Growth of *Listeria monocytogenes* in a Multispecies Biofilm Formed in a Humid Environment With Soluble Protein

By

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ABSTRACT

The primary objective of this research project is to determine the ability of L. monocytogenes to grow as part of a mixed species biofilm in the presence of condensation and protein soils. The secondary objective is to devise a laboratory scale model system to simulate biofilms on surfaces that are often subjected to food soils as well as condensation in food processing areas held at 12 ºC. *L. monocytogenes* maintained greater populations in biofilms exposed to protein soils (from 0.1 to 1.3 Log CFU/cm^2 over a 5 week time period), which simulates an inadequately cleaned surface. This supports our hypothesis that the presence of protein soil will affect the growth of *L. monocytogenes*. High rinse counts (10^5 to 10^6) indicate the ability of these biofilms to act as a reservoir for *L. monocytogenes*, which can then contaminate product as well as food contact surfaces through dripping condensation.

INDEX WORDS: Biofilm, *Listeria monocytogenes*, Humidity, Condensation

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CHAPTER 1

INTRODUCTION

It is nearly impossible to find an environment free from microorganisms. This is especially the case in wet environments, as wet surfaces are well suited to support microbial growth. This growth may occur rapidly as microbes attach to the surfaces and form complex biofilms, which are difficult to control and disinfect. Biofilms are of concern to several industries with operational characteristics that enhance biofilm growth. For example, surfaces in marine drilling operations, such as ship hulls and drilling equipment, are in constant contact with water and microbial life. As a result, researchers in these areas have spent millions of dollars trying to prevent the growth of biofilms, which if left unchecked can lead to corrosion, decrease in pumping efficiency, and contamination [1]. The medical industry has also spent countless research hours developing devices that are resistant to biofilm growth. Indwelling medical devices, such as catheters and heart valves can serve as attachment surfaces for microbes, leading to biofilm formation and subsequent illness. These patients are usually immune compromised and as a result, prevention of biofilm formation is important.

The food industry is also affected by biofilm formation. It is nearly impossible to prevent the introduction of microorganisms in a food processing environment, as most foods have an inherent microbial load. Surfaces in food processing areas provide several advantages for microbial growth. Water and lower temperatures are commonly used in these areas and as a result, surfaces are often continuously or at least frequently exposed

to water in the form of condensation. As surfaces in processing areas come in contact with the food being processed, they collect food soils that can serve to inoculate the surface with microorganisms and provide nutrients to microbial populations already present on the surface. Once these surfaces are inoculated, growth occurs and biofilms rapidly form.

Biofilms in processing environments are hard to completely remove and cause problems for the processors if they are not controlled. Biofilms are resistant to sanitizers commonly applied in process environments [2]. As a result of incomplete removal, portions of the biofilm may remain attached to the surface providing a selective environment for colonization by other microorganisms or serving to contaminate the product as food comes in contact with the contaminated surfaces.

Several microbial pathogens have been implicated in food borne illness, and several of these pathogens can survive as part of the biofilm matrix. For example, *Listeria monocytogenes* survives well as part of a biofilm community and is also responsible for an estimated 2500 serious illnesses and 500 deaths each year [3]. Thus, it is not enough to only eliminate the pathogen from the incoming product. Biofilm formation must also be controlled and prevented if possible.

The ability of *L. monocytogenes* to persist in the biofilm matrix, as well as its ability to cause serious food borne illness should prompt researchers to take an active role in developing models that can adequately determine the nature of the biofilm as well as developing methods of removal and inactivation of the pathogen. Current biofilm models involve laboratory defined media for growth in systems where the surfaces used for biofilm growth are continuously submerged in this liquid. This system has worked well

for studies simulating food contact surfaces, but does not present an accurate picture of biofilms for some surfaces. Many surfaces in food process operations are not in constant contact with an aqueous medium, such as ceilings, and overhead pipes. Water present on processing surfaces is often a result of sporadic condensation that forms as a result of the lowered temperatures characteristic of these operations. The ability of biofilms to adapt to differences in water availability allows them to persist well in these environments.

Nutrient conditions on processing surfaces in food operations also tend to fluctuate rapidly. After cleaning and sanitation, surfaces in a processing plant typically provide low nutrient microenvironments while at the height of processing; these same surfaces can provide extremely high nutrient microenvironments [4]. Thus, some research efforts should be directed toward a different view of biofilm growth as part of an unsaturated system that includes the presence of food soils and condensation.

CHAPTER 2

OBJECTIVES

The overall objective of this research project is to determine the ability of *L. monocytogenes* to grow as part of a mixed species biofilm in the presence of protein soil. An additional objective is to devise a laboratory scale model system to simulate biofilms on surfaces that are often subjected to food soils as well as condensation in food processing areas.

We hypothesize that the presence of protein soil will enhance growth of a cocktail of *L. monocytogenes* strains in a multispecies biofilm with *P. putida* on a stainless steel surface.

CHAPTER 3

LITERATURE REVIEW

Microorganisms form biofilms rapidly on wet surfaces. These microbial communities vary greatly depending on the characteristics of the liquid medium, the surface, and the microorganisms themselves. Several industries have exploited this ability to enhance production of desired microbial end products and to destroy toxins in water system.

3.1 Positive Aspects of Biofilm Growth

Saccharomyces cerevesiae attaches readily to stainless steel wire mesh and polyester sponges. These immobilized cells produce methanol, which can be collected and purified, providing 80 to 98% of the theoretical yield [5]. Cellulase can be collected and purified from *Trichoderma viride*, a fungus, attached to stainless steel meshes. These biofilms produce almost 6 times the amount of the enzyme as a free suspension of the same organism [6]. It is often difficult to obtain production and recovery of products of recombinant genes consistently. Recombinant *E. coli* overproducing β-lactamase can be grown as a biofilm on fiber membranes with almost 10 times the β-lactamase efficiency of a similar suspension culture [7]. Biofilm growth also increases plasmid copy numbers as well as enhances the stability of plasmid products over several hundred generation times [8].

One widespread use of biofilms involves bioremediation and treatment of wastewater. Biofilms in fixed film reactors have been used for polychlorinated

hydrocarbon destruction [9], toluene degradation [10], nitrification and denitrification [11-14], removal of heavy metals [15, 16], and for treatment of phenolic wastewaters [17].

3.2 Negative Aspects of Biofilm Formation

Other industries have spent millions of dollars and countless hours trying to determine the best methods of control and removal of biofilm communities. Marine industries, such as shipping and oil drilling companies, try to prevent formation of biofilms on ship hulls and oil drilling equipment where they cause biocorrosion as well as contamination of oil and decreased pumping efficiency [1]. The medical industry also tries to prevent biofilm formation. Patients experiencing extended stays in the hospital typically have compromised immune systems. As a result, it is especially important to inhibit microbial niches that can serve to complicate medical conditions. *Pseudomonas aeruginosa* biofilms cause cystic fibrosis, while biofilms of other organisms are responsible for infections leading to septicemia in patients with indwelling medical devices such as catheters [18].

Biofilms are of concern in the food processing industry where they can lead to decreases in heat transfer rates, clogging of product lines, corrosion of equipment, and contamination of product and equipment. They also make cleaning and sanitizing of food contact surfaces more difficult. When biofilms are incompletely removed, there is potential for further growth and colonization of equipment and contamination of food.

This review will discuss the steps involved in biofilm formation and the advantages biofilms afford microorganisms, as well as current research involving *Listeria monocytogenes* biofilms and their relevance to the food industry

3.3 Biofilm Formation

A simple definition of a biofilm is a community of microorganisms attached to a surface, but they can be very complex and dynamic systems [19]. The first step in the establishment of a biofilm community involves the formation of a molecular conditioning layer.

