.4092	
LM 3990		0.1245	
Multispecies		-0.5337	
TEMP	0.87	-0.2981	<0.0001
TIME	0.97	-0.1122	<0.0001
TEMP*C	0.99	-	-
TIME*C	0.99	-	-
TEMP*TIME	0.96	-	-
TEMP*Soil	0.98	-	
TIME*Soil	0.92	-	
TEMP*Soil*C	0.96	-	
TEMP*TIME*C	0.99	-	
TEMP*Soil*C	0.99	-	-

Figures 4.1- 4.3. Probability of heat inactivation of *Listeria monocytogenes* Scott A (Figure 4.1), *L. monocytogenes* 3990 (Figure 4.2), and *L. monocytogenes* in multispecies (Figure 4.3) biofilm on Buna- N rubber after heat treatment with soil. Heating temperatures range from 70 to 80 ºC and heating times of 1, 3, 5,and 15 min. There were six replications. Heat inactivation probability plots are represented at 95% (P=.95), and 90% (P=.90) and validated probability level at 90% (VP=.90). The probability levels were derived from the following equation:

$$\mathrm{Ln}(P/P-1) = 19.8811 + \left[0.3718 * \mathrm{Soil}\right] + \begin{bmatrix} 0.4092 * \mathrm{Scott\ A} \\ 0.1245 * \mathrm{LM\ 3990} \\ -0.5337 * \mathrm{Mixed} \end{bmatrix} - 0.2981 * \mathrm{Temp} - 0.1122 * \mathrm{Time}$$

Figure 4.1

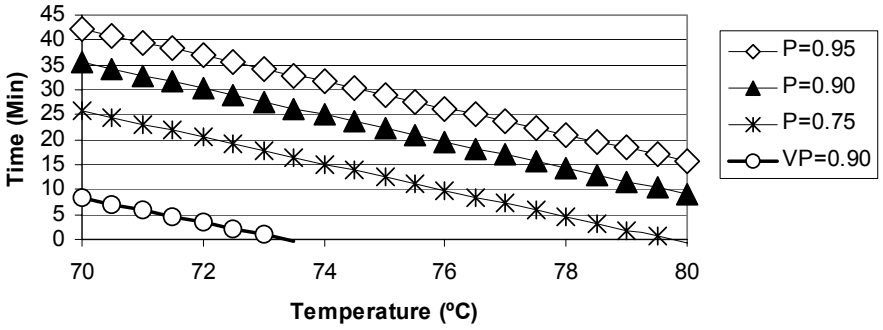


Figure 4.2

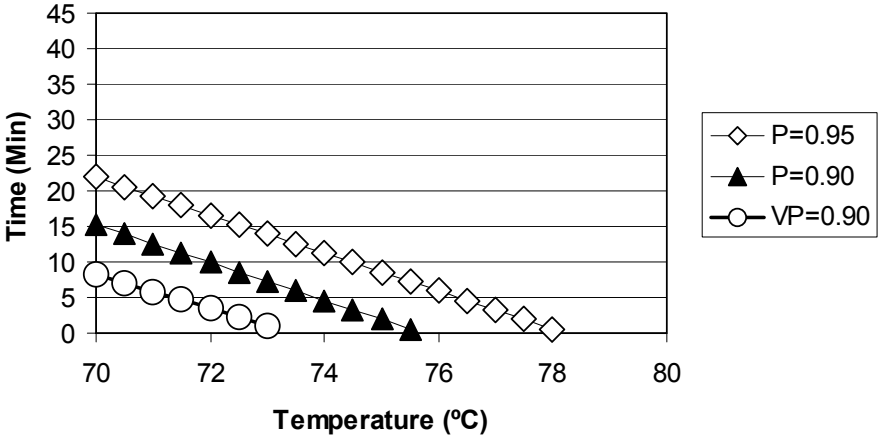
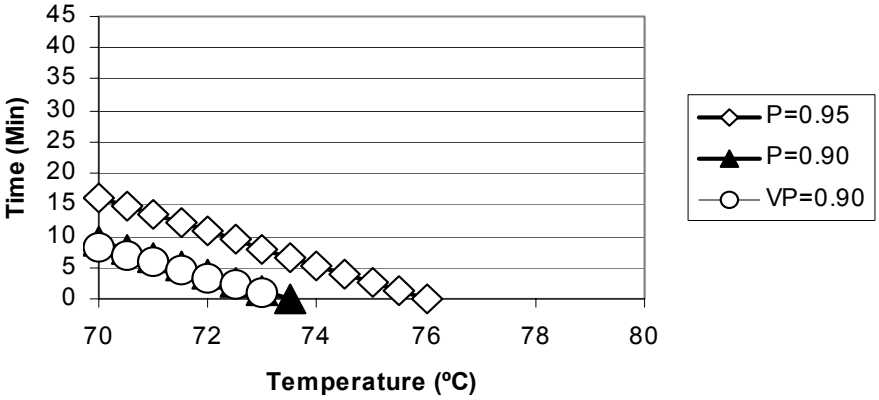


Figure 4.3



Figures 4.4- 4.6. Probability of heat inactivation of *Listeria monocytogenes* Scott A (Figure 4.4), *L. monocytogenes* 3990 (Figure 4.5) and *L. monocytogenes* in multispecies biofilm on Buna- N rubber after heat treatment with low soil (Figure 4.6). Heating temperatures range from 70 to 80 ºC and heating times of 1, 3, 5,and 15 min. There were 6 replications. Heat inactivation probability plots are represented at 95% (P=.95), and 90% (P=.90) levels and validated probability level at 90% (VP=.90). The probability levels were derived from the following equation:

$$\mathrm{Ln}(P/P-1) = 19.8811 + \left[0.000 * \mathrm{Soil}\right] + \begin{bmatrix} 0.4092 * \mathrm{Scott\ A} \\ 0.1245 * \mathrm{LM\ 3990} \\ -0.5337 * \mathrm{Mixed} \end{bmatrix} - 0.2981 * \mathrm{Temp} - 0.1122 * \mathrm{Time}$$

