TRANSFER OF *LISTERIA MONOCYTOGENES* FROM STAINLESS STEEL AND HIGH-DENSITY POLETHYLYENE TO COLD SMOKED SALMON AND *LISTERIA MONOCYTOGENES* BIOFILM COHESIVE ENERGY INVESTIGATION

A Thesis Presented

By

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Food Science

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ABSTRACT

TRANSFER OF *LISTERIA MONOCYTOGENES* FROM STAINLESS STEEL

AND HIGH-DENSITY POLETHYLYENE TO COLD SMOKED SALMON AND

***LISTERIA MONOCYTOGENES* BIOFILM COHESIVE ENERGY**

INVESTIGATION

MAY 2011

FUJIA ZHANG M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by Professor Lynne A. McLandsborough

Listeria monocytogenes is a major concern for the food industry, because it is one of the major agents causing the serious foodborne disease listeriosis. It continues to be a major cause of class I food recalls in the United States. The objective of the first part of this study is to evaluate the effect of hydration level on attached *listeria monocytogenes* on stainless steel/High density polyethylene transferred to food products. Attached cells were prepared on stainless steel/High density polyethylene in diluted tryptic soy broth 1:20 for 5 minutes. Then, attached cells were equilibrated with saturated salt solution at 20 degree C for 24 h (98, 75, 54, and 33% relative humidity; %RH) prior to transferring. Transfer experiments were conducted from inoculated stainless steel/High density polyethylene to cold smoked salmon fillets with a constant pressure of (18kPa) for 30 seconds by using a universal testing machine. The whole experiment was repeated 6 times. The results were analyzed with an analysis of variance by SAS Statistical Analysis Software. The differences between the different RH% and surface conditions (stainless steel and HDPE) were not statistically significant. We observed variation in texture and moisture condition of the cold smoked purchased from a local grocery store.

There was variability in between packages, brands and over the course of storage after opening, and likely contributed to the variability of transfer observed in this set of experiments. The objective of the second part of the research is to study the effect of hydration level on the detachment of *listeria monocytogenes* biofilm growing on stainless steel by using Atomic force microscope. Biofilms were grown on stainless steel in drip flow bioreactor at 32 degree C for 72 h. After this, biofilms were equilibrated over saturated salt solution at 20 degree C for 48 h before the Atomic Force Microscope experiment. The results showed that cohesive energy value of the biofilm increased with biofilm depth. Meanwhile, only square shaped displaced 2.5X2.5 region were able to be visualized after serious of raster scanning under high load which means that moisture condition of *Listeria monocytogenes* biofilm can significantly affect the cohesiveness between Listeria monocytogenes cells and other biofilm materials. These results agree with previous reports on transfer efficiency of *Listeria monocytogenes* biofilm.

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CHAPTER I

LISTERIA MONOCYTOGENES

1.1 Introduction

Listeria is named after Joseph Lister who was a surgeon in Britain (2). It is a bacterial genus which contains six species (2). They are typified by *Listeria monocytogenes* (2, 16, 17, 35*)*. *Listeria monocytogenes* is a gram-positive, rod shaped (1-1.5 um x 0.5 um) bacteria (14). It is ubiquitous in natural environment. It is known as a psychotropic organism which can grow at 4 ℃ or lower (65). It can also survive at a broad pH range (4.3 – 9.6) (65). This makes it has a really impressive ability of resistance to different kinds of environmental stress. It can be found in plant materials, stream water, sewage, soil and food such as cheese (43), milk (20) vegetables, and meat, etc. (30, 59).

1.2 *Listeria monocytogenes* pathogenesis

Listeria monocytogenes is one of the major causal agent of the serious foodborne disease called listeriosis (28). This disease is an important concern for public health all around the world with a clinical mortality rate up to 20-30 percent (46, 56). The disease is due to the ability of *Listeria monocytogenes* to be able to cross the host barriers: intestinal barrier, the blood-brain barrier and the maternal-fetal barrier (66). People who have been exposed to *Listeria monocytogenes* may have vague symptoms, such as fever, vomiting, gastroenteritis and muscle pain (46). However immunocompromised people, including elderly, children, infants, or pregnant women have much higher chance having classical listeriosis which is characterized as meningitis, menigo-encephalitis, fetus infections,

abortions, and/or perinatal infection (2). Every year, there are approximately 500 deaths out of 2500 clinical cases in the United States (2, 10, 66).

Listeria monocytogenes has so many ways to get into food processing environments and often associated with with processing equipment in the (28). *Listeria monocytogenes* has been continually isolated from several of foods and food industry processing plants all around the world (4). Especially the industry related to seafood processing (63).

1.2.1 *Listeria monocytogenes* in food industry

As food industry developed, the presence of human pathogens in food products have been increasingly a great concern as food is processed in larger and larger amounts. *Listeria monocytogenes* is very difficult to totally remove from the food processing environments. One of the reasons is its ability to form biofilm on different kinds of food processing equipment surfaces (37).

Listeria monocytogenes can be attracted to different surfaces which are conditioned with nutrients. Food industry gives good environments for microorganisms to develop biofilm on different processing surfaces. Especially in seafood industry, *Listeria monocytogenes* has become a serious concern as a source of foodborne pathogen. For example, smoked salmon is usually sporadically contaminated with low level of *Listeria monocytogenes*. (63) Under normal hot-smoking and cold-smoking conditions, the number of *Listeria monocytogenes* seems to decrease. It is found that *Listeria monocytogenes* will multiply considerably during storage. The main source of *Listeria monocytogenes* has not been determined.   For now, the most efficient way to eliminate

the contamination is to prevent colonization of the food processing environment and spread to food products (68).

Research has shown that during the storage of cold-smoked salmon, *Listeria monocytogenes* damaged during the smoking process may recover. For hot-smoked salmon, *Listeria monocytogenes* was found to be eliminated during the smoking condition of 65 C for 20 min, and wouldn't recover during storage for 20 days (68, 71). It is possible to use preservatives, to inhibit growth of *Listeria monocytogenes* in smoked salmon. But it is undesirable to use preservatives in smoked salmon. Because the sensory quality and safety of these preservatives should be confirmed before used in food product. In order to prevent colonization in the food processing environment by *Listeria monocytogenes* and spread to the products, HACCP programs should be introduced. All production facilities should be designed to ensure an effective sanitation process. All critical points should be monitored properly for *Listeria monocytogenes* (71).

1.3 Biofilm of Bacteria

1.3.1 Biofilm definition

Biofilm is the matrix-enclosed microbial accretion which adheres to living or non-living surfaces (11). It is a structurally complex, dynamic system with attributes of both primordial multicellular organisms and multifaceted ecosystems (30, 63).

1.3.2 Discovery of Biofilm

When microbiologists could use direct ways to observe the micro-organisms in the real natural environment, an idea that the bacteria prefer to grow on surfaces was came up with hundred years ago. In 1683, Antonie van Leuwenhoek used a microscope to observe the plaque between his own teeth. He also repeated these experiments on two women and

two men who have never cleaned their teeth in their whole lives (11). In his report he wrote to the Royal Society about this observation, he said: "I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving. The biggest sort…had a very strong and swift motion, and shot through the water (or spittle) like a pike does through the water. The second sort…oft-times spun round like a top… and these were far forwards… Moreover, the other animalcules were in such , enormous numbers, that all the water… seemed to be alive" (11). In 1934, Claude Zobell found that the bacteria in marine populations are attracted by the surfaces they usually grow on in form of steady aggregated population. Later on, some scientists examined the biofilm on dental plaque on tooth surfaces and some of them studied the irreversible attachment of the bacteria on the surfaces which constitutes the first stage of biofilm formation using pure culture (11, 21).

1.3.3 The formation of biofilms

Biofilms can exist and grow on both environmental abiotic surfaces (minerals, carapaces, food processing surfaces, floor drains or air water interfaces) and biotic surfaces (plants, microbes, animals). At the most basic level, biofilms are bacteria cells that are attached to a surface in the environment (20, 11, 3, 7, 47). For some bacteria strains, the protective layers of exopolymeric subtances (EPS) outside bacterial cells which can protect them from various environmental stresses are considered more important than their ability to attach to surfaces. EPS can be composed of polysaccharides, proteins and/or DNA (67). It is the interaction of the remarkably elastic polymers of EPS polymers that contribute to the firm attachment of bacterial cells to surfaces. There are two types of biofilm, monolayer and multilayer biofilm (67).