3.3.1 Formation of the Conditioning Film

A surface exposed to an aqueous medium immediately collects proteins and other organic molecules that attach to the surface typically through molecular diffusion [18]. This conditioning film is defined by the surface characteristics as well as the composition of the aqueous medium, and can change the characteristics of the surface as well as influence attachment of microorganisms [20, 21]. Hydrophilic uncharged surfaces are most resistant to adsorption of protein and attachment of bacteria [22]. However, some microorganisms can adhere to both hydrophobic and hydrophilic surfaces by producing different extracellular polymeric substances [23]. Regardless of the surface used solutes in the aqueous medium can change the characteristics of the surface to discourage or promote attachment. For example, skim milk proteins can act on both stainless steel and glass surfaces to discourage attachment of several species of microorganisms regardless of surface roughness [24], and immunoglobulin solutions applied to carbon steel and stainless steel surfaces prevent the attachment of *Pseudomonas sp*. [25]; while surface pretreatment with casein and gelatin enhances bacterial adsorption [26] and treatment of needleless connectors with whole blood proteins enhances bacterial attachment [27]. Also, negatively and positively charged surfaces both collect net negative charges, which could influence microbial attachment [18]. In addition, a change in orientation of

macromolecules, which can be large and bear multiple attachment sites, to the subsurface provides a range of hydrophobicities and charges throughout the surface [28]. This information proves useful to those in the food processing industry where control and prevention of biofilm formation are critical. Thus, formation of the conditioning layer is a dynamic and complicated process that can obscure surface properties as well as influence microbial attachment.

3.3.2 Microbial Attachment

The conditioning layer is established rapidly after a clean surface is immersed in a solution, and microorganisms become attached to these molecules. Bacteria arrive at the conditioned surface through chemotaxis in response to nutrient gradients, diffusion, convection, and sedimentation, as well as incidental interactions with the surface as they are carried through the bulk fluid [29, 30]. Thus, the microbial characteristics of the bulk fluid play a role in determining the microbial characteristics of the biofilm. Bacteria present in high numbers may out-compete microbes present at lower concentrations for surface area. On the other hand, microorganisms in low concentrations, or those subject to high flow rates or turbulent conditions, may use surface colonization as a means to increase their numbers as the substrate provides a stationary position to reproduce and send daughter cells into the bulk phase. Attachment occurs rapidly and continuously in response to environmental conditions that support the transition to the attached state [19, 31]. Adsorption and attachment of microorganisms is primarily due to the interaction of chemical groups in the conditioning film with bacterial appendages as well as bacterial surface proteins [32-35]. This attachment is initially reversible as microbes can be rinsed off, but then becomes more stable and eventually is characterized as an irreversible

attachment as bacterial polymers are produced to support the biofilm matrix [18, 19, 26, 36]. A complex interaction between microorganisms and the adsorbed conditioning layer sets the foundation for a complex biofilm matrix.

3.3.3 Growth and Establishment of the Biofilm Matrix

Once microorganisms attach to a surface, they start to proliferate and produce microcolonies in which attached cells and daughter cells are surrounded by biopolymers that help stabilize the biofilm matrix and microenvironment. These biopolymers generally consist of extracellular polymeric substances (EPS) created primarily of polysaccharides containing carboxyl groups excreted in response to attachment [37]. EPS makes up between 50 and 90% of the biomass in a biofilm and serves to stabilize the matrix by allowing the attached microorganisms to maintain their sessile environment over time [38]. Production of EPS can be stimulated, as is the case when *Pseudomonas putida* is exposed to toluene or when some *Pseudomonas spp*. are exposed to low water activity [37, 39]. Biofilm formation involves numerous interactions, which lends support to the idea that biofilm structure is far from completely understood.

3.3.4 Biofilm Structure

Biofilm microstructure depends on a variety of conditions present in the system. Fluid flow and hydration are important to biofilm structure. In general, biofilms that form in fully hydrated systems, where they are submerged within the fluid, have a mushroom-like appearance with cells growing from the surface into the fluid phase. Channels exist around these EPS encapsulated cell stalks that allow convective transport of nutrients as well as metabolic end products through the matrix [4, 37]. Biofilms that are not continuously exposed to the fluid phase, perhaps many in food processing environments,

tend to grow as groups of irregularly spaced microcolonies [4]. As the biofilm matures the thickness varies and, in concert with microbial metabolism, allows the formation of gradients of oxygen and carbon dioxide creating anaerobic or microaerophillic areas [18, 26, 39]. This can create a niche for other organisms that may not be able to exist in the medium under normal oxygen tensions [18]. The flow of the bulk fluid affects biofilm structure, as biofilms grown under turbulent flow conditions tend to be filamentous while biofilms grown under laminar flow conditions tend to grow as patches of cell clusters separated by empty spaces. This may be a result of shear stress on the biofilm [40]. EPS provides other advantages to the biofilm, which will be discussed later.

3.3.5 Detachment and Desorption

In a mature biofilm, it is a common occurrence for cells and portions of the biofilm to detach or become desorbed and reenter the bulk fluid. Desorption is the opposite of attachment, and results in a loss of cells from the substratum. Detachment occurs when there is a loss of biofilm matrix. The loss of organisms and biofilm material may be due to the build up of toxic end products, which makes the biofilm hazardous to the bacteria and, initiated through quorum sensing, they detach and find new areas to colonize. There are three primary mechanisms for detachment; sloughing, erosion, and release of daughter cells [26]. Sloughing generally occurs in thicker biofilms in high nutrient conditions and involves a loss of a large portion of the biofilm. In this case, cells surrounded by portions of the biofilm matrix are released from the film into the fluid phase. Erosion is the continual loss of small portions of the biofilm over time usually due to shear stress [26]. This shear stress can cause portions of the biofilm to be left behind if the microorganisms initially colonizing the surface are strong enough to resist removal

[41]. The remaining attached biofilm can aid recolonization efforts of new organisms, while the eroded portions can serve to create new biofilms elsewhere or can clog spray nozzles or other equipment. As the biofilm matures, daughter cells are produced, and they can either remain with the biofilm, or be released contaminating the bulk fluid and possibly forming biofilms in other locations. Detachment and desorption are all characteristic of a mature biofilm and at some point cell death is inevitable.

3.3.6 Cell Death

Cell death can occur as a result of from starvation, build-up of toxic metabolites, or introduction of a toxin (such as a biocide) into the system [26]. As the biofilm thickens upon maturation, diffusion of nutrients from the bulk fluid into the inner portions of the biofilm slows. As a result, cellular metabolism is reduced and cells become smaller. This may eventually lead to death as substrates at the surface are used up with little influx of nutrients. The bacteria can become stuck in the nutrient depleted matrix and essentially starve. This leads to cell lysis within the biofilm. Cell lysis is an advantage for other cells as it releases nutrients, which are used by surrounding microorganisms. However, death of inner cells can loosen the foundation of the biofilm causing detachment into the bulk fluid. If one species dies off, this can completely change the dynamics of the biofilm as other organisms may be depending on the metabolism established by the now dead species. Even with this starvation process, biofilm growth offers many advantages to the bacteria over planktonic existence.

3.4 Advantages of Life In a Biofilm v. Planktonic Existence

Attached cells are phenotypically different from their planktonic counterparts. Genes involving EPS production are often up-regulated as a result of attachment [42], and Rice

et. al found that *Pseudomonas aeruginosa* has a lag time in growth phase during the transition between planktonic and sessile growth, which is consistent with phenotypic change occurring upon attachment [43]. Research using several different species of microorganisms shows major differences in gene regulation as a result of attachment to a surface. For example, *Pseudomonas putida* increases production of 15 proteins and decreases 30 proteins [44]. Expression of 38% of *E.coli* genes were found to differ as a result of attachment, and *Listeria monocytogenes* was shown to up-regulate 22 proteins while down regulating 9 of the 550 proteins surveyed [45, 46]. Most of these proteins are involved in stages of cellular metabolism indicating a complex pattern of gene regulation in response to attachment to surfaces. Regulation of these metabolic genes may be in response to differences in nutrient conditions at the surface. Existence as part of a biofilm offers many advantages over planktonic growth. Probably the most important advantage of attached growth as part of a biofilm involves nutrient availability.