Figure 4.4

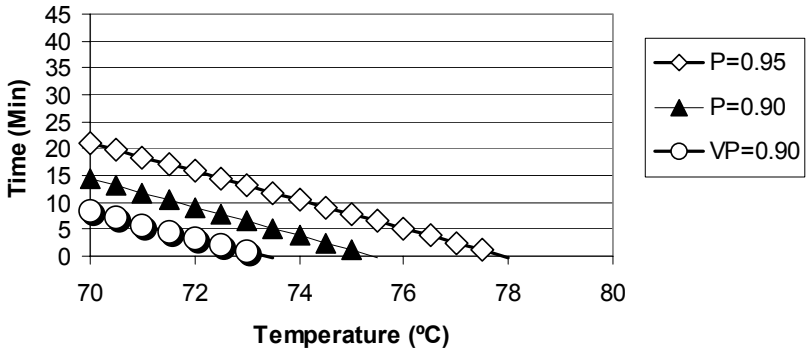


Figure 4.5

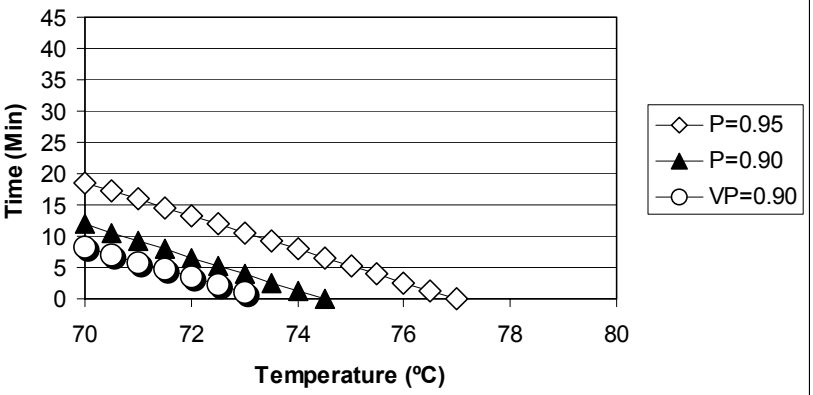
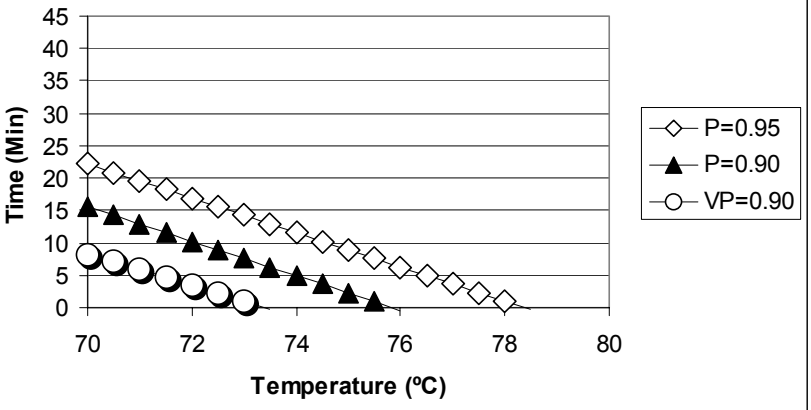


Figure 4.6



CHAPTER 5

PROTEIN EXPRESSION OF NON-HEAT AND HEAT STRESSED BIOFILM OF *LISTERIA MONOCYTOGENES* AND SUBSEQUENT BIOFILM FORMATION ON STAINLESS STEEL [1]

---

[1] Chmielewski, R.A.N. and J.F. Frank 2003. To be submitted to the *Journal of Food Protection*

ABSTRACT

Hot water sanitation is commonly practiced in the food industry. The purpose of this study was observe the effects of repeated sublethal heating on the biofilm production of *L. monocytogenes* Scott A and 3990 and related protein expression. *L. monocytogenes* Scott A heated at 70ºC for 3 min showed a five fold increase in biofilm production compared to non-heated *L. monocytogenes* Scott A. *L. monocytogenes* 3990 did not show such an increase in biofilm production. The proteomic analysis of *L. monocytogenes* 3990 showed changes in expression of 19 proteins using two dimensional-SDS PAGE, of which 3 proteins were up-regulated and 16 were down-regulated as a consequence of heat stressed. The proteomic analysis of heat treated *L. monocytogenes* Scott A showed that there were 18 protein spots expressed in the 2D-SDS PAGE of which 2 spots were up-regulated and 16 were down- regulated as a result of sublethal heat stress. The increase in biofilm production following sublethal heating emphasizes the importance of the complete removal of microbial cells and polymeric substances from surfaces during cleaning and sanitation.

INTRODUCTION

The transition of bacterial cells from the planktonic to the biofilm mode of growth is genetically regulated (4, 10, 17, 19). Physiological changes including the formation of pili and other surface proteins occur in bacterial cells when growth transitions from planktonic to biofilm producing state (18, 21, 24). Proteomic analysis shows differential protein expression of *L. monocytogenes* and *B. cereus* in the transition from planktonic and biofilm producing mode is associated with the response to environmental stresses such as nutrient and oxygen depletion. Nineteen proteins associated with biofilm production are up-regulated. These include catabolic ornithine carbamoyl transferase (cOTase), L-lactate dehydrogenase (lct E) and YhbH, a member of the σ^{54} transcription protein (18, 24). Both cOTase and lct E are enzymes that occur in anaerobic metabolism. YhbH, a member of the σ^{54} factor, is responsible for inducing a response to environmental stresses (18, 24).

Cross protection in bacterial cells can be induced in response to environmental stresses such as starvation, osmotic shock, heat shock, and oxidative shock (9, 12-14, 23, 25). Stephens (23) observed the increase thermotolerance of *L. monocytogenes* in high osmotic (NaCl) conditions and noted increased stability of ribosomal (30S) subunits whereas in the presence of Mg^{++} destabilized the 30S subunits and *L. monocytogenes* was more heat sensitive. Stephens (23) suggested that not only heat shock proteins were responsible for thermotolerance but also ionic shock protein. Other studies (2, 3, 6, 13, 20) demonstrated that the exposure of *Listeria* to temperatures above optimal growth conditions, known as heat shock (ca.45-50ºC) triggers the synthesis of heat shock protein

(hsp) resulting in increased thermotolerance. This heat shock condition is transient and is known as adaptive thermotolerance (3). Heat resistance of bacteria in starved conditions was demonstrated by several studies. Lou et. al (15) observed increased heat resistance of *L. monocytogenes* Scott A after starvation in a broth system. Jorgensen (13) had similar observation in *Pseudomonas fluorescens*. Protein analysis showed that seven proteins were induced and up-regulated upon heat shock treatments; these proteins were not identified by sequencing (20). However, sequencing of the three proteins induced by osmotic shock revealed that the proteins AppA, Cte and YvyD were up-regulated. AppA is an acetate kinase, Cte is a general stress protein induced in stationary phase (5)and YvyD protein similar to YhbH protein and is responsible for inducing a response to environmental stresses such as nutrient depravation, ethanol, heat. These proteins are both regulated by σ^B (18, 24)

Biofilms of *L . monocytogenes* have been produced on many surfaces including stainless steel, glass, rubber, plastics and microtiter plates (1, 8, 11) in continuous flow and static systems. In this study, glass wool was used as the substratum as it provides a large surface to volume ratio and allows the separation of the biofilm biomass from the planktonic cells. To date, no study has been done to show the effects sublethal heating on biofilm production. Therefore, the purpose of this study was to analyze the effect of repeated sublethal heating on the biofilm production of *L. monocytogenes* and related protein expression.

MATERIALS AND METHODS

Cultures

L. monocytogenes Scott A and 3990 were selected for use in this study because preliminary study indicated increased heat resistance and high biofilm producing abilities. Cultures were activated from frozen beads (Microbank, Prolab diagnostics, Austin , TX) in Tryptic Soy broth (TSB) (Difco brand, Becton Dickson, Sparks, MD) for 18 h at 32ºC.

Biofilm on glass wool

Biofilms were grown on glass wool because glass wool provides a large surface area allowing the harvesting of large quantity of cells for investigative studies on the effect of sublethal heating on subsequent biofilm formation on stainless steel surfaces and to study the corresponding protein expression. Glass wool was prepared by washing in deionized water, dried and autoclaved. Fifty grams of the sterile glass wool was added to 500ml of 1:10 diluted TSB (3g/L) inoculated with 0.1% of *L. monocytogenes* Scott A or 3990 strains. The inoculated glass wool was incubated at room temperature (25 +/- 2 ºC) on a rotary shaker (150rpm). After 4 h, the inoculated glass wool was rinsed with phosphate buffer (0.01M, pH 7.0) to remove unattached cells and sterile diluted TSB was added. Incubation continued at 25ºC. Biofilm was washed and replenished with diluted TSB daily for 7 days. After 7 days, cells were harvested from glass wool and divided into two portions. One portion was used in heating study and the other portion was used for proteomics analysis. This experiment was repeated twice with triplicate samples.