1.3.3.1 Monolayer biofilm

A monolayer biofilm is formed by the attachment of single cells on the surface. The monolayer biofilm is considered as a single layer of cells adhered to surfaces. When cell surface interactions are more important than the interactions between bacterial cells, monolayer biofilm will be the favored one. It has been described by scientists that the monolayer formation occurs in two steps. Firstly, most of the bacterial cells attaching to the surfaces will break loose from the strength that tethers them to the surfaces. This stage is called transient attachment. The bacterial cells which still attach to the surfaces undergo the transition from transient to irreversible attachment (11, 60, 40).

1.3.3.2 Multilayer biofilm

If the bacteria attach as clusters of cells or if a monolayer biofilm remodels to form clusters, a multilayer biofilm, that is defined as one in which the bacterium is attached both to the surface and to neighboring bacteria, is formed. It might form on the abiotic environmental surface, an air-water interface, or the surfaces of another organism. The bacterial cells in multilayer biofilms produce different kinds of components which compose the biofilm matrix. There are so many environmental signals have been identified as the guidance for the formation of multilayer biofilm, including mechanical signals (such as sheer stress), nutritional and metabolic cues, inorganic molecules, host-derived signals, and/or the presence of antimicrobials (41).

Data from numerous researches suggest that flagellum plays an important part in the bacterial cells sensing the surfaces and the subsequent formation of biofilms (31, 40, 29, 62, 63). From the study of *Pseudomonas. aeruginosa*, the positive regulator of biofilm matrix synthesis named AlgT was found having the ability to inhibit flagella gene

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expression (31, 57). In some strains of *Vibrio. cholera* mutants which are lack of completed functional flagellum, observation shows increase in transcription of genes that are involved in the synthesis of biofilm matrix and significant increase in biofilm matrix synthesis. The mutants without flagella motor can't produce biofilm matrix even with the presence of environmental signals (40). It is possibly that when the surface interacts with flagella, the drag force of the flagella motor will increase. As a result, a signal will be transduced. When the flagella motor is not present, the signal can't be transduced (24). In biofilm formation of *Bacillus subtilis*, the protein EpsE binds flagellar switch protein FliG and inhibits it from interacting with flagellar motor thus immobilizing the flagellum. When the condition changes to an unfavorable status for biofilm formation, EpsE is released from the flagellum and in turn the bacterium can become motile again (5).

The amount of nutrients in the bacterium living environment has a great influence to the orientation of multilayer biofilm formation. For example, *Salmonella enterica* serovar Typhimurium will form multilayer biofilm during nutrient limitation environments; while *V. cholera* will form multilayer biofilm under the nutrient-rich environments. There are so many nutrient signals which will affect the multilayer biofilm formation, including glucose and catabolite repression, indole, polyamines, etc (20, 19). Both the presence of iron and phosphate an either enhance or repress biofilm formation depending upon the type of bacteria. Iron which mostly resides stably in ferric oxide hydrate complexes or tightly binds either to specialized extracellular iron carrier proteins or to small molecules known as siderophores, is one of the most important inorganic molecules which provide the signals and activate multilayer biofilm formation. Due to the type of bacteria, the limitation of iron can lead to either increased biofilm formation

or repressed biofilm formation (15, 39). Phosphate is another important inorganic molecule which contributes to the multilayer biofilm formation. Scientists both found positive and negative effect on biofilm formation from the limitation of phosphate in environment (39). For many pathogenic organisms, the host-derived molecules which can provide signals that can activate the synthesis of components resisting to the host-derived molecules, might promote bacterial biofilm formation (11).

1.4 Bacteria cells adhesion and biofilm formation initiation

Normally, for the bacteria cells to adhere to surfaces, they should firstly know the accurate direction where surfaces are located. The planktonic bacteria cells will release protons and molecules acting as signal carriers while they are moving around in fluid environment. The signal molecules will diffuse radially away from the individual cell in the fluid. Some scientist speculate that if there is a sharply rise of concentration of either protons or signal molecules on one side of the cell, that side should be close to a surface or interface. Due to this signal, the bacterial cell can find its way to attach to surfaces (11). Scientists also used phase contrast and confocal light microscopy to directly observe living cells and confirmed that the planktonic cells are attracted to surfaces. The cells would across the surface before they settle down in a place and initiate their adhesion behavior (11). If the surface is already adhered with cells, the subsequent cells would roll away from the adherent cells from the same species. As a result, a monolayer of bacterial cells forms on the colonized surface. These observations clearly showed that the bacterial cells can sense their proximity to surfaces, and this proximity signals elaborate species-specific behavior patterns (68).

After initiation of their adhesion behavior, the biofilm formation process will be activated. In order to strengthen the attachment to surfaces, the bacterial cells will produce exopolymeric substances (EPS) (12). This EPS also contribute to the adhesion to other bacterial cells, and it will progress the reversible attachment stage to the irreversible adhesion phase of biofilm formation. So many researchers have been working on observation of the up-regulation of specific genes and the actual beginning of EPS production of the modified bacterial strains which contains reporter genes. Research data from *Pseudomonas aeruginosa* have shown that the up-regulation of the EPS synthesis genes is mediated by a sigma factor which also upregulates a wide variety of other genes (57). As a result, it can produce a biofilm phenotype which is significantly different from the phenotype of planktonic cells of the same species. They also compare the proteins in the cell envelope fraction of cells in the biofilm phenotype, with proteins in the same fraction of planktonic cells. Result showed that there are 30-40% of these proteins are different (57). So we know that at least in one organism and might be in most bacteria, the biofilm cells are significantly different from their planktonic counterparts (12).

1.5 Classification of adhesive structures in monolayer biofilm

There are three types of adhesion structures that have been found during monolayer biofilm formation.

1.5.1 Preformed adhesions

The flagellum and pili are involved in preformed adhesions. The phenotypes of aflagellate and paralyzed nonmotile bacterial mutants are difficult to reconcile (62). The mutants who have a paralyzed flagellum are often unable for bacterial attachment on surfaces. But bacteria without flagellum can successfully contribute to develop

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monolayer formation to the multilayer biofilm formation. It is considered that the flagellum can help bacteria to overcome long-range repulsive forces between bacterium and the surfaces. From the research in *V. cholera*, the mutant which was unable to produce the integrate flagellum can still contribute to the formation of monolayer biofilms, while the mutant without flagellar motor is completely defective in monolayer formation, thus the flagellar motor seems to play an important role in formation of bacterial monolayer biofilm. This phenomenon indicates that flagellar motor plays an important role in monolayer biofilm formation.

For gram-negative bacteria adhesion to surfaces, the retractable pili are commonly required. It is believed that the pili, which are able to retract against great force, can pull bacteria either onto or along surfaces by attaching to the surface and retracting (72).

1.5.2 Conditionally synthesized adhesions

Retractable pilus can mediate the transient attachment in many bacteria. But this kind of attachment is reversible. There are some factors which will turn this transit attachment into permanent attachment have been discovered. For example, the change of *Pseudomonas fluorescens* from reversible attachment to irreversible adhesion status is mediated by a large secreted protein called LapA, which associates with the bacterial cells surface. It is known that secretion of LapA is inhibited by RapA, which is a phosphodiesterase that degrades the second messenger cyclic diguanylate monophosphate. So scientists have an assumption that cyclic diguanylate monophosphate enables secretion of LapA.

The bacteria *Caulobacter crescentus* can best describe the transition from transient to permanent adhesion of monolayer biofilm. A protease will break down the

flagellum of cells which contributes to the attachment. Then a holdfast composed of oligomers of N-acetyglucosamine, which is known as the world's strongest "bond", will be synthesized and take flagellum's place. This polysaccharide ensures tight adhesion to the surface.

1.5.3 Specific adhesions

There are also some special cases that bacterial cells can specifically adhere to their mammalian host cells to form monolayer biofilm. After the reversible attachment of pathogenic cells to their host cells, they will produce a specific component which will attach to the receptors on the host cells surfaces. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* can produce a surface protein called invasion. It can bind to a glycoprotein named β1 integrin which is found on the surface of M cells. The

enterohemorrhagic and enteropathogenic strains of *E. coli* have an ability to transfer their own bacterially delivered receptor into mammalian cells, which do not have the specific receptors on the surface, by using their type III secretion system (13).

1.6 Biofilm matrix composition

The biofilm matrix is believed as a highly hydrated (up to 97% water) sponge-like structure. We can find membrane vesicles, DNA, surfactants, exopolysaccharides, lipids, glycolipids, and different ions in the biofilm matrix. It is likely that during different conditions and times of the biofilm maturation, the biofilm components which are more important to the biofilm function will be different. Exopolysaccharides are thought to be a major component of biofilm matrices. In most studies, scientists found that with the absence of exopolysaccharide synthesis and export, surface attachment of bacterial cells would be always observed. There are some general exopolysaccharides synthesized by

different species of bacteria. Furthermore, some bacteria can synthesize and export many kinds of exopolysaccharides (8, 18).