3.4.1 Nutritional Advantages

Microorganisms in the planktonic phase are often nutrient limited as they have difficulty seeking nutrition, especially in nutrient limited environments characteristic of clean food processing areas. The conditioning layer on a surface concentrates nutrients especially in low nutrient environments [47]. The EPS also traps nutrients from the bulk fluid allowing them to diffuse into the biofilm or preventing them from diffusing away into the bulk fluid. This may provide the microbes with an improved supply of nutrients depending on the characteristics of the liquid phase [48]. As mentioned previously, a slower growth rate and cell lysis occurs within the biofilm and these can also be advantageous as slower growing cells can survive better in lower nutrient conditions, and

the released cellular components of lysed cells are typically held within the matrix where they have nutritional value for surviving microbes.

Most biofilms are composed of many species. One microorganism can break down complex molecules that another microorganism cannot. In this way, the complex organization of different species within the biofilm can provide building blocks for growth. Some organisms within the biofilm can degrade EPS in order to supply metabolic building blocks [37]. Once a nutritional niche is established based on the characteristics of the biofilm, other microorganisms are excluded by means of competition for nutrients or colonization space, or other microorganisms can be included as a niche may exist for them where there was not one previously. These nutritional interrelationships, as well as the characteristics of the bulk fluid and EPS produced, define the microbial characteristics of a biofilm.

3.4.2 EPS: The Protective Barrier

The presence and dynamic character of EPS provides other advantages to the microbes. EPS allows the microorganisms to establish a favorable microenvironment and maintain this growth niche over a period of time [37]. The EPS structure has also been shown to hold water and therefore can protect microbes within the matrix from desiccation and rapidly changing environmental conditions [4, 38, 39, 49, 50]. In fact, the structures of *Pseudomonas putida* biofilms are very stable with changes in available water, which commonly occurs in areas where biofilms are not continuously exposed to a fluid phase [4].

Not all microorganisms produce EPS, and those that do not can use the EPS structure provided by other microbes. For example, *L. monocytogenes* does not produce an

extensive EPS network after attachment, but takes advantage of other microorganisms that do such as *Pseudomonas spp*. [51, 52]. In fact, 10 environmental isolates of *Pseudomonas spp*. stimulated the growth of *L. monocytogenes* possibly due to the protective effects of the EPS [53]. Some strains of *Pseudomonas putida* degrade triclosan and other phenolic compounds, which may confer protection from microbial inactivation to other organisms within the matrix [54]. EPS surrounding cells can also be removed during sloughing, and may provide protection for these planktonic cells as a result of growth within the biofilm matrix. These results show the ability of the EPS structure to act as a barrier against undesirable conditions.

3.4.3 Biofilms and Antimicrobial Resistance

The resistance of biofilms to biocides and cleaning has been well documented, and this resistance can be due to the failure of the sanitizer or disinfectant to penetrate the biofilm, the microbial stress response, the heterogeneity of the biofilm, as well as quorum sensing within the matrix [55].

Biofilms of *Listeria monocytogenes* are resistant to nisin and ciprofloxin at concentrations that inactivate planktonic cells. This is a result of incomplete penetration of the biofilm [56, 57]. This phenomenon has relevance to the food industry where the presence of *L. monocytogenes* has been linked to many serious outbreaks of food borne illness. *Pseudomonas putida* biofilms are resistant, as a result of incomplete penetration, to nonfoaming acidic and liquid hypochlorite sanitizers commonly used for disinfection in the food industry [2]. Others have shown that small molecules, such as some antibiotics, rapidly diffuse through the EPS matrix, which implies that some other

inherent resistance mechanism must be responsible for persistence of the microorganisms [58].

When microorganisms are stressed, especially via nutrient limitation, they exhibit lowered metabolic rates. These cells exhibit slow growth and as a result, may decrease in size. This results in a decrease in uptake of nutrients and can also result in a decrease in the uptake of antimicrobial agents leading to a dose that is ineffective in inactivating microorganisms.

Biofilms are heterogeneous systems in which the activity of one organism can confer protection to others. Some microbes actively degrade certain organic compounds and can protect other microbes within the environment [54]. It has been postulated that molecules entering into the polysaccharide matrix are inactivated by interactions with the matrix itself. For example, *Pseudomonas aeruginosa* biofilms are resistant to tobramycin as a result of EPS binding that restricts penetration. This can protect inner organisms regardless of species. Resistance by this mechanism can lead to microbial exposure to biocides in small enough doses to select for a resistant population [59].

Microorganisms produce compounds that, when present in high enough concentrations, can act to signal other microorganisms. This signaling mechanism is called quorum sensing. Since microbes within the matrix are in close proximity to one another, and because diffusion is slower through a biofilm matrix than through aqueous media, these molecules are required in lower concentrations. Thus, the killing off of upper layers of the biofilm can act as a signal to inner cells, through quorum sensing, that can lead to detachment and thus survival of a portion of the biofilm. Thus one can see the

importance of understanding biofilm growth and control especially in the food industry where cleaning procedures may be inadequate to destroy or inactivate microorganisms.

3.5 Microbiology and Pathogenesis of *L. monocytogenes*

L. monocytogenes is a rod-shaped, gram-positive organism ubiquitous in the environment. It is able to persist throughout a wide pH range and can survive temperatures from 4 to 37 ºC. Under optimum pH and temperature conditions, *L. monocytogenes* can rapidly multiply [60, 61].

In general, healthy adult populations infected with *L. monocytogenes* are asymptomatic, or show slight flu-like symptoms [62]. There are several predisposing factors for infection and subsequent illness upon ingestion of *L. monocytogenes*. People with compromised immune systems, such as those with serious illnesses such as cancer or AIDS, those taking drugs for immune suppression, young children, pregnant women, and people over the age of 60 [63]. After *Listeria monocytogenes* infection, susceptible individuals typically develop central nervous system infections such as miningoencephalitis, meningitis, or septic infections [64]. In pregnant individuals, *L. monocytogenes* can cross the placenta causing death of the fetus [62]. This organism's prime route of host infection appears to be the crossing of the intestinal mucosa through induced endocytosis into endothelial cells. Once through the host barriers, *Listeria* produces listeriolysin O and phospholipases, which allow the microbe to enter host cytoplasm and begin replication [61]. It is a facultative anaerobe, facultative psychrotroph, and its ability to grow slowly at refrigeration temperatures, and under low oxygen conditions make *L. monocytogenes* a concern in the food industry where it is primarily seen as a result of post process contamination [65]. *L. monocytogenes* can

survive and grow rapidly in biofilms consisting of other organisms present in food process environments, such as *Pseudomonas spp* [51-53].

3.5.1 *L. monocytogenes* and Food Borne Illness

L. monocytogenes has been implicated in numerous food borne outbreaks involving many different food products. In 1979, 23 hospitalized patients who consumed lettuce, carrots, and radishes were diagnosed with listeriosis [66]. In 1981, commercially prepared coleslaw was implicated in a Canadian outbreak [67]. Pasteurized milk was implicated in a 1983 outbreak of listeriosis in Massachusetts [68]. Mexican style cheese led to an outbreak in Southern California in 1985 [69], and consumption of chocolate milk in Illinois led to an outbreak of listeriosis [70]. Hot dogs were linked to 101 cases of listeriosis in 22 states from late 1998 to 1999 [71]. Since these outbreaks, regulations on the presence of *L. monocytogenes* in foods have been strengthened, and the number of outbreaks has declined. However, according to the Centers for Disease Control in Atlanta, *L. monocytogenes* infections still cause an estimated 2500 serious illnesses and 500 deaths each year [3]. In addition, *L. monocytogenes* outbreaks have been associated with soft cheeses, ice cream, salami, undercooked chicken, cream, blueberries and strawberries, nectarines, lettuce, and alfalfa [64]. This is only an incomplete list. Persistence of *L. monocytogenes* in food processing environments, especially as part of a biofilm community, makes it of concern to food processors [63].