Heat treatment

Biofilm on glass wool was rinsed in phosphate buffer (0.01M, pH 7.0), drained and added to a sterile 1L glass beaker containing preheated phosphate buffer. The biofilm on the glass wool was heated at 70ºC for 3 min. obtaining an internal temperature in the glass wool of 67ºC. The glass wool was removed, rapidly drained and submerged into cold (4ºC) Tryptic soy broth with yeast extract (TSB-YE, Tryptic Soy Broth, (30g/L) and yeast extract (6g/L) (Difco brand, Becton Dickson, Sparks, MD). The glass wool was incubated at 25ºC on a rotary shaker (150 rpm) for 18-24h. Then the glass wool-containing biofilm was transferred to diluted TSB with Fraser broth supplement SR156E (Oxoid, Ogdenburg, NY) for 1 day at 25 ºC and then transferred into diluted TSB with incubation at 25 ºC and daily media changes for a total of 7 days. The heating and culturing process was repeated three times before the biofilm was harvested. This experiment was repeated twice with triplicate samples.

Harvesting of cells

Biofilms (heated and unheated) on glass wool were rinsed in phosphate buffer (0.01M, pH 7.0) with vigorous shaking to remove unattached cells. The glass wool was aseptically removed from the flask and placed in a sterile filtered stomacher bag and drained. Glass-wool was resuspended in phosphate buffer and stomached for 3 min. The filtrate from both replicates were combined into one sample as the quantity of protein accumulated was marginal for separation. The filtrate was centrifuged at 9500 g for 25 min at 4ºC . The pellet was washed three times in Tris-EDTA buffer. One portion of the

cells were stored on cryoprotectant beads (Microbank™, Prolab diagnostics, Austin, TX) and frozen at -80C, the other portion was used for protein extraction.

Protein extraction

Proteins were extracted from heated and unheated Scott A and 3990 cells. Protein extraction is in preparation for protein separation using two-dimensional gel electrophoresis. The washed pellet was resuspended and sonicated (Sonic dismembrator, model 150, Fisher Scientific, Pittsburgh, PA) three times for 2 min at 4ºC using a tip setting at power 6 and 50% pulse duration. The cell suspension was prepared for protein extraction using a Biorad ReadyPrep™ sequential protein extraction kit (Biorad, Hercules, CA). The suspension was centrifuged (9000g, for 25 min) three times in three respective reagents containing 40mM Tris buffer and tributyl phosphine (TBP). The supernatant from each extraction was collected and precipitation in 3 volumes of ice-cold acetone, then incubated for 2 h at -20 ºC then centrifuged for 25 min at 9000 g. The protein pellet was resuspended in rehydration buffer (8 M urea, 2% CHAPS, 10mM dithiothertol (DDT), 0.2% biolyte, and 0.001% bromophenol bluel) and frozen at -20ºC. The protein assay was performed using BCA protein assay (BCA protein assay kit 23225, Pierce, Rockford, IL). Samples were re-extracted using PlusOne™ 2-D clean up protein extraction kit (Amersham Biosciences, Piscataway, NY).

Sample preparation for staining and electrophoresis

The protein extract from heat treated and unheated (control) samples were resuspended in 200 µl rehydration buffer containing Cy ™3 DIGE Fluor (λ_{ex}= 553 nm, λ_{em}=572 nm) and Cy ™ 5 DIGE Fluor (λ_{ex}= 648 nm, λ_{em}=669 nm) (400pmol/ 50 ug,

Cyanine dye DIGE Fluors, Amersham Biosciences, Piscataway, NY) to a final protein concentration of 5.0 μg/ml or a total of 40 μg per sample per gel. The control sample was fluorescently labeled with Cy 3 and the heat treated samples with Cy 5. Both the control and treated samples were combined and used to rehydrate the gel acrylamide strip (Biorad, Hercules, CA) .

Two dimensional gel electrophoresis

Isoelectric focusing Samples were rehydrated in isoelectric focusing (IEF) unit (Biorad, Hercules, CA) using the active rehydration protocol at 500V recommended by the manufacturer. Isoelectric focusing was performed for 7 h using a seven step holding and linear ramping program starting with 500v and ending with 8000 V .

Second dimension electrophoresis The second dimension separated proteins by molecular weight using the Ettan ™ Dalt II (Amersham Biosciences, Piscataway, NY) vertical gel electrophoresis system with 8-15 % gradient polyacrylamide gel. The sample strips were equilibrated in two buffers(6 M urea, 2% (w/v) sodium dodecyl sulphate (SDS), 0.375M Tris-HCL, pH 8.8, 20% (v/v) glycerol one containing 130 mM DDT with bromophenol dye (0.001% (v/v)) and the other buffer containing 135mM iodoacetamide and rinsed in 1 X running buffer (25 mM Tris, 192mM glycerin and 0.2% (v/v) SDS). The strips were gently rocked on a rotary shaker for 15 min in each buffer. The SDS unit was powered at 30 m-amps then to 50 m-amps for 4 hr.

Analysis of protein patterns

The Typhoon 9400 laser scanner (Amersham Biosciences, Piscataway, NY) was used to capture the image of the fluorescent labeled gels. The image (Fig. 3B) from each gel shows both the control sample (green) and heat treated sample (red). Each of the samples were viewed independently by selecting the individual excitation and emission wavelengths (Fig. 3C). The images were automatically optimized using Image Quest software (Amersham Biosciences, Piscataway, NY). DeCyder software used algorithms to select protein spots, filter dust and quantify the peak height, area and volume of the protein spots from the fluorescent labeled gel images. After imaging, gels were destained, fixed and restained with SYPRO™ Ruby dye (Amersham Biosciences, Piscataway, NY)

Surface preparation

Stainless steel surfaces were prepared for biofilm formation by biofilm cells (non-heated and sublethally heated) previously harvested from glass wool. Biofilms were formed on stainless steel for quantitative measurement. Stainless steel coupons 10 cm^2 (5x 2 cm) were degreased in acetone, sonicated for 60 min at 55ºC in alkali detergent (Micro™,International products corp., Burlington, NJ)) soaked overnight for about 18 h, rinsed with deionized water, sonicated for 20 min in acid cleaner (Zep formula 3586, Zep manufacturing, Atlanta, GA, 30 ml/L or phosphoric acid, 15ml/l) and rinsed in deionized water.

Biofilm formation

L. monocytogenes Scott A and 3990 and, heat treated Scott A and 3990 biofilm cells were each activated from frozen beads (Microbank™) in Tryptic Soy Broth (Difco brand, Benton Dickson, Sparks, MD) for 18 h at 32ºC. Biofilms were produced by immersing stainless steel surfaces in diluted TSB (1:10) inoculated with the appropriate culture, followed by incubation for 4 h at 25ºC, rinsing with phosphate buffer, transferring to diluted TSB (1:10) and incubation for 48 h at 25 ºC.