1.7 Biofilm community structure

Biofilms can be divided into two types. (i) Those that shows mushroom-like structures separated by voids and low surface coverage. (ii) Those with sheet-like compact layers and high surface coverage (11). Medium composition, presence of surfactants, various types of motility and quorum-sensing effects are the main determination of biofilm architecture. Studies have shown that the initial biofilm community structure unit is microcolony (11). Microcolonies are inconsecutive matrix-enclosed bacterial cells communities. It might contain one species, or many species of cells. In thin biofilms, microcolonies are arranged horizontally, while in thick sessile biofilms, they will form vertical arrays. Microcolonies are usually composed of 10-25% bacterial cells and 75-90% EPS matrix, depending on the bacterial species in microcolonies. The bacterial cells in the biofilm matrix lack of motility. They usually form a mushroom-like shaped structure. Scientists usually find the bacterial cells in the 'crown' of the mushroom. It is also found that the closer to the core of the microcolony, the denser the matrix will be. Computer modeling showed that decreases in surface motility would lead to the irregular topologies, while increases in surface motility would lead to the flat topology of biofilm formation (11). A study of *P. aeruginosa* showed that diverse carbon sources can induce changes in bacterial motility on surfaces (57). In one study, succinate was used as the carbon source in the medium for the growth of wild type *P. aeruginosa*, which produced flat biofilms with this carbon source.. When glucose was used as the carbon source in the medium, the biofilms had irregular topology.

Microscopic direct observation has confirmed this prediction of computer analysis results. It was thought that the biofilm was accretions of cells in an amorphous matrix which made up a simple vertical vector (6). In 1988, research data showed that the beta-lactam antibiotics penetrate the entire thickness of the biofilm in 90 seconds, however there was no significant death of bacterial cells in the biofilm. After the direct observation of the architecture of the biofilm by using confocal microscopy, scientists found the true water channel system which is the real access for the penetration of the solutes (11).

1.8 Microorganisms in food industry

The development of biofilms can be found on all kinds of surfaces in any environment where the viable organisms are. In the food processing industry environment, it is usually easy for bacteria to attach the processing surfaces with different kinds of nutrients which are contained in the food ingredients. They are transported to the processing surfaces by turbulent flow of the bulk liquid or by diffusion. Numerous reports have shown the persistence of foodborne pathogens on food contact surfaces in food processing plant, including *Listeria monocytogenes* , *Yersinia enterocolitica* , *Campylobacter jejuni*; Escherichia coli O157:H7 and etc. Some strains of *Listeria monocytogenes* have been reported to remain in those food processing plants for even 10 years or more (6).

Pathogens, attaching to the food contact surfaces, which lead to the formation of biofilm in food processing systems are always undesirable and harmful. The first report which improve that it is possible for foodborne bacteria to develop biofilm in the food contact surfaces under appropriate condition has been published (Zottola et al., 1981)

1.8.1 Sources of the contamination

Improperly cleaned and sanitized equipment, air-borne microflora, fllors, waste water pipes, bends in pipes, rubber seals, conveyor belts, stainless steel surfaces, etc., are the most common and major sources involving in the contamination and biofilm accumulation of food industry (61). In milk processing plant, the system called Cleaning-in-place (CIP) procedure is commonly applied. But the defect of CIP procedures is that it can't avoid the accumulation of microorganisms on the food processing equipment surfaces which lead to the formation of biofilm.

Studies have shown that bacteria prefer a conditioned surface. The deep channels and crevices on stainless steel surfaces have the ability to trap the bacteria cells. It has been observed that *Listeria monocytogenes* produced attachment fibrils when they attach to stainless steel surface (73). Scientists also found that electrostatic, hydrophobic interactions viz. and exopolymer interactions contribute to the attachment of *Listeria monocytogenes* on different kinds of surfaces (26). Those bacteria producing biofilms on food processing surfaces are usually the reason to cause post processing contamination which leads to lowered shelf life of the food product (15, 28, 26).

1.8.2 Controlling and removing

In this era of emphasis on food safety, an effective method for cleaning and sanitation is imperative for the high-volume food processing and preparation operations. It has been proved that removing bacterial biofilm from food processing surfaces is really difficult. So, the cleaning and sanitation procedure used in food industry should be cost-effective and exhaustive. With the help of an efficient cleaning program, the accumulation of particulates and bacterial cells on food processing surfaces and the

formation of bacterial biofilms will be eliminated. A bad cleaning practice will increase the risk of biotransfer from food processing environment to the food products (28).

Even with the best cleaning and sanitizing practices available, the particulates and bacterial biofilm are not likely to be removed completely. It is because the food processing equipment design should be taken into consideration. Researchers found the smoothness and quality of the equipment used in food processing environment were the same important as cleaning practices. But, It is unavoidable the cleaning procedure will cause some damages (scratches, deep channels, crevices), which are the good place for biofilm formation, on the food processing equipment surfaces. Stainless steel had better hygienic properties by resisting damage caused by the cleaning procedure (58, 51).

Equipments type, proper accessories, correct construction, process layout and process automation are also the factors which can affect the biofilm formation and transfer of bacterial cells in the food processing environment. There are physical, chemical and biological methods available to eliminate the biofilms in the food processing environment (14).

Bacterial cells in biofilm are much more resistant to environmental pressure than their planktonic counterpart. In order to detach cells on biofilms, the exopolymeric matrix should be broken down by either chemical or mechanical methods. Before chemical treatment, the microorganisms should be eliminated as many as possible by mechanical methods. Without the mechanical cleaning procedure prior applied, the slime, which is good for the formation of biofilm, will be left on the crevices on the food processing surfaces (20, 11).

Some chemicals like EDTA which contains chelating agents can bind the calcium and magnesium ions on the cell membranes. As a result, the outer membranes of the bacterial cells will be destabilized. So the bacterial cells can be easily detached. Another way to detach the cells on biofilms is to break down EPS which is the major component of biofilm matrix. Oxidant such as iodine or chlorine can enable the detachment of bacterial biofilms from food processing surfaces. Especially for *Listeria monocytogenes*, 200 μg g−1 monolaurin in vacuum packaged samples significantly reduced the numbers of 5 *monocytogenes* (49, 33, 49, 48, 74, 75, 76).

Before cleaning and sanitizing with chemical methods, physical treatment should be applied to the food processing environment. Super-high magnetic fields (Okuno et al., 1993; Pothakamury et al., 1993), ultrasound treatment (Jeng et al., 1990; Pitt et al., 1994; Qian et al., 1997) are always used to eliminate biofilms on food processing surfaces. Electrodes which is made of silver, carbon and platinum was proved to have the ability to kill planktonic bacterial cells with low electric currents of 200 and 400 μA. Even with the treatment of exclusive methods, the traditional way of cleaning (brushing) should not be neglected. Nowadays, bacteriocins and specific enzymes are used as inhibitors to eliminate the adhesion of bacteria on food processing surfaces.

1.8.3 HACCP

The HACCP concept was developed in the 1950s by the National Aeronautics and Space Administration (NASA) and Natick Laboratories for use in aerospace manufacturing under the name "Failure Mode Effect Analysis." It was used to make sure that the food used in space should be absolutely free of pathogens (69). Later, HACCP was applied in food industry to provide management to protect consumer's health. *Listeria*

monocytogenes is a major target of HACCP. Because *Listeria monocytogenes* was considered as the greatest hazard through environmental contamination, the test samples in food industry should be taken from ceilings, hoses, equipment surfaces, floors, and drains (69). In order to prevent *Listeria monocytogenes* from entering food processing system, all these places should be cleaned and sanitized properly (25, 40, 69).

1.8.4 Bacterial transfer to foods

Because bacteria can be attracted by different food processing surfaces and develop biofilm in a very condition. Cross contamination of food products in a food processing operation environment become a major concern to consumers and food manufacturers. It is one of the most important contributing factors in foodborne illnesses. A lack of understanding in many food processing and food service settings concerning the transmission and growth of pathogens can cause foodborne outbreak situations. The same situation could happen during household environment. Foodborne disease outbreak data collected by the Centers for Disease Control and Prevention from 1998 to 2002 show that the contamination during production and processing, or cross-contamination in the kitchen was the important factor in 1/3 of outbreaks which have been identified in U.S. A variety of knowledge gaps of how pathogen spread among food processing environment and processed products have been identified in risk assessments. Microorganisms in foods may be derived from the raw materials, ingredients, personnel, or the work environment. .