3.5.2 *L. monocytogenes* In a Mixed Species Biofilm

Most natural biofilms are composed of multiple species, and *L. monocytogenes* will grow readily in these consortia [52]. Biofilm formation may be enhanced under nutrient limiting conditions as cells typically have empty receptors on their surfaces that aid

attachment [51]. Food processing plants are good examples of environments with fluctuating nutrient levels. During processing, food soils can build up on surfaces providing a high nutrient level for surrounding microbes. After cleaning and sanitation procedures, which typically occur daily and focus on food contact surfaces, the processing environment provides relatively low nutrient conditions. Studies with *L. monocytogenes* show increased attachment in minimal laboratory media [72]. As a result, it can be inferred that *L. monocytogenes* may show enhanced attachment in some food process environments. Several studies indicate that *L. monocytogenes* survives better when *Pseudomonads* are present as primary colonizers [51-53, 73, 74]. Food processing plants with their lack of adequate cleaning, residual water, and sometimes lower temperatures often provide optimum conditions for the attachment and growth of *Pseudomonas* [74]. It follows that *L. monocytogenes* may become integrated into biofilms with *Pseudomonas spp.* as the primary colonizer in food processing areas. Three days of growth of *L. monocytogenes* as a biofilm under processing condition can provide a product inoculum level high enough to be of concern from a pathogenic standpoint [75]. This can lead to a high contamination level in raw product that comes in contact with contaminated surfaces, as well as finished product when surfaces are inadequately cleaned and sanitized.

3.5.3 Current Research on *L. monocytogenes* Biofilms

Much current research has as its objective to determine the ability of *L. monocytogenes* as a biofilm itself or as part of a multispecies biofilm (often in concert with *Pseudomonas*) to resist heat, desiccation, and sanitizers. Heat treatment studies indicate that *L. monocytogenes* is resistant as part of a biofilm [76]. *L. monocytogenes* is

resistant to many different antimicrobial agents including nisin and ciprofloxin, as well as monolaurin and acetic acid [56, 57, 77]. *Pseudomonas* biofilms are resistant to tobramycin, triclosan, phenolic compounds, chlorine solutions, piperacillin, imipenem, ofloxacin, as well as nonfoaming acidic sanitizers and liquid hypochlorite sanitizers [2, 54, 58, 59, 78]. Surface attachment studies have demonstrated that not all strains of *L. monocytogenes* will attach in the same way to different surfaces, but that *L. monocytogenes* readily integrates itself with other primary colonizers to live within the matrix produced by the other organism. Since one species of microorganism can provide protection to other microbes in the biofilm, it is entirely possible that this wide range of *Pseudomonas* resistance could benefit *L. monocytogenes* grown in concert with *Pseudomonas*.

3.6 Research Needs

Current research is somewhat lacking for several reasons. First, most research involving *L. monocytogenes* biofilms uses strains that have been cultivated in the laboratory for many generations. It is possible that these strains adapt to exist best under laboratory conditions, and so the response of laboratory strains to experimental conditions may not give an accurate representation of the microbial dynamics inherent in environmental populations. Therefore, these research results may not adequately represent microbial dynamics in processing environments. As previously stated, different strains of *L. monocytogenes* have shown differential attachment abilities [79]. Inoculations with a cocktail of several strains should provide a better model of the species by taking variances due to strain differences into account.

Most laboratory research involves the use of a single microbial species (i.e. *L. monocytogenes*) to model biofilms in the environment. As previously stated, environmental biofilms are typically composed of multiple species and strains of organisms present in the environment. Since *L. monocytogenes* exists in a multispecies biofilm, future studies should model multispecies biofilm communities.

Biofilm research typically involves complete submersion in laboratory media for the entire growth process, and this media may not represent food processing environments. Biofilms continuously exposed to an aqueous phase are not representative of some food processing environments [4]. Some surfaces are exposed to water, frequently in the form of condensation as a result of low processing temperatures. Condensation on these surfaces can harbor microorganisms that may be of pathogenic significance. For example, *L. monocytogenes* has been isolated from condensate in ready-to-eat facilities as well as in dairy plants [80, 81]. These surfaces are usually not food contact surfaces and are often overlooked during cleaning and sanitation procedures.

As a result of inadequate cleaning, they may develop layers of dirt and food soils. Laboratory models of biofilm growth often exclude the presence of characteristic food soils. Nutrients are a defining aspect of biofilm formation, so food soils should be included in biofilm models that have relevance to the food industry. This is especially important for inactivation studies as sanitizers and cleaning treatments may interact with food residues giving an inadequate picture of inactivation.

3.7 Conclusion

The ability of microorganisms to form biofilms and survive despite removal and inactivation efforts, underscores the importance of research in this area. Biofilms are of

importance in the food industry where they can cause loss of time and money by contamination of product. The presence of *L. monocytogenes* in food processing environments, its ability to become integrated in the biofilm matrix, and its ability to cause serious illness and death, make it a good subject for research. Thus, the current literature shows a need for research into multispecies biofilms, including *L. monocytogenes*, in the presence of food soils typical of a food processing environment.

CHAPTER 4

EXPERIMENTAL METHODS

4.1 Humidity Chambers

High humidity chambers were prepared for biofilm experiments by autoclaving Nalgene 250 mm desiccators at 121° C for 15-20 minutes. Chambers were then filled with approximately 500 mLs of sterile water and a sealant was applied at the desiccator seams to prevent water loss by evaporation. They were then placed in an incubator at 12° C for 24 hours to allow temperature and humidity equilibration within the chamber.

4.2 Stainless Steel

New stainless steel coupons (type 304 #4B finish) measuring 2 x 5 cm were rinsed in acetone to remove adhesives. All steel coupons were sonicated in an alkaline solution (2% Microsoap – International Properties Corporation, Burlington, NJ) for 1 hour at 80° C. They were rinsed in deionized water and sonicated in a solution of 15% phosphoric acid for 20 minutes at 80° C. The coupons were then rinsed in deionized water and autoclaved for 15 minutes at 121° C.

4.3 Cultures

Environmental isolates of *P. putida*, as well as five strains of *L. monocytogenes* were obtained from the culture collection at the Department of Food Science and Technology at the University of Georgia. These cultures were stored at –80° C on cryoprotective beads. They were activated in TSB at 25° C and subcultured once. Since the experimental temperature was to be 12° C, cultures were then transferred to 12° C

and subcultured once before inoculation to allow acclimation. A flow diagram of this procedure is presented in Figure 1.

4.4 Biofilm Preparation

P. putida was inoculated into 1 L of 3.0 g/L Tryptic Soy Broth, (0.1 of normal strength referred to as 10% TSB) to provide an initial inoculum of 1%. The inoculated broth was then dispensed into sterile test tubes each holding 1 10 cm^2 stainless steel coupon. Inoculated coupons were incubated at 12° C for a 4 hour attachment period. The coupons were then rinsed in sterile phosphate buffer to remove unattached and loosely attached cells and transferred to fresh 10% TSB. Incubation at 12 Celsius continued for 48 hours. Growth of *P. putida* biofilms was confirmed through microscopic examination of 2 stained samples per replication. A flow diagram of this procedure is presented in Figure 2.

After the 48 hour incubation of the *Pseudomonas* biofilms, the coupons were removed, rinsed in sterile phosphate buffer, and placed in sterile test tubes. An inoculation cocktail was prepared by placing 1% of each of 5 environmental isolates of *L. monocytogenes* into 10% TSB providing an initial inoculum of 5%. This cocktail broth was then dispensed in 25 mL aliquots into the test tubes containing the previously inoculated steel coupons. The cocktail broth was plated on Listeria Selective Agar in duplicate to determine an average inoculation level of 7.59 Log CFU/mL after 5 replications. The coupons were then incubated at 12° C for 4 hours. After the attachment phase, the coupons were removed, rinsed in sterile phosphate buffer, and placed in sterile test tubes containing fresh 10% TSB. Incubation continued for 48 hours at 12° C. Growth of *L. monocytogenes* within the biofilm was confirmed through staining with a

fluorescein-labeled antibody specific to *Listeria*. This procedure has been used to consistently develop multispecies biofilm communities by several researchers in our laboratory, and a flow diagram of this procedure is presented in Figure 3.