Microscopy

Listeria biofilms formed on stainless steel were stained with 10ug/ml of Bactrace ® fluorescein-labeled affinity antibody to Listeria (Kirkegard & Perry Laboratories, Gaithersburg, MD) and incubated for 20 min in the dark, rinsed and counter stained with 10 ug/ml Concanavalin A conjugated with Texas Red ® for 20 in the dark. Biofilms were observed using Nikon Eclipse 600E microscope at 400X magnification. The area of the microscopic field imaged was 37,800 μm^2 (0.0378000mm^2). Images were captured using an Optronics camera (Goleta, CA) and Magnafire CCD camera and analyzed using image tool (University of Texas Health Science, San Antonio). Images were converted into black and white by thresholding (Adobe Photoshop, Adobe system, Inc, San Jose, CA) so that cells were white and stainless steel was black. Black and white pixels were calculated using image tool. The white pixels represented percent area covered by biofilm. Ten images were obtained per coupon. There were five replications for each culture type for a total of 50 images per culture.

RESULTS

Sublethal heating of biofilm cells induced increased production of biofilm for *L. monocytogenes* Scott A but not strain 3990 when the strains were subsequently grown on stainless steel surfaces. Unheated *L. monocytogenes* (Scott A and 3990) biofilms showed similar level of biofilm development on stainless steel, 7.8 % and 8.8 % biofilm respectively (Fig.5.1). Heat stressed biofilm cells of Scott A exhibited a five fold increase in biofilm production (39%) compared to the Scott A control (7.8%). However, no such changes in biofilm production were evident between the heated and unheated 3990 biofilms. Images of the non-heated and sublethally heated treated biofilms of Scott A and 3990 on stainless steel surfaces (Fig.5.2) demonstrate the increased density and surface coverage of biofilm from the heat stressed Scott A biofilm cells (Fig. 5.3).

The 2-D PAGE analysis (Fig.5.3) showed the changes in expression of 19 proteins for heat stressed *L. monocytogenes* Scott A biofilm cells and 18 proteins for heat stressed *L. monocytogenes* 3990 biofilm cells. The proteins from both strains (Scott A and 3990) were in the molecular weight of 15 to 95 Kda and most were separated in the acidic range except for 3 proteins (spots # 465,474 and 466 for Scott A and 751, 752 and 754 for 3990). The comparison of protein spots of the control and the heat stressed *L. monocytogenes* Scott A and 3990 biofilms (Fig. 5.3) show that there were significant differences in protein expression before and after heat stress.

One protein (spot no. 637) expressed by Scott A control was absent in the heat stressed Scott A while 14 spots expressed in the Scott A control and were repressed (-) in

heat stressed biofilm cells (Tb. 5.1 and Fig. 5.3 and 5.4). Three proteins repressed in Scott A control biofilm cells were induced in the heat stressed Scott A biofilm cells. Of the proteins induced by heat treatment, spot numbers 932, 644 and 958 showed a 10.4, 3.8 and 2.2 fold increase in protein expression (Tb 5.1).

Most of the proteins separated by SDS-PAGE were in the acidic range (pI 4.5-6.8) except for three spots (spot number 465, 466 and 475) which were isolated in the alkali range (pI 9.3) (Fig. 5.5). These proteins were not expressed by stressed biofilm cells. One protein (spot 956) was expressed in the *L. monocytogenes* 3990 control but was absent in heat stressed *L. monocytogenes* 3990 (Fig. 5.5). This protein spot has similar (pI and MW) to the protein (spot 637) in *L. monocytogenes* Scott A control (Fig. 5.3). There were four spots expressed in *L. monocytogenes* 3990 control were induced in heat stressed 3990 biofilm cells. These proteins (spot nos. 1321, 1395, 639 and 863) showed a 4.5, 4.5, 3.2 and 2.3 fold increase respectively (Fig. 5.6).

Twelve proteins were repressed in heat stressed *L. monocytogenes* 3990 (Tb. 5.2). The most abundant protein of these spots was spot no. 956 which was showed a 17 fold decrease after heating (Tb. 5.2 and Fig. 5.5).

Spot 644 on the *L. monocytogenes* Scott A SDS-PAGE was a protein down regulated after heat stress (Fig.5.4). That protein was also absent in the *L. monocytogenes* 3990 SDS-PAGE (Fig. 5.5). The proteins (spot 851 and 316, Figure 5.4 and 5.6) that were expressed in the SDS-PAGE of *L. monocytogenes* Scott A controls were also expressed in the heat stressed biofilm cells but were shifted to a lower molecular weight. Shifts in molecular weight is usually associated with co-translational

or post-translational modification of a glycosylated or phosphorylated group and are associated with thermal stability (18) however, the shifts in protein molecular weight of heated unexplicitly demonstrated the opposite effect.

Tentative identification of the protein samples showed that proteins down regulated were associated with virulence protein, metabolic enzymes, general stress and heat shock proteins. The data on protein expression is not complete so no connection could conclusively be made between increased biofilm production and corresponding protein expression.

DISCUSSION

In the food industry hot water rinsing for soil removal and sanitation is a common practice. The inadequate cleaning and removal of microbial cells may lead to the increased biofilm formation and polysaccharide accumulation. Although cells within a biofilm may be killed by a sanitation process, the remaining biofilm polymers may also become harborage for subsequent microbial contamination. In this study repeated sublethal heating triggered a five fold increase in biofilm production for heat stresses *L. monocytogenes* Scott A while there was no significant increase in biofilm production in the 3990 strain. Increase biofilm production could have significant implications in sanitation processes practiced in the food industry. Increased biofilm production can lead to increase in biofilm cells survival. Studies (3, 6, 12, 13) indicate that prior heat shock increases the thermotolerance of *L. monocytogenes*. There are increases in thermotolerance associated with different heating menstra, for example, reheated milk, sous-vide products and meat emulsions increased thermotolerance with D 60- values

ranging from 0.5- 8.32 min (6, 7, 9, 15, 16). *L. monocytogenes* in biofilm (8) as well as stationary phase cells are more thermotolerant (15) however, differences in heat resistance of *L. monocytogenes* between strains and within serovars have been demonstrated (22).

Proteomic analysis of *L. monocytogenes* Scott A and 3990 indicated that most proteins expressed were between 15 and 95 Kda and were in the acidic range (pI 4.5-6.8) except for three spots (Figure 3C and 4C) which were in the alkali range (pI 9.3). The alkali proteins were expressed in the unheated biofilm cells only and not after heat stress. Proteomic analysis (18, 20, 24) indicates that proteins expressed when *L. monocytogenes* cells transitions from planktonic to the biofilm state are in the acid range (pI = 4.6-5.8) with molecular weights of 23.9- 57.6 Kda. While the most common heat shock protein expressed by *L. monocytogenes* is 17.6Kda with a pI of 5.1. Some proteins which are known to be expressed when *L. monocytogenes* is subjected to heat above 30ºC are heat shock protein (Rpos H), β-galactocidase, Prf A protein, which is a co-regulator of σ^B that that transcribes prf-A dependent virulence gene cluster (hly, actA, plcA, inlA).

In conclusion, sublethal heating of *L. monocytogenes* Scott A biofilm cells induced a five fold increased production of biofilm for but, this did not occur in the 3990 strain. The protein expression of unheated and sublethally heated *L. monocytogenes* (Scott A and 3990) biofilm cells were different between the unheated control and heat stressed biofilm cells. However, no conclusive links could be made between the biofilm production and protein expression due to insufficient data. The finding of increased

biofilm production from sublethally heat *L. monocytogenes* 3990 biofilm cells emphasizes the importance the of controlled effective cleaning for the removal of polymeric substances and biofilm cells from stainless steel surfaces.