1.9 Affection adhesion strengths on bacterial transfer from surface to food

Biofilm is not a stagnant system. The development of biofilm also include detachment of the cell from the biofilm. Most of the detachment s in biofilm were due to the detachment

of single cells (7). Cell-to-cell and cell-to-surface adhesion strength play an important role in bacterial transfer from surface to food. There are many factors which can affect direct surface-to- surface transfer of biofilm. Two events appear to be the major processes which are involved during the transfer. First, the internal structure of the biofilm should be broken down. So the cells can detach from the first surface. Then, the adhesion between the cell and the second surface should occur (78). There is a transfer model showing the importance of the mechanical properties of the biofilm and the interaction forces between the biofilm and surfaces. When the two surfaces are in contact, the biofilm will be compressed. As a result, there will be shearing force and normal stress among the biofilm structure. These stress will help collapse the biofilm. When the two surfaces get apart, the tensile stress between the biofilm and receptor surface would contribute to the dislocation of the biofilm (78,79, 80).

In many surface-to-surface transfer studies, the hydration state of the biofilm was found to be an important factor that affects the transfer efficiency of the biofilm (80). From the formal work in our lab, we proposed that there are two factors causing the increased transfer from dried *Listeria* biofilms (50, 51, 52). First, the drying will decrease the cell-to-cell and cell-to-surface adhesion forces. Second, if the moisture in the food is higher than in the biofilm, the efficiency of transfer of dried cells will increase due to capillary forces. We also believed that drying of *Listeria* biofilms weakens adhesion forces to surfaces or the cell-to-cell adhesion forces and proposed this to be one of the mechanisms of biofilm detachment and propagation to cause the spreading of bacterial throughout the processing environment (50, 51, 52).

1.10 Conduct scan-induced abrasion to measure cohesive energy

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In order to measure the cohesive energy of biofilm, researchers have developed a proper method by using Atomic Force Microscope. They took a proper size of nonperturbative topographic image of the biofilm at low applied setpoint (1 nN). Then they zoomed into a specific smaller subregion and repeated raster scanning at a much higher load for specific times. Then the non-perturbative image of the original biofilm region was taken. This process was repeated several times within the same region. With the topographic changes of the biofilm during raster scanning, the average depth was measured. This whole process was repeated in order to confirm the reproducibility of this method.

CHAPTER II

OBJECTIVES

The general objective of this project will be to understand the environmental and biological factors associating with *Listeria monocytogenes* biofilm detachment from food processing surfaces.

Objective 1: Study the macroscopic factors which affect the efficiency of transfer of adhesive *Listeria monocytogenes* from simulated food processing surfaces materials under for different relative humidities to a targeted food sample (cold smoked salmon fillet)

Objective 2: Study the cohesive characteristics of biofilm of *Listeria monocytogenes* using Atomic Force Microscopy at different relative humidities.
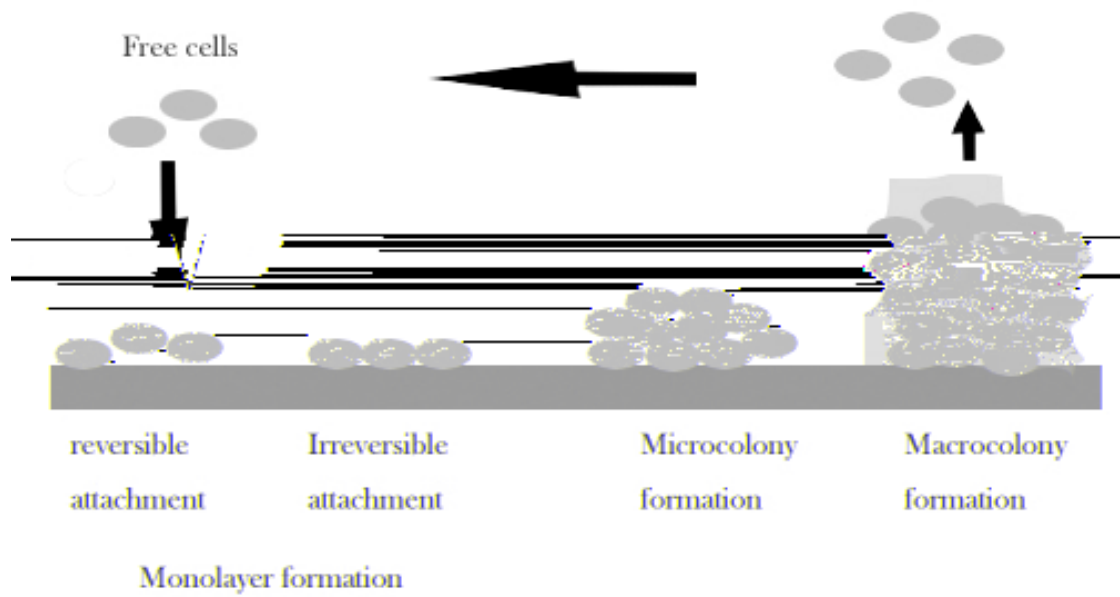
Figure 2.1 Bacterial biofilm development. Diagram adapted from
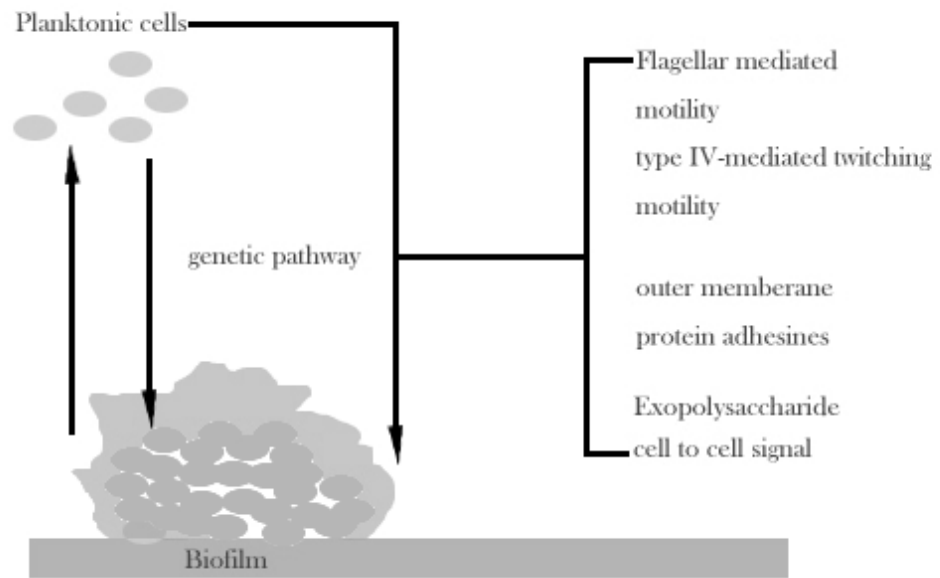McLandsborough et al (36).

Figure 2.2 Overview of factors important in biofilm formation. Diagram adapted from Pratt et al (47).

CHAPTER III

CHARACTERIZE THE INFLUENCE OF RELATIVE HUMIDITY TO THE 'EFFICIENCY OF TRANSFER' OF ADHESIVE *LISTERIA MONOCYTOGENES* TRANSFER FROM STIANLESS STEEL AND HDPE TO COLD SMOKED SALMON

3.1 Introduction

Listeria monocytogenes is always a serious safety issue for food processing industry due to its ability to survive in forms of biofilm during the food processing environment. Many kinds of food products have been reported associating with *Listeria* outbreaks, including ready to eat meals and hotdogs (26, 64, 44, 42, 34, 45). Many scientists have been investigated into *Listeria monocytogenes* biofilm formation in food processing equipment surfaces. Results have been published regarding listerial transfer from processing surfaces to food samples and vice versa (38, 55, 27, 1). The hydration level, surface roughness, contact pressure and duration time have already studied well on some ready-to-eat meat samples (38, 55, 27, 1). But recent researchers have paid little attention on the transfer properties by using cold smoked salmon fillet as the food sample. In our lab, Andres Rodiguez used to use bologna and hard salami as the food samples to investigate the factors which affect the *Listeria monocytogenes* biofilm efficiency of transfer (EOT). The objective of this study was to characterize the influence of relative humidity on the transfer of adhesive *Listeria* monocytognes cells from simulated food processing surfaces to cold smoked salmon fillets.

3.2 Materials and methods

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3.2.1 Cold smoked salmon fillet

Cold smoked salmon fillets were purchased from the regular grocery store once a week. In order to minimize the effect from cold smoked salmon fillet condition, the fillets were purchased fresh for every round of experiments. Cold smoked salmon fillets were cut into rectangle shape with sterilized knives. Each fillet were kept in sterilized culture dish.