Control coupons were placed in sterile 10% TSB, incubated for 4 hours, rinsed in sterile phosphate buffer, incubated in fresh 10% TSB for 48 hours, rinsed in sterile phosphate buffer, incubated in sterile 10% TSB for 4 hours, rinsed in sterile phosphate buffer, and incubated for an additional 48 hours in fresh 10% TSB to confirm that contamination did not occur in the experimental samples.

4.5 Biofilm Growth and Analysis

After the 48 hour incubation period with the *L. monocytogenes* cocktail, coupons were removed and rinsed in sterile phosphate buffer. One side of each coupon was swabbed with a sterile sponge soaked in a 5-7% sodium hypochlorite solution to inactivate microorganisms. The same side was then swabbed with a 10% solution of sodium thiosulfate to neutralize the bleach solution, and the coupons were rinsed in sterile phosphate buffer to remove residual sodium thiosulfate. Cleaning one side of each coupon was a necessary step as the coupons would be placed cleaned side down on the desiccator plates. A method for standing the coupons up vertically could not be used as condensation would run down the coupon. Staining with Hoescht (diluted 1:1000) indicated that this was an appropriate method for biofilm removal of one side of the coupon without affecting growth on the uncleaned side.

Two coupons per replication were then placed, cleaned side down on the sterile desiccator plate and incubated at 12° C for desired time. Two coupons per replication were placed in large petri dishes, cleaned side down, and 0.1 mL of chicken serum was

applied. Chicken serum was chosen to simulate protein soils in ready to eat processing plants. The serum was spread using a Teflon plate spreader to coat the entire surface, but not disrupt the biofilm structure. The protein-treated coupons were then placed protein side up, on the sterile desiccator plate and incubated at 12° C for the desired time. Two coupons per treatment per replication were analyzed for each time point. A separate desiccator was used for each time point and each treatment to provide each replication, and a flow diagram of this is presented in Figure 4.

Coupons were removed at specified time intervals, and samples were analyzed for *L. monocytogenes*. Two coupons per replication were each placed in a plastic bottle with 3 grams of glass beads (425-600 microns in diameter) and 10 mLs of a 0.1% solution of Tween 80 made in 0.1% peptone water. The bottles were shaken for 1 minute to dislodge biofilm. Tween 80 was used as a surfactant to assist removal of protein and biofilm from the surface of the coupons. Tween 80 at much higher concentrations than those used in this study does not inactivate microorganisms [82]. The glass bead method used here is adequate for biofilm removal and subsequent recovery of microbes within the biofilm [83]. The resulting liquid was serially diluted in 0.1 % peptone water, plated on Listeria Selective Agar, and incubated at 32° C for 48 hours. A detection limit was calculated as some of the rinses consistently provided very low colony counts. If plates contained 1-10 colonies, the data value was entered as 1 CFU/cm^2. Plate counts above 20 CFU/mL were entered as the actual data value. This limit was chosen as the dilution scheme only allowed for statistically accurate counts above 20 CFU/mL. Two coupons per replication were analyzed immediately after 48 hour incubation with *L. monocytogenes* to give an initial average *L. monocytogenes* count of 6.34 Log CFU/mL after 5 replications.

Corresponding rinses were also plated to obtain day zero rinse values of 5.58 Log CFU/mL after 5 replications. All samples in this experiment were plated in duplicate and 5 replications of each data point were performed.

CHAPTER 5

EXPERIMENTAL RESULTS AND DISCUSSION

5.1 Humidity Chambers – Experimental Design

Before the effect of protein on *L. monocytogenes* biofilms could be studied, chambers were designed to simulate humid environments typical of food processing areas. We set several requirements for these humidity systems, including size and characteristics of condensation. One chamber was required for each treatment (controls, biofilms with protein soil, and biofilms without protein soil) and each treatment time. These chambers must fit in one incubator, as a result, chamber size was important. Next, we wanted to achieve consistent condensation on each coupon. This condensation should be dispersed evenly on each coupon and must be resident on the steel over a 5 week time period.

Nalgene autoclavable desiccators (250 mm) were the correct size to provide separate chambers for each treatment and time point. Since the interactions between *L. monocytogenes* biofilms and protein soils were the primary focus of these experiments, initially a single species biofilm composed of five strains of *L. monocytogenes* was used. After 5 replications at 12º C, it was determined that these strains did not produce repeatable biofilms at 12° C. Two out of 5 replications showed *L. monocytogenes* growth, while populations did not produce biofilms in 3 out of 5 replications. As a result, the incubation temperature for these experiments was set at 25° C.

Room temperature fluctuated between 23° and 27° C, which led to differences in condensation between desiccators during the incubation periods. Desiccators at the top of the incubator collected less condensate than those placed at the bottom, and large condensate drops formed inside the lids of the chambers with very little condensation forming on the steel coupons. When the desiccators were moved, these drops fell on some of the coupons causing inconsistent replications. Modification to the chamber designs was attempted to overcome this problem.

The first modification was to use 125 mm filter paper discs to line the sides of the desiccator. The paper was held in place by the metal plate with the bottom of the filter paper in contact with the water (Fig. 5). The paper pulled water up from under the plate to achieve consistent humidity throughout the desiccator. This method did not work as expected. After several days' incubation, the filter papers were too saturated to remain vertical and often bent over and came in contact with the steel coupons. This design seemed inadequate after two replications.

The second modification was to add insulation to the humidity chambers in order to keep the temperature inside the incubator consistent and to encourage uniform condensation to form on the coupons. Strips of rubber foam weather seal (3/4 " wide, 5/16 " thick) were placed on the lid of the desiccators (Fig 6). We believe that this prevented fluctuation in temperature at the top of the chamber allowing condensation to form evenly on the coupons and surfaces within the desiccators.

However, since most processing environments are held at lower temperatures, and since biofilms found in these areas are rarely composed of a single species, we ultimately decided that a mixed species biofilm of *P. putida* and a cocktail of *L. monocytogenes*

strains would be used. After several replications, we determined that the combination of microbes produced repeatable biofilms at 12° C. As a result, the experimental parameters were changed to include *P. putida* and the 12° C incubation temperature. The lower temperature would also allow condensation that met our requirements to form within the unmodified desiccators.

5.2 Results of Sample Analysis

Table 1 summarizes data for 5 replications of each treatment at each time period by giving the mean of duplicate plate counts (Log CFU/cm^2) and standard deviations between replications at each time point. Day 0 values were obtained after the 48 hour incubation with *L. monocytogenes* once one side of the coupons was cleaned. These counts show the numbers of *L. monocytogenes* present in the biofilm before treatment with protein to provide a base level for comparison. All uninoculated controls showed no growth and are not included in Table 1.

Table 1 in Appendix A provides the statistical output based on the GLM procedure of SAS using data points from all treatments (biofilm, biofilm rinse, biofilm with protein, and biofilm with protein rinse) at all time points. At alpha = 0.05, time and treatment showed significance, and an interaction between time and treatment was significant. A high value for R^2 shows good fitness for the model. The t grouping indicates that counts from each treatment were significantly different with the biofilm with protein rinses having the highest mean population of *L. monocytogenes* and the biofilm without protein rinse having the lowest mean population of *L. monocytogenes*.

Graphs of each treatment plotted separately at sampling times are provided in Figure 7. Letters at the top of each bar group time points into significantly different groups, and

the statistical analysis is presented in Tables 2-5 in Appendix A. Statistical analysis for the biofilm without protein treatment indicates that time was a significant factor for this treatment (see Appendix A). Time as a variable was separated into 3 time groups which is presented in: days 7 and 21 with the highest mean population of *L. monocytogenes*, days 3, 28, and 35, and days 14 and 28 with the lowest mean population. This analysis supports the idea that growth is cyclic within the biofilm without protein treatment. Statistical analysis of biofilm with protein treatments indicates that time is also a significant factor for this model, with time divided into 3 groups presented in: days 7 and 21 with the highest mean populations, days 3 and 21, and days 3, 14, 28, and 35 with the lowest mean populations of *L. monocytogenes*. Time also proved significant in biofilm without protein rinses, segregating the data into 3 time groups presented in: days 7, 21, and 28 with the highest mean populations, days 14 and 21, and days 3 and 35 with the lowest mean populations. Statistical analysis indicates that time is not a significant factor for data within the biofilm with protein rinse treatment. Figure 7d. provides a graphical representation of L. monocytogenes populations at each time point.