References

1. **Blackman, I. C., and J. Frank.** 1996. Growth of *Listeria Monocytogenes* as a biofilm on various food processing surfaces. J. Food Prot. **59:**827-839.

2. **Bolton, D. J., C. M. McMahon, A. M. Doherty, J. J. Sheridan, D. A. McDowell, I. S. Blair, and D.Harrington.** 2000. Thermal inactivation of *Listeria monocytogenes* and *Yersinia enterocolitica* in minced beef under laboratory conditions and in sous -vide prepared minced and solid beef cooked in a commercial retort. J. Appl. Microbiol. **88:**626-632.

3. **Bunning, V. K., R. G. Crawford, J. T. Tierney, and J. T. Peeler.** 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella typhimurium* after sublethal heat shock. Appl. Environ. Microbiol. **56:**3216- 3219.

4. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847-867.

5. **Duche, O., F. Tremoulet, A. Namane, T. E. L. G. Consortium, and J. Labadie.** 2002. A proteomic analysis of the salt stress response of *Listeria monocytogenes*. FEMS Letters **215**.

6. **Farber, J. M., and B. E. Brown.** 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. Appl. Environ. Microbiol. **56:**1584-1587.

7. **Farber, J. M., and P. I. Peterkin.** 1999. *Listeria monocytogenes* in meat products, p. 505-564. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria*, Listeriosis, and Food Safety. Marcel Dekker, Inc, New York.

8. **Frank, J., and R. Koffi.** 1990. Surface-adherence growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J. Food Prot. **53:**550-554.

9. **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.

10. **Heilmann, C.** 1996. Molecular basis of intracellular adhesion in the biofilm forming *Staphylococcus epiderrmidis*. Mol. Microbiol. **20:**1083-1091.

11. **Helke, D. M., and A. C. L. Wong.** 1994. Survival and growth characteristics of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and buna-N rubber. J. Food Prot. **57:**963- 968, 974.

12. **Jorgensen, 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.

13. **Jorgensen, 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 shock-induced protein in *Listeria monocytogenes*. J. Appl. Bacteriol. **80:**216-224.

14. **Knabel, S. J., H. W. Walker, P. A. Hartman, and A. F. Mendonca.** 1990. Effect of growth temperature and strictly anaerobic recovery on survival of *Listeria monocytogenes* during pasteurization. Appl. Environ. Microbiol. **56:**370-376.

15. **Lou, Y., and A. Yousef.** 1996. Resistance of *Listeria monocytogenes* to heat after adaptation to environment stresses. J. Food Prot. **59:**465-471.

16. **Mackey, B. M., and N.Bractchell.** 1989. The heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. **9:**89-94.

17. **McLean, R. J. C., M. Whiteley, D. Stickler, and W. C. Fuqua.** 1997. Evidence of autoinducer activity in nauturally occurring biofilms. FEMS Microbiol. Lett. **154:**259-263.

18. **Oosthuizen, M. C., B. Steyn, D. Lindsay, V. S. Brozel, and A. v. Holy.** 2001. Novel method for the proteomic investigation of dairy-associated *Bacillus cereus* biofilm. FEMS Microbiol. Lett. **194:**47-51.

19. **Pesci, E. C., J. B. J. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg, and B. H. Iglewski.** 1999. Quinolone signaling in the cell-cell communication system of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. **96:**11229-11234.

20. **Phan-thanh, L., and T. Gormon.** 1995. Analysis of heat and cold shock proteins in *Listeria* by 2-dimensional electrophoresis. Electrophoresis **16:**444-450.

21. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of Escherica coli biofilm formation:roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285-293.

22. **Sorqvist, S.** 1994. Heat resistance of different serovars of *Listeria monocytogenes*. J. Appl. Bacteriol. **76:**383-388.

23. **Stephens, P. J., and M. V. Jones.** 1993. Reduced ribosomal thermal denaturation in *Listeria monocytogenes* following osmotic and heat shocks. FEMS Microbiol. Lett. **106:**177-182.

24. **Tremoulet, F., O. Duche, A. Namane, B. Martinie, T. E. L. g. consortium, and J. C. Labadie.** 2002. Comparison of protein patterns of *Listeria monocytogenes* grown in biofilm or in plantonic mode by proteomic analysis. FEMS Microbiol. Ecol. **210:**25-31.

25. **Walsh, D., J. J. Sheridan, G. Duffy, I. S. Blair, D. A. McDowell, and D. Harrington.** 2001. Thermal resistance of wild-type and antibiotic resistant *Listeria monocytogenes* in meat and potato substrates. J. Appl. Microbiol. **90:**555-560.

Table 5.1. Proteins induced or repressed in heat stressed *L. monocytogenes* Scott A biofilm cells.

Spot identification number [a]	Approx. pI [b]	Approx. molecular mass (Kda)	The volume ratio of protein spots induced(+) or repressed (-) by heat [c]
637	4.5	45	-21.28
851	4.1	25	-1.19
994	5.0	20	-3.44
318	4.1	78	-10.02
682	4.9	40	-6.45
465	9.0	60	-43.12
822	5.2	28	-5.81
601	5.0	38	-8.22
661	5.2	40	-3.16
379	4.9	75	-8.72
474	9.1	60	-65.5
510	4.7	55	-4.76
466	9.1	60	-81.11
932	4.1	22	10.44
450	5.2	60	-8.35
958	5.1	20	2.18
644	5.1	32	3.81
1088	6.5	15	-5.28

[a] spot number indicated on the gel, Figure 3

[b]pI and molecular mass are determined by protein standards on 2D gel

[c] (+) protein spots significantly ($P< 0.05$) induced in *L. monocytogenes* after heat stress; (-)protein spots significantly ($P< 0.05$) repressed in *L. monocytogenes* after heat stress.

Table 5.2. Protein induced or repressed in heat stressed *L. monocytogenes* 3990 biofilm cells.

Spot identification number [a]	pI [b]	Molecular mass (Kda)	The volume ratio of protein spots induced(+) or repressed (-) by heat [c]
956	4.8	48	-17.2
528	4.6	76	-16.1
588	4.7	74	-9.3
1264	4.3	30	-3.2
1205	5.1	35	-17.2
1201	5.0	35	-3.6
1477	5.1	23	-2.8
1032	4.9	40	-4.1
1395	4.1	25	4.5
751	9.0	60	-36.7
863	4.6	50	2.3
841	4.6	50	-1.6
752	9.0	60	-43.0
638	4.5	70	1.5
1206	5.3	35	-17.1
754	9.0	60	-52.6
639	4.6	60	3.2
1321	4.2	28	4.5

[a] spot number indicated on the gel, Figure 4

[b]pI and molecular mass are determined by protein standards on 2D gel

[c] (+) protein spots significantly ($P< 0.05$) induced in *L. monocytogenes* after heat stress; (-)protein spots significantly ($P< 0.05$) repressed in *L. monocytogenes* after heat stress.

Figure 5.1. Biofilm formation on stainless steel surfaces showing percent biofilm coverage. Biofilm were grown in 1:10 diluted TSB incubated at 25 ºC. The following are the designated codes for the cultures. LMSA= *L. monocytogenes* Scott A, control; LM3990= *L. monocytogenes*, strain 3990, control; HLMSA= heat stressed *L. monocytogenes* Scott A; HLM3990= heat stressed *L. monocytogenes*, strain 3990. *L. monocytogenes* cells were stained with Bactrace ® fluorescein-labeled affinity antibody to Listeria and the biofilm polymers were stain with Concanavalin A conjugated with Texas Red ®. Letters that are different show significant differences (p= 0.05).