3.2.2 Slide preparation

Stainless steel coupon (type 304 with a 4b finish), high-density polyethylene (manufactured by Amherst Machine, Amherst, Mass) was used as imitation of food processing surface material during the experiment. The total surface area of the stainless steel coupon was 54 cm2 with a contact-transfer surface area of 5.4 cm2. The total surface area of the high-density polyethylene (HDPE) was 29.8 cm2 with the contact surface area of 7.7 cm2. In order to remove the grease, the stainless steel and HDPE were soaked in acetone for 10 minutes and rinsed with distilled water. Then the slides were cleaned with ethanol and rinsed thoroughly with distilled water. At last, all the slides were kept in the boiling distilled water for 10 minutes. Before the experiment, all the slides were kept individually in 50 ml centrifuge tubes and autoclaved for 20 min at 121 °C (50, 52).

3.2.3 Bacteria strains

Four strains of *Listeria monocytogenes* (LM10, LM21, LM27, and LM29) were stored under -75 °C in TSBYE with the glycerol to a final concentration of 12.5%. Frozen TSAYE slants were prepared monthly. The culture was transferred to the slants and

incubated under 32 °C for 24 hours and stored under 4℃. Before each experiment, the four working cultures were transferred from the slants to 10 ml of TSBYE. The incubation was under 32°C for 18 hours (50, 52).

3.2.4 Bacterial cells attachment and transfer slides preparation

Attached cells on stainless steel and high-density polyethylene surfaces were prepared. Four *L. monocytogenes* strains (LM10, LM21, LM27 and LM 29) were incubated individually in TSBYE medium for 18 hours at 32 °C. After 18 hours' incubation, a mixture was made by adding 1% of the 18 hours' growth from LM10, LM21, LM27 and LM29 to 1 liter of TSBYE.   Thirty milliliter (30 ml) this mixture was added to each 50 ml centrifuge tube containing a clean, sterilized stainless steel coupon/high-density polyethylene. All the stainless steel and high-density polyethylene slides were soaked in the bacterial mixture for 5 minutes under room temperature then moved to clean and sterilized 50 ml centrifuge tubes for later use (50, 52).

3.2.5 Relative Humidity control

In order to equilibrate the attached cells on the surfaces with different relative humidity (%RH), four saturated salt solutions at 20 °C (sodium chloride (NaCl) 75% RH, potassium sulfite (K_2SO4) 98% RH, magnesium chloride $Mg(NO_3)_2$ 54% RH, and magnesium nitrate $MgCL_2$ 33% RH, Sigma-Aldrich, St. Louis, Mo.) were used. The inoculated slides with attached bacterial cells were equilibrated over salt solutions 98, 75, 54 and 33% RH) at 20 °C in the walk in incubator in desiccators for 24 hours (50, 52).

3.2.6 Transfer experiment

Transfer equipment named QTS Materials Evaluation System (Brookfiled Engineering, Middleboro, Mass) was used to provide constant pressure (18 kPa) and contact time (30 min) between the inoculated slides surfaces and the food samples. The inoculated stainless steel and high-density polyethylene slides were held by the custom cell and brought into contact with the surface of cold smoked salmon fillet. After specific time of contact, the cold smoked salmon fillets were moved to stomach bags which contain 30 ml of buffer peptone water. The bags would be agitated by stomacher for 1 min on high speed mode (Seward stomacher blender, UK). Then the samples were plated on TSAYE with proper dilution by using spiral plater (Spiral Biotech, Norwood, MA.). All the plates were incubated at 32°C for 24 hours in a walk in incubator. The plates were counted by Q-count (Spiral Biotech, Norwood MA) with the unit of CFU/ml. Then the transferred cell levels with the unit of CFU/cm2 area were calculated by the contact surface of the inoculated stainless steel and high-density polyethylene slides. For each experimental replication, sterile stainless steel was used as negative controls to make sure there was no contamination on the original slides and the cold smoked salmon fillet samples

For this transfer experiment, we wanted to get the data for efficiency of attached cell transfer (EOT) from slides surfaces to food samples. (50, 52) We needed to know the original amount of *Listeria monocytogenes* on the stainless steel and high density polyethylene slides before the transfer experiment. Additional stainless steel and high-density polyethylene slides were treated similarly as the transfer slides. All these slides were kept in a 50 ml of centrifuge tube with 30 ml of buffer peptone water and 3 grams of glass beads with diameter of 710-1180 microns (Sigma-Aldrich). After 1 minute strong shaking, cells were removed from the inoculated slides surfaces into buffer

peptone water. They were spiral plated immediately on TSAYE. The original cell level
(CFU/cm2) on the slide surface was calculated.

$$\text{EOT} = \frac{cell.level.transferred.to.food.sample}{original.cell.level.on.the.control.slides}.$$

3.2.7 Experimental plan and data analysis

The whole experiment was repeated 7 times, and each parameter of the transfer experiment was performed in duplicate. The data were collected and used to calculate the EOT value. Then all the data were organized in Excel and analyzed by SAS Professional Statistical Analysis Software (SAS Institute, Cary, N.C.). Because of heterogeneous variance, the EOT data were log transformed before the statistical analysis.

3.3 Results and Discussion

The objective of this research is to investigate the affection of *Listeria monocytogenes* attached cells %RH on the transfer of biofilm from stainless steel and high-density polyethylene to cold-smoked salmon slices   In this experiment, we used cold-smoked salmon. In order to equilibrate the attached cells on stainless steel and high-density polyethylene slides with different RHs of 33% 54% 75% 98%, each set of slides were placed in a desiccators with 4 saturated salt solutions at 20 ℃ for 24 hours. Formal research in our laboratory has imaged *Listeria monocytogenes* in this situation with Atomic Force Microscope (51). Results show that *Listeria* does not colonize the entire stainless steel surface. It grows as individual cells or microcolonies with EPS on the slide surface (51).

In this research, we found that there was a similar trend in that greater transfer was observed from stainless steel as the adherent cells were eqiuilabrated at lower %RH (Fig 3.1), however the differences between the different relative humidities and surface conditions (stainless steel and HDPE (Fig 3.1 and 3.2) were not statistically significant. We observed variation in texture and moisture condition of the cold smoked purchased from a local grocery store. There was variability in between packages, brands and over the course of storage after opening, and likely contributed to the variability of transfer observed in this set of experiments.

Previous work used bologna salami and American cheese (50, 51, 52) which are food products that are much more consistent composition and texture, thus we were able to observed increased transfer with decreasing relative humidity (50, 51, 52). In a previous study (50, 52), our lab observed that the % RH of the biofilm grown on slides is critical in forming capillary forces that hold the biofilm together. This was also observed with bacterial cells dried from stainless steel to bologna (manuscript in preparation). However this supposition can't explain the differences in EOT between bologna (mean EOT = 3) and hard salami (mean EOT = 0.35; P < 0.01) (50). Due to the different composition and moisture levels between bologna and hard salami, we have a hypothesis that the water in the food may create a liquid bridge or "capillary neck" between the dried biofilm and the food. Therefore, the water in the food may be the major parameter in the biofilm transfer (50). From the past research (50), we proposed that if the moisture in the food is higher than in the biofilm, the biofilm transferred to food will be increased (50). This observation was not confirmed using salmon fillets. However, we believe this may be due to the inherent variability between fillets and packages of salmon fillets.

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Therefore, any differences in transfer due to different relative humidity's may be less than differences in transfer due to product variability.

Table 3.1 *L. monocytogenes strains*

L. *monocytogenes* Lab designation no.	CU ISOLATION No.[a]	Lineage	Source
LM10	FSL-N1-304	II	LAB STOCK
LM21	FSL-J1-225	I	LAB STOCK
LM27	FSL-C1-109	I	LAB STOCK
LM29	FSL-C1-122	I	LAB STOCK

[a] **Strains were obtained from M. Wiedmann, Cornell University (CU)** (77)