Statistical analysis of surface associated treatments (biofilms with and without protein) show time to be a significant factor, which supports the idea of a cyclic increase and decrease in growth within the biofilm. There are several possible explanations for this. First, this may show a pattern of biofilm growth that involves sloughing of microbes as populations get too crowded, or as toxic end products build up. Sloughing of microbes is only partially supported as corresponding rinse values are high, but not high enough to explain the decreases.

Another explanation for fluctuating populations of *Listeria* is the build up of toxic end products. In general, *Pseudomonas* produces proteases during late exponential and stationary phases [84]. In fact, production of proteases by *Pseudomonas spp*. is enhanced at lower temperatures with high concentrations of proteases being produced at temperatures as low as 5° C [85]. Proteolysis results in the production of amino acids which are further broken down, by deaminases and other enzymes, into low molecular weight products such as ammonia, amines, and sulfides [84]. These compounds may build up in the experimental biofilms held at 12° C as *Pseudomonas* uses available protein, causing conditions within the biofilm to become more alkali. *L. monocytogenes* is resistant to alkaline conditions which can even initiate cross protection against heat [86]. Thus, the survival of *L. monocytogenes* as part of the *Pseudomonas* biofilm may actually be enhanced through stresses created within the microenvironment.

L. monocytogenes in biofilms subjected to protein soils did not appear to decrease as rapidly or as dramatically as those in biofilms not subjected to protein. Thus, it appears that populations of *L. monocytogenes* within biofilms exposed to chicken serum were much more stable than within the biofilm alone. Rinse counts with soil were on average 2-3 logs higher than biofilm rinses without protein soil, and remained above 5 log CFU/cm^2 over all time points. As condensate from dirty surfaces drips onto food contact surfaces or into the product, contamination can occur. Both treatments (biofilm and biofilm with protein) show that populations of *L. monocytogenes* can survive well in multispecies biofilms that are not continuously submerged. This suggests that unclean surfaces subject to condensation can provide relatively stable environments for biofilm

growth and that these biofilms can act as reservoirs leading to product contamination, for example, through condensate.