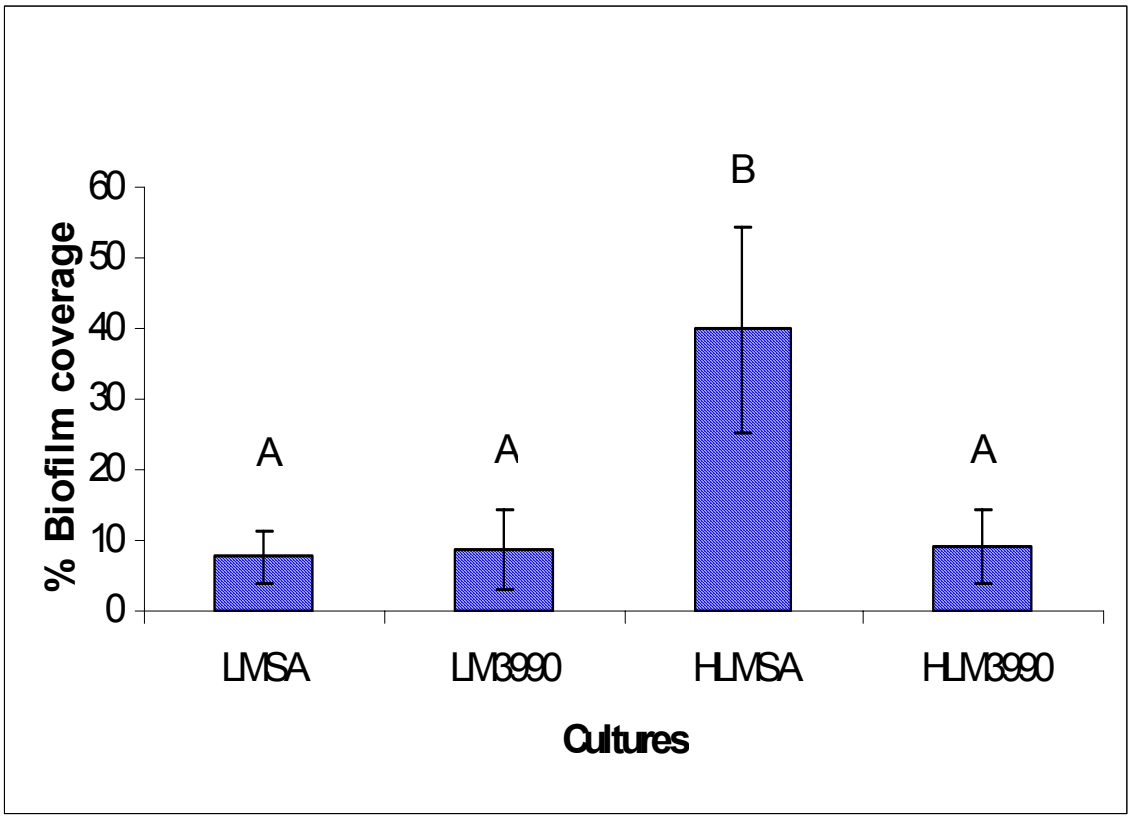

% Biofilm coverage
60
50
40
30
20
10
0
A
A
B
A
LMSA
LM3990
HLMSA
HLM3990
Cultures

Figure 5.2. Photomicrographs of *L. monocytogenes* biofilm on stainless steel surfaces. Cells were stained with Bactrace ® fluorescein-labeled affinity antibody to Listeria and the biofilm extracellular polymeric substances were stain with Concanavalin A conjugated with Texas Red ®. A. represents *L. monocytogenes* Scott A control (LMSA); B. represents heat treated *L. monocytogenes Scott A* (HLMSA); C.. represents *L. monocytogenes*, strain 3990, control (LM3990) and D. represents heat treated *L. monocytogenes*, strain 3990 (HLM3990).

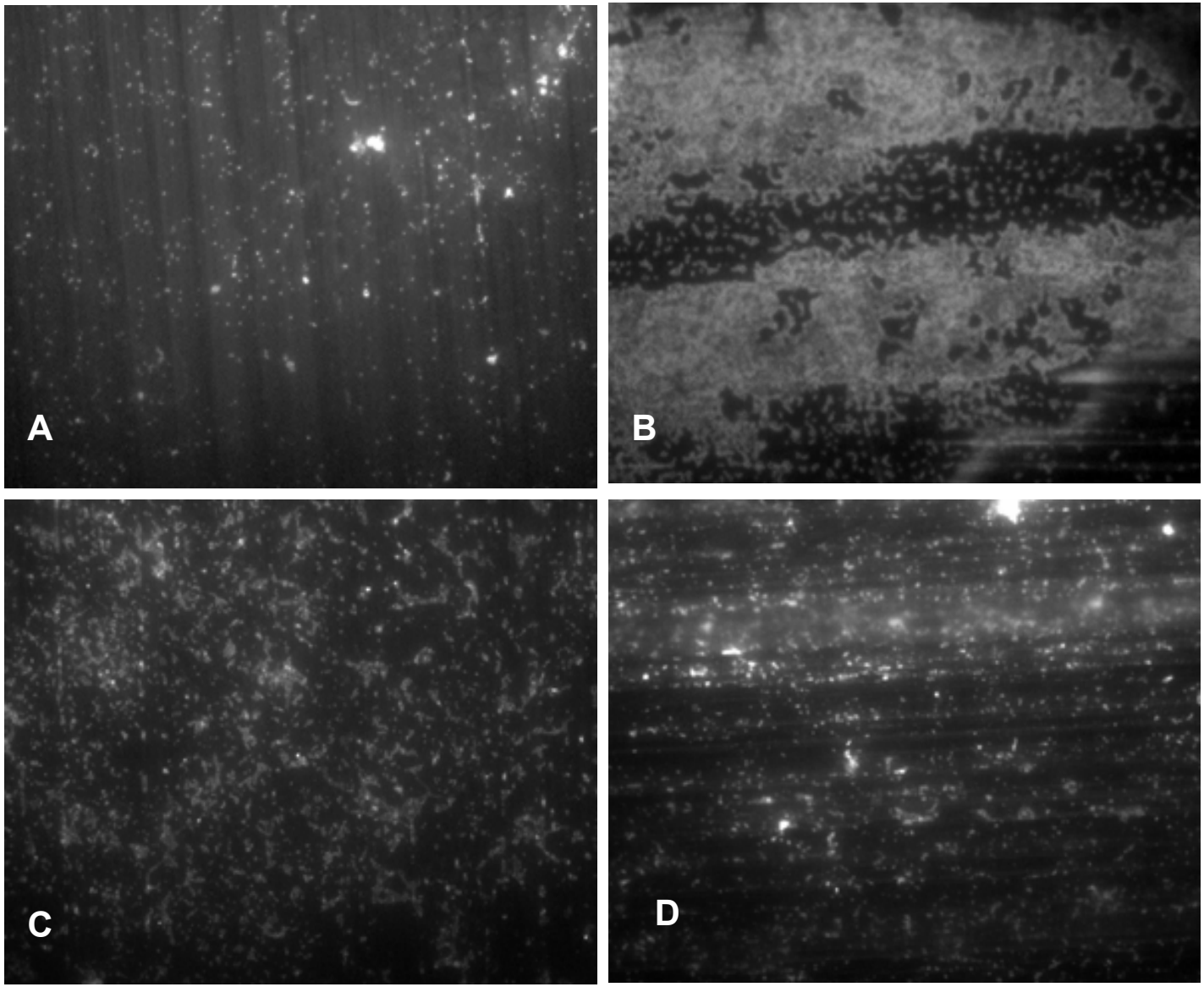

A
B
C
D

Figure 5.3. SDS-PAGE images showing expressed proteins of *L. monocytogenes* Scott A .