Figure 3.1 Effect of Relative Humidity of *L. monocytogenes* dried films on stainless steel slides on efficiency of transfer to cold smoked salmon. Error bars represent the standard error of the mean (n=6). No statistical differences were observed.
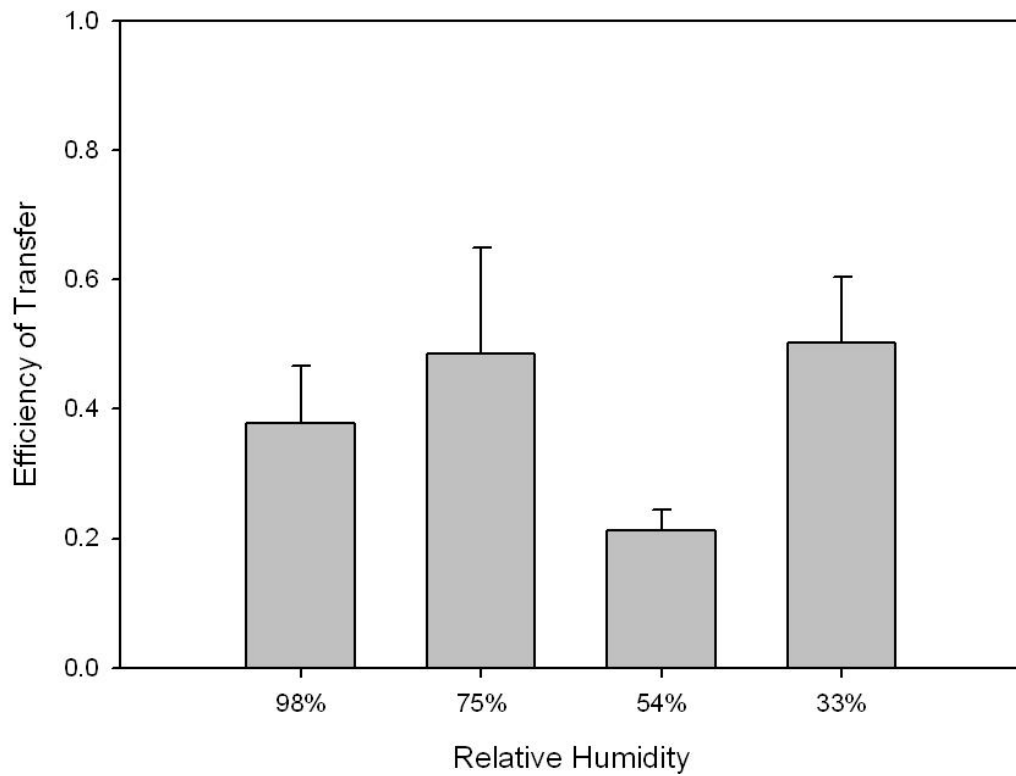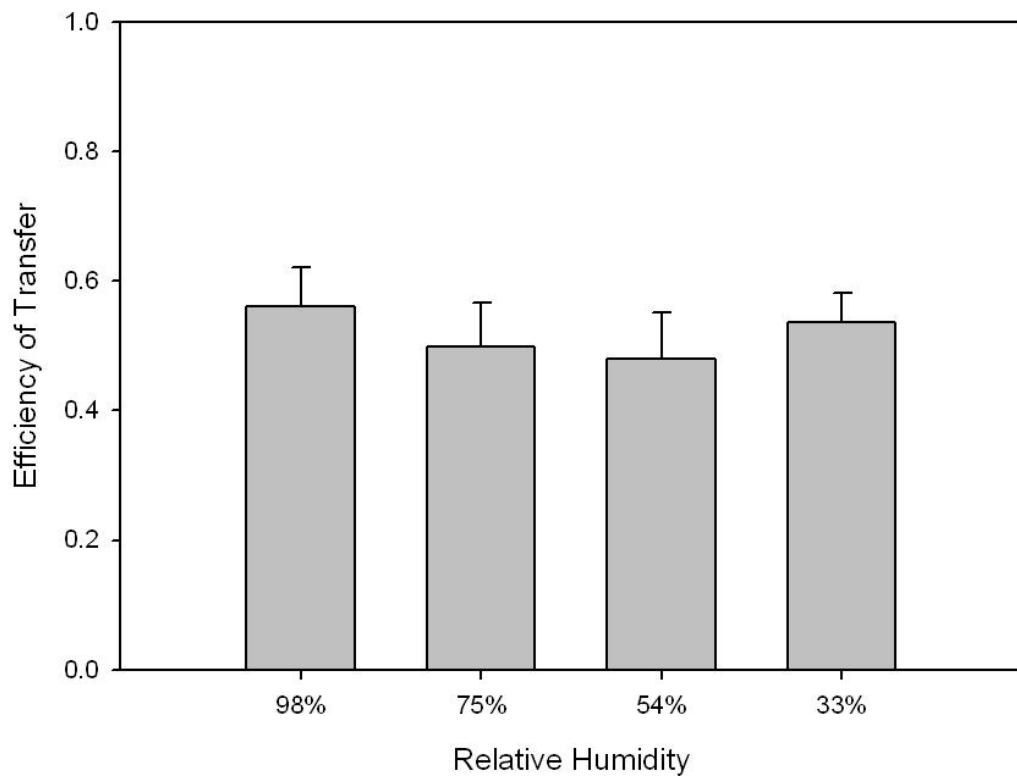
Figure 3.2 Effect of Relative Humidity of *L. monocytogenes* attached cells on HDPE slides on efficiency of transfer to cold smoked salmon. Error bars represent the standard error of the mean (SEM) (n=6).

CHAPTER IV

COHESIVENESS MEASUREMENT OF *LISTERIA MONOCYTOGENES*

BIOFILM BY USING ATOMIC FORCE MICROSCOPE

4.1 Introduction

It is important to understand all aspects of biofilms in order to better control the growth of desired biofilms and eliminate the undesired biofilms. Many publications have been reported the factors that contribute to the formation of bacterial biofilm, however the principles of detachment of cells from biofilm has not been well investigated.

Atomic Force Microscopy (AFM) is a very high resolution type of scanning probe microscopy. The AFM is one of the foremost tools for imaging, measuring and manipulating matter at the nano-scale. Francois Ahimou have reported a method for measuring the dissipation of frictional-energy on moist bacterial biofilm during abrasion via a raster-scanned tip under an elevated load by using Novel Atomic Force Microscopy (9, 32, 54). They also quantified the volume of detached biofilm via before/after topographic image comparisons. This method has been shown to be a reproducible method to assess the "micro-rheological" characteristics of a hydrated biofilm. Atomic Force Microscope has been used to image and measure the frictional forces of different material surfaces (81). With the frictional response collected from the repeated scanning with variable loads by using AFM, researchers can get the information on the viscoelastic and viscoplastic properties of a material. In the past, some AFM studies have shown some biofilm properties under a dried conditions Then researchers developed a reproducible method to use Atomic Force Microscope to measure the cohesive energy under aqueous condition. They used Atomic Force Microscope to apply groups of raster

scanning under an elevated loading force. The volume of detached biofilm will be quantified by comparing the topographic change of the images which are taken by the AFM (81). In order to calculate the cohesive energy of biofilm, the frictional energy dissipation that does not contribute to biofilm displacement but being instead lost as heat (W_h) should be collected by AFM.

The objective of this work is to use AFM to measure the cohesive energy of *L. monocytogenes* biofilms on stainless steel slides and to evaluate the influence of water upon cohesive energy.

4.2 Material and Methods

4.2.1 Bacterial strains

For this study, *L. monocytogenes* Scott A (lab designation LM21) was selected to product a single strain biofilm study. Cultures were stored under -75 ℃ in tryptic soy broth with 0.6% yeast extract (TSBYE) with the glycerol to a final concentration of 12.5%. Working slants were prepared from frozen stock monthly. The culture was transferred to a trypic soy agar slant with 0.6% yeast extract (TSAYE) and incubated at 32 ℃ for 24 hours and then stored at 4 ℃. Before each experiment, the working culture was transferred from the slant to 10 ml of TSBYE. The incubation was under 32 ℃ for 18 hours.

4.2.2 Drip flow bioreactor

A low shear biofilm was grown in a modified drip flow bioreactor (model DF 202, BioSurface Technologies Corp., Bozeman, MT). The modified Welshimer's broth (MWB) is used as the growth medium for the biofilm formation. Before the experiment, the drip flow bioreactors and rest of the accessories are installed and autoclaved for 20 minutes. After 18 hours incubation at 32 ℃, 1 ml of LM21 inoculum is transferred into each channel with 15 ml of MWB and stainless steel slides in it. The reactor is pre-cultured for 24 hours under 32 ℃. After the initial adhesion stage, continuous flow was started after 24h by placing the drip flow reactor on a stand with 10° angle and pumping MWB through at a flow rate of 0.75 ml/min per channel for 48 hours at 32 ℃ (22, 23, 53).

4.2.3 Biofilm distribution and confocal imaging

Before we started the AFM experiment, a proper distribution of *Listeria monocytogenes* biofilm for the detachment experiment was necessary. In order to visualize the distribution of *Listeria monocytogenes* biofilm produced in Drip Flow Bioreactor, we used SYTO9 Green to stain the bacterial on biofilm. SYTO9 Green stain (2 μL) was mixed thoroughly with 1 mL distilled sterilized water. The diluted stain was added to biofilm grown on stainless steel slides for 5 min in the dark. Sterilized water was used to gently wash the slides twice. The stained slides were kept in dark before confocal image..