Figure 1. Culture Preparation

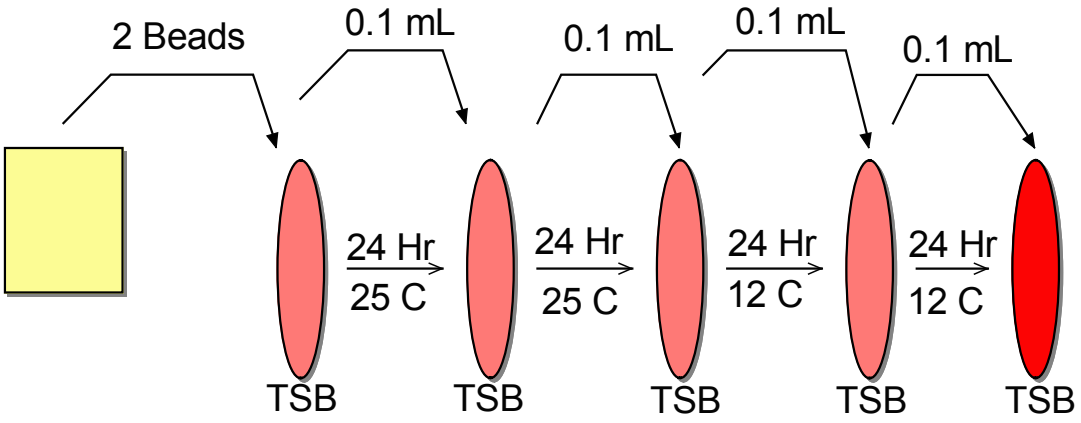

Figure 2. Preparation of P. putida biofilms

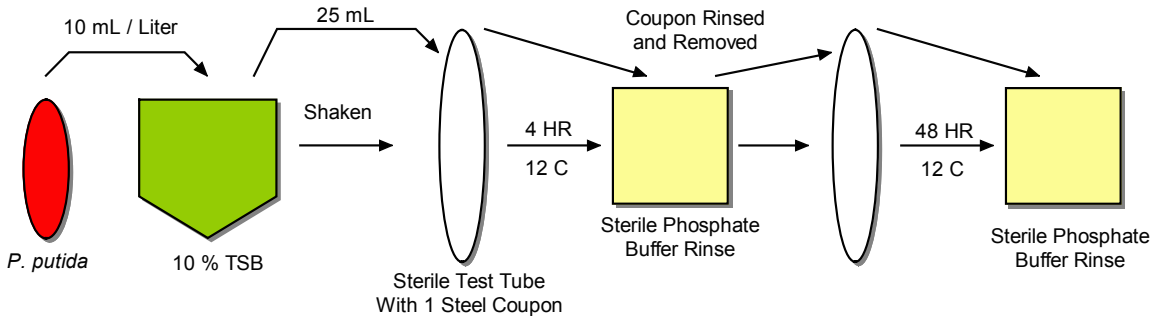

Figure 3. Preparation of L. monocytogenes biofilms

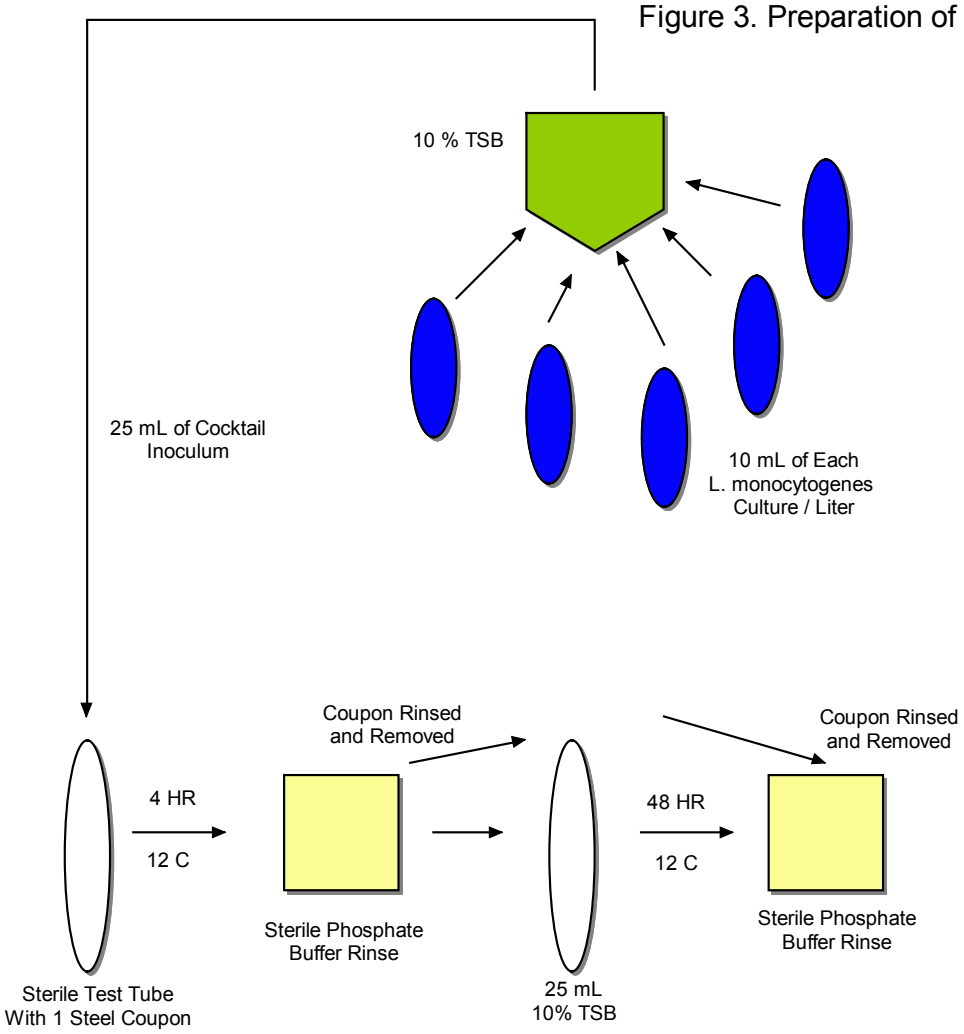

Figure 4. Coupon Treatment After Biofilm Formation

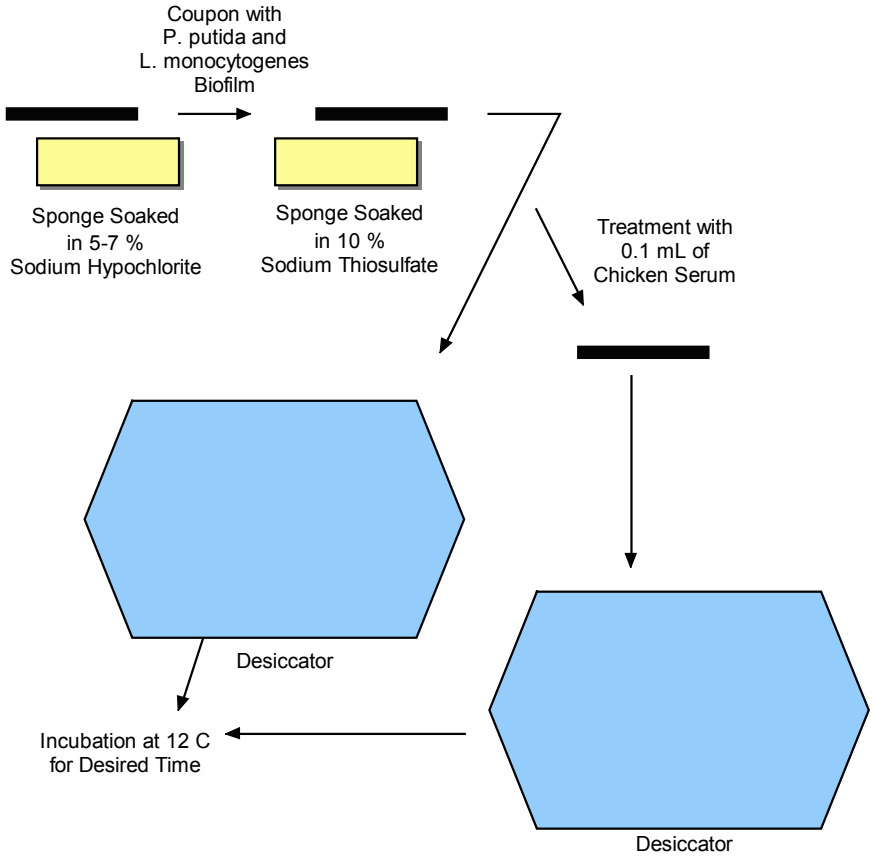

Figure 5. Preliminary Desiccator Design With Filter Paper Lining

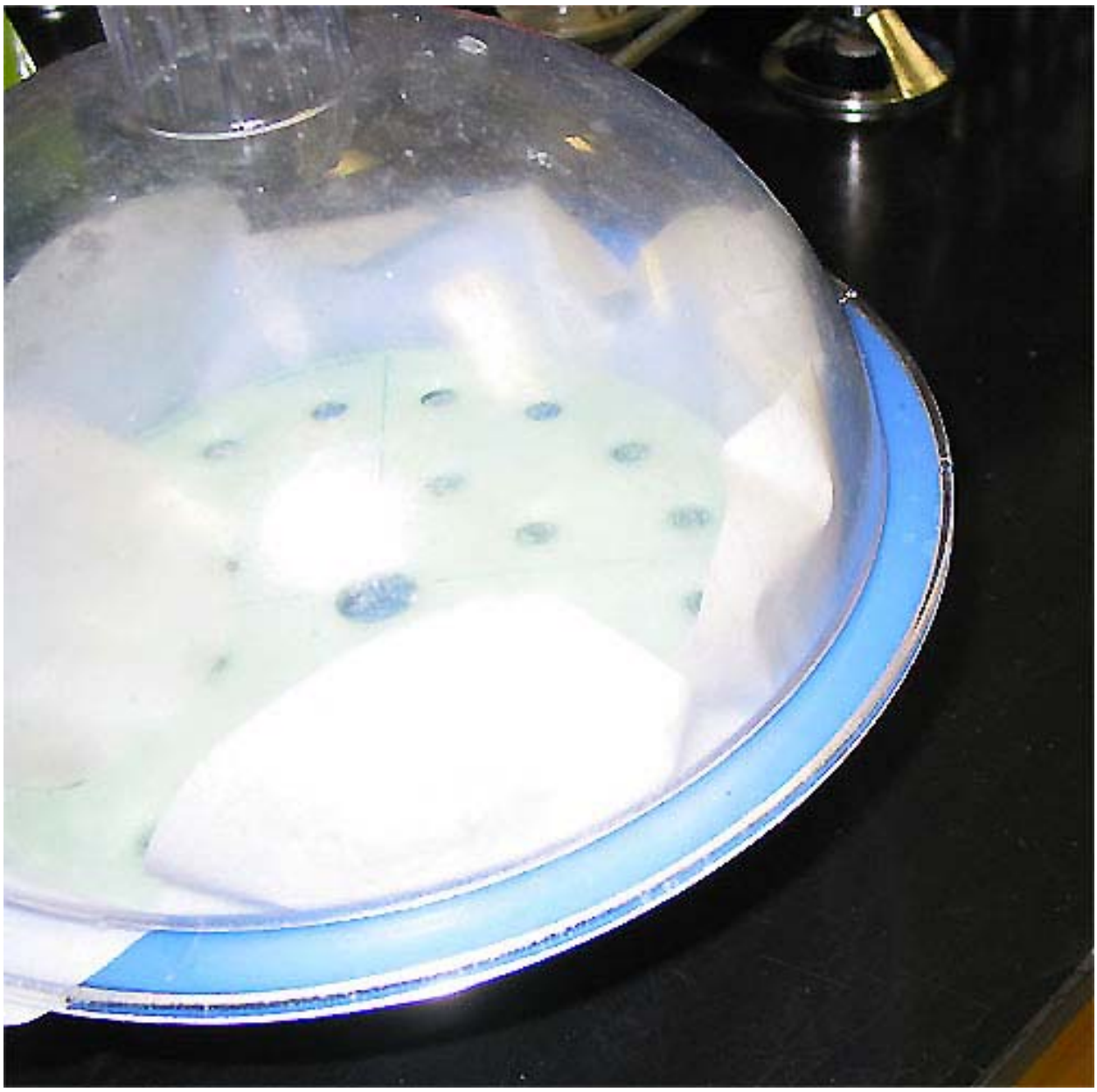

Figure 6. Preliminary Desiccator Design Including Insulation

Table 1. *L. monocytogenes* Populations in Biofilms With and Without Protein (Log CFU/cm^2)

Day	0	3	7	14	21	28	35
Biofilm (average)	6.34	3.73	5.08	3.13	4.77	3.64	4.10
Biofilm (standard deviation)	0.51	0.35	0.44	0.48	0.40	0.34	0.38
Biofilm with Protein (average)		4.45	5.17	4.34	4.89	4.19	4.24
Biofilm with Protein (standard deviation)		0.25	0.52	0.58	0.43	0.22	0.30
Biofilm Rinse (average)	5.58	0.88	5.31	4.80	5.14	5.54	0.82
Biofilm Rinse (standard deviation)	1.11	0.16	0.66	0.48	0.12	0.26	0.17
Biofilm with Protein Rinse (average)		6.50	6.23	6.08	5.50	5.72	5.80
Biofilm with Protein Rinse (standard deviation)		0.29	0.68	0.31	0.34	0.60	0.89