A. represents proteins expressed by *L. monocytogenes* Scott A control and stained with Cy ™3 DIGE Fluor (λ_{ex}= 553 nm, λ_{em}=572 nm). B. represents proteins expressed by heat stressed *L. monocytogenes* Scott A stained with Cy ™ 5 DIGE Fluor (λ_{ex}= 648 nm, λ_{em}=669 nm).

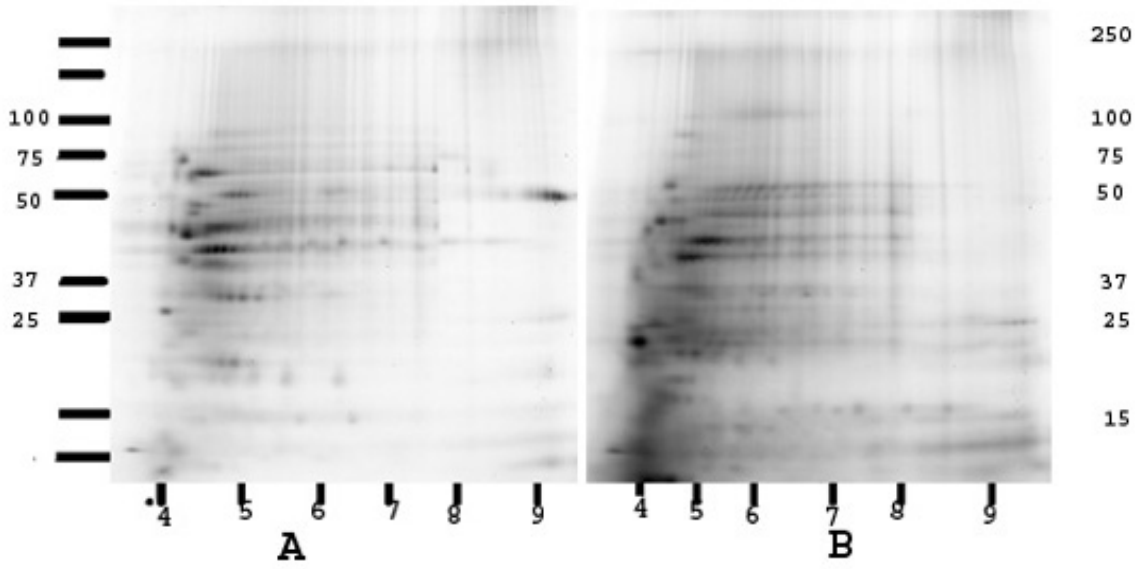
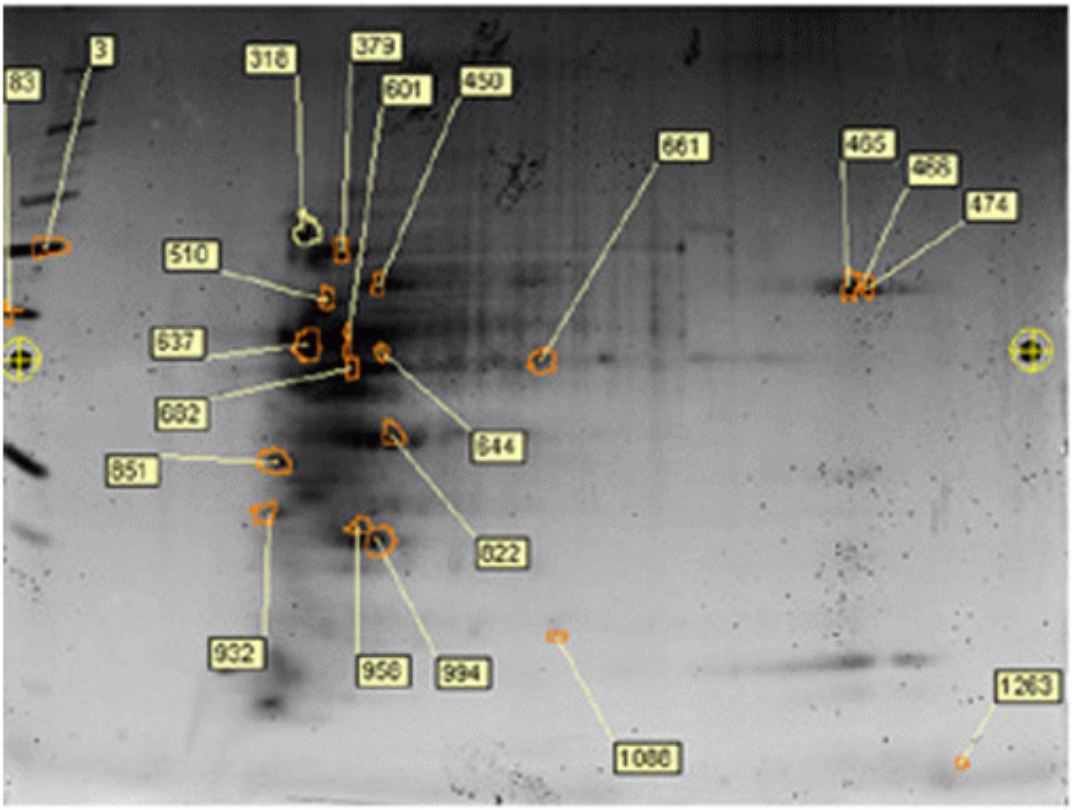

100
75
50
37
25
4
5
6
7
8
9
A
4
5
6
7
8
9
B
250
100
75
50
37
25
15

Figure 5.4. SDS-PAGE images showing expressed proteins of *L. monocytogenes* Scott A (control and heat stressed) stained with Sypro Ruby ™ dye. Labeled spots represent changes in protein expression due to heat stress.


3
83
318
379
601
450
661
485
488
474
510
537
682
844
851
822
932
956
994
1080
1263

C

Figure 5.5. SDS-PAGE images showing expressed proteins of *L. monocytogenes* 3990 . A. represents proteins expressed by *L. monocytogenes* 3990 control and stained with Cy ™3 DIGE Fluor (λ_{ex}= 553 nm, λ_{em}=572 nm). B. represents proteins expressed by heat stressed *L. monocytogenes* 3990 stained with Cy ™ 5 DIGE Fluor (λ_{ex}= 648 nm, λ_{em}=669 nm). C. represents *L. monocytogenes* 3990 (control and heat stressed) stained with Sypro Ruby ™ dye. Labeled spots represent changes in protein expression due to heat stress.