4.2.4 Slides preparation for AFM imaging and cohesiveness measurements

After growth at 32 C for 72 hours, stainless steel slides containing biofilms were placed in desiccators containing saturated salt solutions and allowed to equilibrate for 48h under 20 C. (sodium chloride (NaCl) 75% RH, potassium sulfite (K2SO4) 98% RH, magnesium chloride Mg(NO3)2 54% RH, and magnesium nitrate MgCL22 33% RH, Sigma-Aldrich, St. Louis, Mo.).

4.2.5 Atomic Force Microscopy

All AFM experiments were performed with a Veeco CP-II Atomic Force Microscope with a large area scanner (100μm) in conjunction with a AFM image processing software. Images of topography (height in nanometers) and friction force (raw units of volts) were collected as the tip was scanned across the sample surface under feedback-maintained constant vertical deflection of the cantilever (in nanometers, converted to applied loads via multiplication by the calibrated cantilevers). Rectangular shaped cantilever supplied by '*TED PELLA, INC.*'. The scan velocity was in the range of 5μm/s to 10μm/s.

4.2.6 Cantilever calibration

In order to accurately measure Lateral Force Microscope (LFM) data from the deflection of the AFM cantilever, the actual spring constant of the cantilever was needed. The 'Reference cantilever method' (84) was used measure the actual spring constant of the cantilever used for each in the raster scanning experiment. To do this, reference cantilevers were purchased (Veeco) and mounted onto a magnetic AFM sample disk. The reference cantilevers each came with a calibrated spring constant (Kref). The cantilever to be calibrated was placed close to the reference cantilever (Fig 4.1A). The cantilever to be calibrated was then moved onto the hard surface of the AFM chip and the

deflection sensitivity was measured by using contact mode (S_{hard}) (Fig 4.1B). To measure the deflection sensitivity on the reference cantilever (S_{ref}) the cantilever was aligned with the reference cantilever so that and the deflection sensitivity was measured using contact mode (Fig 4.1C). The length of the calibration cantilever (L) and the offset between the tip of the cantilever and the end of the reference cantilever (ΔL) was measured using image analysis and the following equation was used to calculate the actual spring constant of each cantilever.

$$K_{final} = K_{ref} \times \frac{(S_{ref} - S_{hard})}{S_{hard} \times (1 - \Delta L/L)^3}$$

(1)

AFM images representing the actual condition of 5μm x 5μm and 2.5μm x 2.5μm biofilm region were taken with the calibrated cantilever

4.2.7 Frictional Force calibration

After the calibration of the AFM cantilever, a pre-cleaned AFM calibration grating was used to measure frictional force. Measurements were made during multiple experiments and with multiple cantilevers under identical conditions before and after each biofilm abrasion experiment to ensure that the AFM probe state was unchanged during the course of each experiment. This was done by gradually increasing loading force on a 2.5X2.5 um region on Silicon wafer surface. In all cases, the plot of raw friction force (volt) versus the loading force of the probe (nN) was well reproduced by a linear fit (Fig 4.4). The slope of the linear plot ($m_{silicon}$) was assumed to be equal to the 'to be determined' apparatus coefficient multiplied by the actual dimensionless friction coefficient obtained ($\mu_{silicon} = F_f/F_n$ (0.19±0.1) averaged from data obtained by Buenviaje

etal. (82) and Putman et al. (83). F_f is the frictional force and F_n is the total normal force due to applied and additive adhesive loads. So, the frictional force collected from the experiment was converted from volt into nN by using following equation:

$$\mu_{silicon} = F_f/(m_{silicon}/0.19) \quad (2)$$

4.2.8 Raster scanning acquisition

In order to image and measure the cohesive energy, a contact mode rectangular cantilever was used. *Listeria monocytogenes* biofilms on stainless steel were placed in different saturated salt solutions to control the %RH of the biofilm. Using AFM, topography data of 5 µm X 5 µm biofilm region at a low applied (~0nN) loading force were collected. After finding an appropriate area with confluent biofilm growth, a 2.5 µm X 2.5 µm sub-region was re-imaged and repeated raster scanning at with a high pressure load of 100nN . This high load raster scanning was repeated four times then, the loading force was reduced to ~0nN to data from the original 5 µm X 5 µm region was collected to evaluate the influence of the high pressure abrasion and then a second series of four 2.5 µm X 2.5 µm high pressure load raster treatments. The changes in topography after each raster treatment series was determined by subtraction to obtain the height reduction of the sub-region after four raster abrasions. The entire process was repeated five times within a specific biofilm region with 20 raster scans under high load. AFM image processing software was used to measure the mean height reduction of the 2.5 µm X 2.5 µm subregion (81). To determine the total frictional energy dissipated during one group of raster abrasion (W_T) the following equation was used:

$$W_T = 2dnxF_f \quad (3)$$

Where (d) represents the length of each scan line,(n) number of scan lines per raster, (F_f) is the frictional force (as determined experimentally) and (x) is the number of raster abrasion scans. In order to obtain the energy dissipation that did not contribute to the displacement of biofilm (W_h), the biofilm sample was scanned under increasing applied loads (81). We observed displacement only occurred at 10 nN. So the frictional energy dissipation that produces biofilm displacement during each raster scan was calculated by subtracting the value measured during the first scan. W_h is given by the following equation. (F_A : frictional force when biofilm displacement does not occur)

$$W_h = 2dnF_A \quad (4)$$

The cohesive energy (ε_{coh}) will be calculated using following equation, where V is the volume of biofilm displaced per group of raster abrasion. W_T is the total frictional energy dissipation during a succession of raster scans (81).

$$\varepsilon_{coh} = (W_T - W_h)/V \quad (5)$$

4.2.9 Volume of displaced biofilm material

In order to determine the volume of displaced biofilm material during AFM scanning, the nonperturbative 5X5µm topographic images, each following four raster abrasions, were subtracted to obtain the topographic changes that had occurred during the four scans at high loads (81). The average depth of abrasion was measured from each different image by using Image Processing software. The volume of displaced biofilm was calculated by multiplying the mean height reduction by the raster scan area.

4.2.10 Raw friction force collection

The raw friction force with the units of volts was determined from one-half of the difference between retrace (right to left) and trace (left to right) 512X512 pixels LFM images. Friction force was measured by using Image Processing software. All friction peaks were fit with a Gaussian distribution to determine the mean values (81).

4.3 Results and Discussion

In order to perform AFM on biofilms, it was important to determine the appropriate conditions of biofilm growth to obtain biofilms with areas of confluency greater than 5 μm x 5 μm. Initial experiments were performed with confocal microscopy to screen for conditions. Biofilms grown on stainless steel in a drip flow reactor at 32C for 72h produced the greatest cell coverage of stainless steel (Fig 4.2). The structure observed was similar to other research in our laboratory: *L. monocytogenes* does not colonize the entire stainless steel surface. Instead it grows as cells or microcolonies with EPS on slides surfaces (51). However, the microcolony surface coverage under these conditions were great enough to easily find areas of confluence that was large enough to allow a 5 μm x 5μm area of growth. These conditions were selected for AFM analysis.

The objective of this research is to investigate the effect of %RH on cohesive energy of *Listeria monocytogenes* biofilms developed on stainless steel coupon using AFM. Initial work was done to optimize imaging of *L. monocytogenes* biofilms contact AFM (Fig 4.3 A and B). Images typically showed biofilms developed with what appears to be a coating of extrapolymeric substances, in agreement with previous AFM imaging performed in our laboratory (51).

The method used in this research is a well-established method to determine the cohesive energy of biofilm over a defined volume of material that is removed using AFM (81).The advantage of this method is that it incorporates all of the energy contributing to the displacement of the biofilm in the calculation of cohesive energy, in a reproducible manner..

Prior to measuring biofilm cohesiveness, all cantilevers were calibrated with 'Reference Cantilever method before each experiment (Fig 4.1).   In order to measure biofilm cohesiveness, a 5 µm X 5µm biofilm region was scanned by AFM using low loading pressure (Fig 4.4A) and then a series of high load raster scanning was then performed on a smaller region with an area of a 2.5 µm X 2.5 µm (Fig 4.4b). The changes in topography after each raster treatment series was determined by subtraction of the 5x5 µm biofilm region to obtain the height reduction of the sub-region after four raster abrasions (Fig 4.4C).   An example of images obtained from biofilms after equilibration at 33% RH can be seen in Fig 4.5. After the first raster scans, the a 2.5 µm X 2.5 µm area of scanning becomes visible, and becomes more defined after each 4 raster scan (Fig 4.5A). These images present that the depth of abrasion increased with raster scan number. The cumulated biofilm displaced volume was plotted against the scan number (Fig 4.5B). The mean volume displacement per scan of *Listeria monocytogenes* biofilms which were stored at 33% RH was 0.16 µm^3.