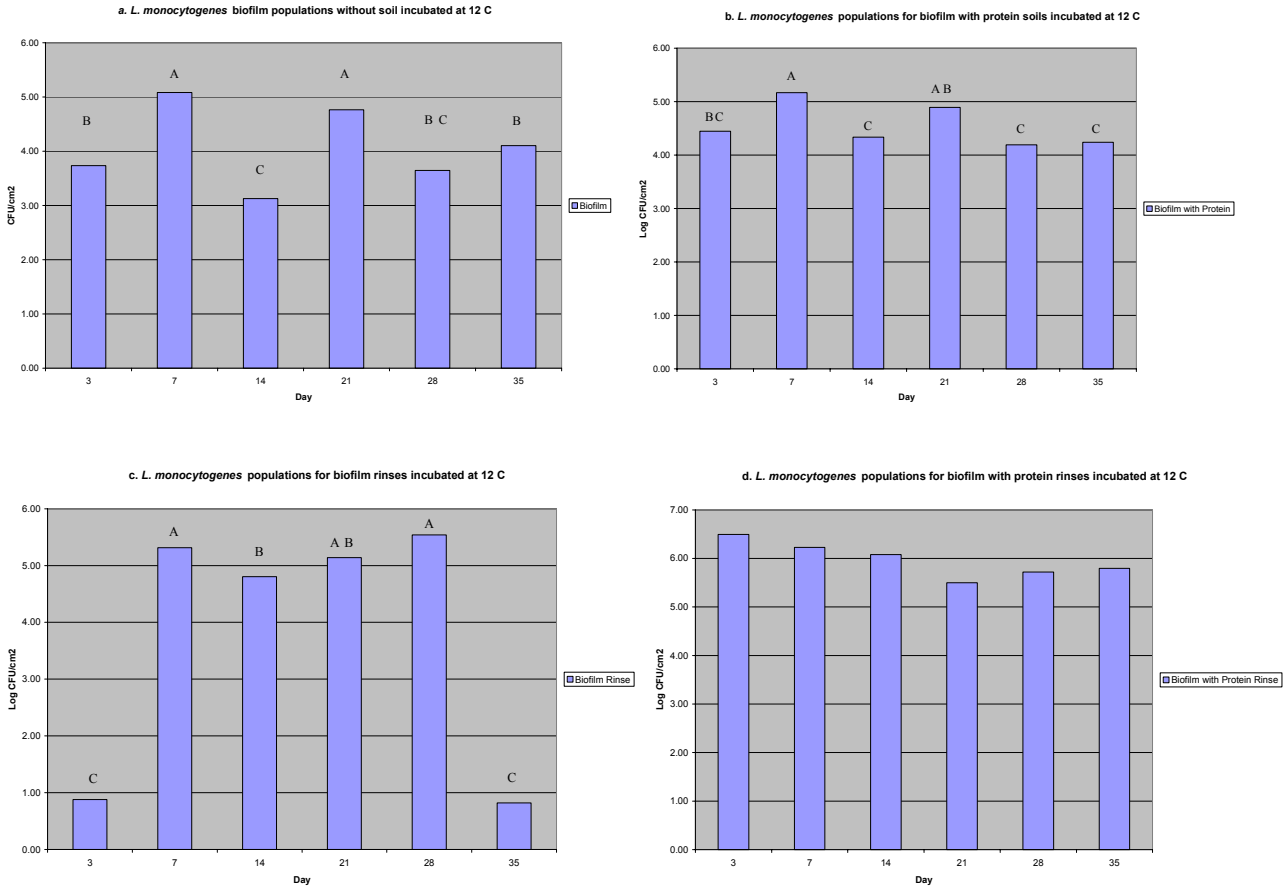

Figure 7. Populations of *L. monocytogenes* in Biofilms With and Without Protein (including rinse data) as Determined by Viable Counts on Listeria Selective Agar

CHAPTER 6

SUMMARY AND CONCLUSIONS

A new method of biofilm growth was developed to simulate surfaces that collect condensation in food processing environments. These areas are often infrequently or incompletely cleaned as they are usually not food contact surfaces. Growth of L. monocytogenes on these surfaces is associated with product contamination problems. This model system demonstrates the ability of *L. monocytogenes* to survive in a multispecies biofilm on stainless steel surfaces that collect condensation. *L. monocytogenes* maintained greater populations in biofilms exposed to chicken serum, which simulates an inadequately cleaned surface. This supports our hypothesis that the presence of protein soil will enhance the growth of *L. monocytogenes*. High rinse counts indicate the ability of these biofilms to act as a reservoir for *L. monocytogenes*, which can then contaminate product as well as food contact surfaces through dripping condensation. The microbial profile of incoming product is not the only aspect of food processing that must be understood. It is also important for food processors to better understand biofilm growth in areas subject to soil and condensation as these biofilms can lead to loss of money and time due to contaminated product.

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APPENDIX A. STATISTICAL OUTPUT

Table 1. Statistics for all treatments

Dependent Variable: cts

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	236.3037730	10.2740771	53.27	<.0001
Error	96	18.5140616	0.1928548		
Corrected Total	119	254.8178346			

R-Square	Coeff Var	Root MSE	cts Mean
0.927344	9.570074	0.439152	4.588808

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	5	43.8192675	8.7638535	45.44	<.0001
trt	3	87.4962079	29.1654026	151.23	<.0001
time*trt	15	104.9882976	6.9992198	36.29	<.0001

t Grouping	Mean	N	trt
A	5.9843	30	Biofilm with Protein Rinse
B	4.5454	30	Biofilm with Protein
C	4.0760	30	Biofilm
D	3.7495	30	Biofilm without Protein Rinse

Table 2. Statistics for biofilm treatment by time

Dependent Variable: cts

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	13.45318800	2.69063760	16.67	<.0001
Error	24	3.87448400	0.16143683		
Corrected Total	29	17.32767200			

R-Square	Coeff Var	Root MSE	cts Mean
0.776399	9.857508	0.401792	4.076000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	5	13.45318800	2.69063760	16.67	<.0001

t Grouping	Mean	N	time
A	5.0824	5	7
A	4.7656	5	21
B	4.1014	5	35
B	3.7342	5	3
C B	3.6442	5	28
C	3.1282	5	14

Table 3. Statistics for biofilm with protein treatment by time

Dependent Variable: cts

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3.89874097	0.77974819	4.73	0.0038
Error	24	3.95980040	0.16499168		
Corrected Total	29	7.85854137			

R-Square	Coeff Var	Root MSE	cts Mean
0.496115	8.936259	0.406192	4.545433

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	5	3.89874097	0.77974819	4.73	0.0038

t Grouping	Mean	N	time
A	5.1684	5	7
B A	4.8914	5	21
B C	4.4448	5	3
C	4.3356	5	14
C	4.2388	5	35
C	4.1936	5	28

Table 4. Statistics for biofilm rinse treatment by time

Dependent Variable: cts

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	127.6164395	25.5232879	189.99	<.0001
Error	24	3.2241620	0.1343401		
Corrected Total	29	130.8406015			

R-Square	Coeff Var	Root MSE	cts Mean
0.975358	9.775199	0.366524	3.749533

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	5	127.6164395	25.5232879	189.99	<.0001

t Grouping	Mean	N	time
A	5.5412	5	28
A	5.3144	5	7
B A	5.1398	5	21
B	4.8028	5	14
C	0.8796	5	3
C	0.8194	5	35

Table 4. Statistics for biofilm with protein rinse treatment by time

Dependent Variable: cts

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3.83919667	0.76783933	2.47	0.0609
Error	24	7.45561520	0.31065063		
Corrected Total	29	11.29481187			

R-Square	Coeff Var	Root MSE	cts Mean
0.339908	9.313763	0.557360	5.984267

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	5	3.83919667	0.76783933	2.47	0.0609

t Grouping	Mean	N	time
A	6.5824	5	3
B A	6.2280	5	7
B A C	6.0810	5	14
B C	5.7968	5	35
B C	5.7184	5	28
C	5.4990	5	21