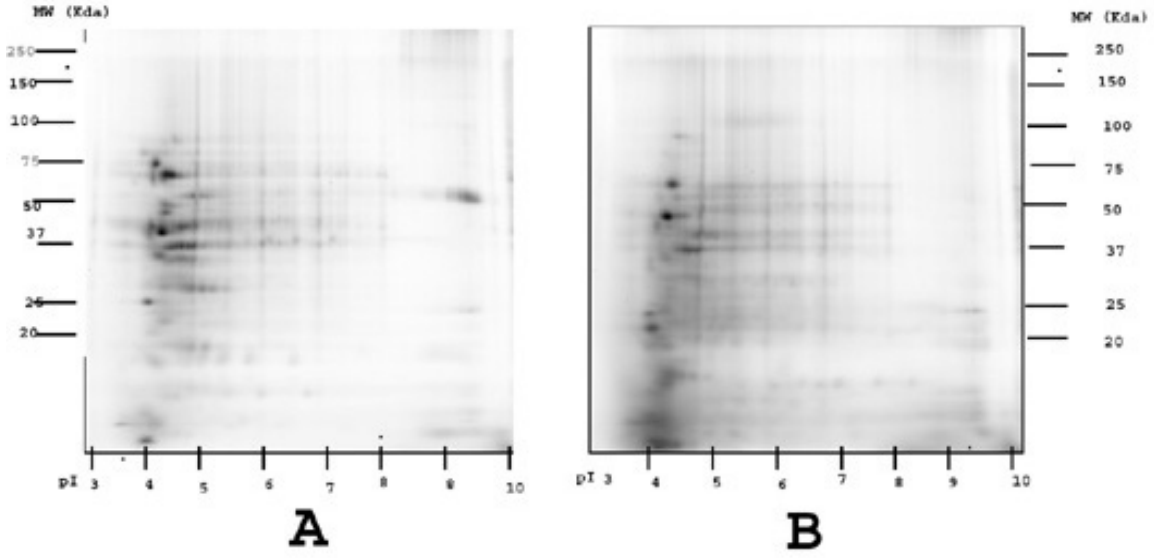
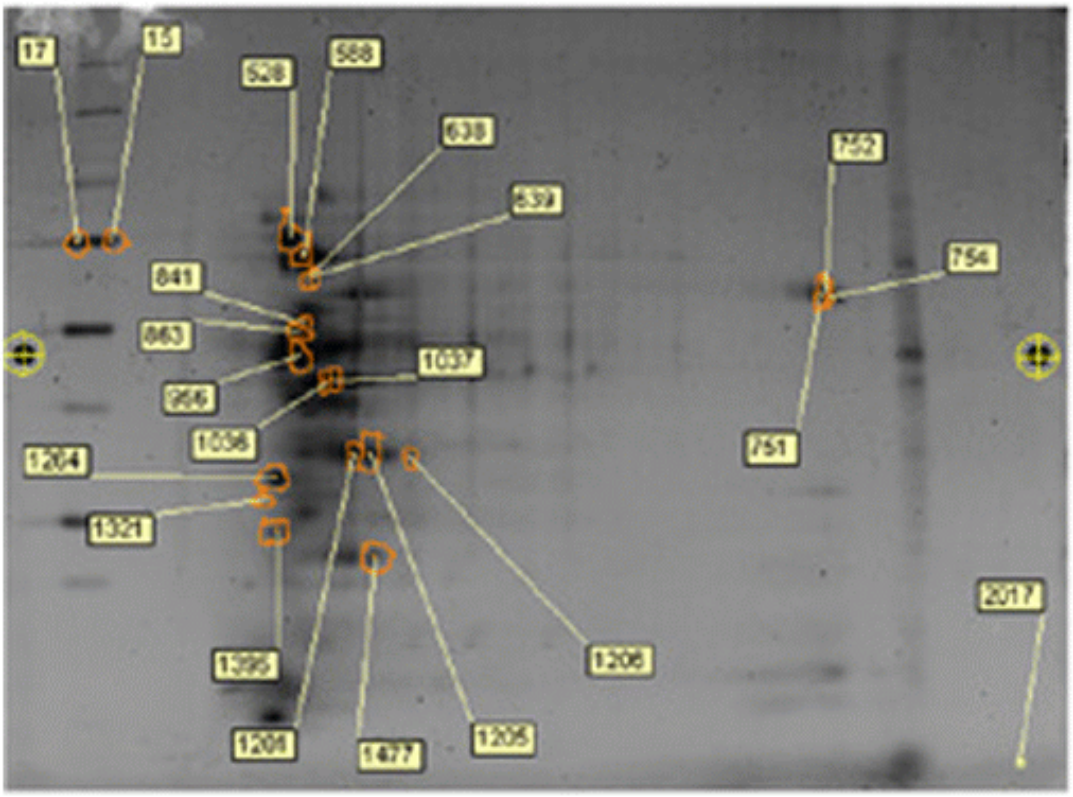

MW (Kda)
250
150
100
75
50
37
25
20
pI 3 4 5 6 7 8 9 10
A
MW (Kda)
250
150
100
75
50
37
25
20
pI 3 4 5 6 7 8 9 10
B

Figure 5.6. SDS-PAGE images showing expressed proteins of *L. monocytogenes* 3990 (control and heat stressed) stained with Sypro Ruby ™ dye. Labeled spots represent changes in protein expression due to heat stress.


17
15
528
588
638
752
539
754
841
863
1037
956
1036
751
1204
1321
2017
1206
1205
1208
1477

C

CHAPTER 6

SUMMARY

A predictive model for heat inactivation of *listeria monocytogenes* biofilm on stainless steel.

- A predictive model was developed that showed differences in heat resistance of *L. monocytogenes* biofilm cells in monoculture and within multispecies biofilm. The *L. monocytogenes* 3990 biofilm was more heat resistant in the presence of soil than *L. monocytogenes* Scott A and the multispecies biofilm containing *L. monocytogenes*, *Pseudomonas* spp. and *Pantoea agglomerans* on stainless steel surfaces.
- The validation study shows that at the 50% probability level of *L. monocytogenes* inactivation, the predictive model with 3990 strain was conservative in its estimate of *L. monocytogenes* biofilm inactivation. The five-strain cocktail survived better than the Scott A strain and exhibited similar survival as the mixed culture.
- The Scott A model was not a conservative predictor of *L. monocytogenes* inactivation in a biofilm after heat treatment, so, this model would have limited use.
- The mixed culture biofilm showed similar inactivation as the validation model with a slightly more conservative prediction at the higher levels of heat treatment. This mixed culture model adequately assessed the risk of *L. monocytogenes*

biofilm inactivation and can be used in situations of low risk of *L. monocytogenes* exposure to product.

Heat inactivation predictive model for *listeria monocytogenes* biofilm on buna-n rubber.

- The predictive model for heat inactivation of *L. monocytogenes* biofilms on rubber provides for two prediction situations in the presence of soil, the fairly conservative prediction using the heat resistant *L. monocytogenes* Scott A strain and the less conservative predictions based on *L. monocytogenes* 3990 and *Listeria* in a multispecies biofilm.
- For the low soil condition, heat inactivation of the Scott A and 3990 strains paralleled the validated model while the *L. monocytogenes* in a mixed culture biofilm was conservative in its predictions.
- These predictive models could be used as a guide to apply hot water sanitation when chemical sanitation is ineffective.

Protein expression of non-heat and heat stressed biofilm of *l. monocytogenes* and subsequent biofilm formation on stainless steel

- The protein expression of heat stressed *L. monocytogenes* (Scott A and 3990) biofilm grown on stainless steel indicated changes in the proteins expressed before and after heat stress.
- Biofilm formation on stainless steel indicated that heat stress induced an increase in the production of biofilm for *L. monocytogenes* Scott A.