In order to determine how much of the cohesive force was due to frictional force (Fa), the frictional force was measured after the calibration of AFM cantilever on a pre-cleaned AFM calibration grating. Measurements were made during multiple experiments and with multiple cantilevers under identical conditions, before and after each biofilm abrasion experiment to ensure that the AFM probe state was unchanged during the course of each experiment. This was done by gradually increasing loading force on a 2.5x2.5 µm region on Silicon wafer surface. In all cases, the plot of raw friction force (volts) versus the loading force of the probe (nN) was well reproduced by linear fit (Fig 4.6).

Once raw data were collected, friction force (nN) measurements were converted from the raw friction force (V). The raw friction force with the units of volts was determined from one-half of the difference between retrace (right to left) and trace (left to right) from 512X512 pixel LFM images (Fig 4.7). The volume of biofilm material, the energy contributing to the biofilm displacement and the cohesive energy were calculated (Table 4.1). The values of cohesive energy of *Listeria monocytogenes* biofilm developed under 33%RH were plotted as functions of scan number (Fig 4.8). The cohesive energy value of the biofilm increased while the scan number increased. This could indicate that outer EPS layers of *Listeria monocytogenes* biofilm are more loosely associated with one another, while the deeper biofilm matrix materials are more tightly associated with each other and therefore have higher cohesive energy value. This phenomenon was also observed in other researchers' study in biofilm cohesiveness with AFM (81).

In order to evaluate the effect of different relative humidity's on the cohesiveness of *L. monocytogenes* biofilm which were developed on stainless steel, biofilm were equilibrated respectively with 33%, 54%, 75% and 98% RH. After 20 times of high load raster scanning, a topography image of the 5 μm x 5 μm biofilm range were collected and the linear analysis was conducted individually for each image (Fig 4.9). The biofilms equilibrated at 33% RH showed cell removal and allowed for calculation of cohesiveness, however, the biofilm that were equilibrated at 54%, 75% and 98% RH barely showed any of the biofilm height reduction (Fig 4.9 B, C and D). This small amount of height change at is within the error of the instrument or may have due to due to removal of EPS. Since no volume displacement occurred, the cohesive energy of the biofilms at higher relative humidity's could not be calculated. However, this does indicate that the cohesive energy

required to remove biofilm materials with higher moisture levels was greater than we could put into the system using our AFM.

From these results, we can clearly see that RH% plays an important role in effecting the cohesiveness of *Listeria monocytogenes* biofilm growing on stainless steel surfaces. Previous *Listeria monocytogenes* biofilm transfer experiment in our lab showed a similar phenomenon that transfer number increased with decreasing relative humidity (50, 51, 52). In addition, a better way of controlling the development level of *Listeria monocytogenes* biofilm and its relative humidity during the experiment is critical for improving the consistency and precision of our experiment.

Such research in the future can improve our understanding of *Listeria monocytogenes* biofilm cohesiveness properties and help to design new strategies for manipulating *Listeria monocytogenes* biofilm development.

Table 4.1 Summary of results obtained from two independent biofilms grown on stainless steel slides with MWB media.

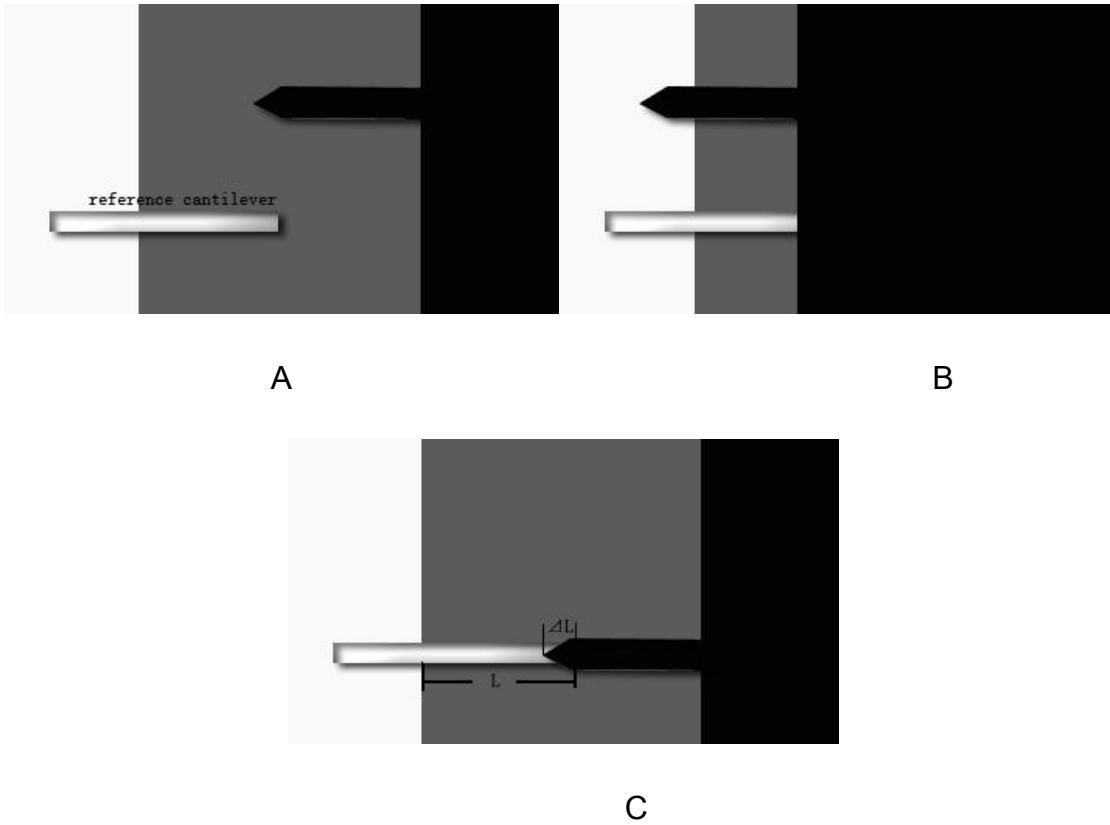| Sample	Scan No.	Vol displaced After 4 raster Scan (μm^3)	Friction Force (nN)	Cohesive Energy ($pJ/\mu m^{3)}$)
	4	0.863125	4.00525	22.001
	8	0.336	3.9437	55.579
Biofilm 1	12	0.562	6.2973	54.67
	16	1.01	4.5921	21.776
	20	0.357	7.5348	103.811
	4	1.055	1.3208	5.851
	8	0.71875	1.7622	11.733
Biofilm 2	12	0.56075	2.7743	24.28
	16	0.4013125	3.736	46.195
	20	0.3715625	3.9096	52.286

Figure 4.1 Reference cantilever procedure. (A) Initial alignment reference
cantilever. (B) Deflection sensitivity on hard surface. (C) Measurement on
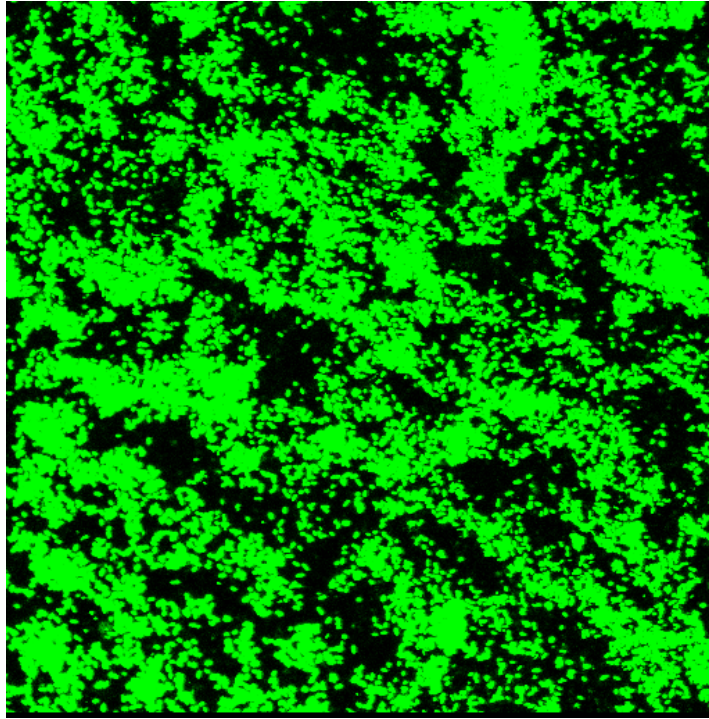reference cantilever

Figure 4.2 Confocal microscope picture of 72 hours *Listeria monocytogenes* (LM21) biofilm on stainless steel coupon which is stained by SYTO 9 green fluorescent nucleic acid stain.
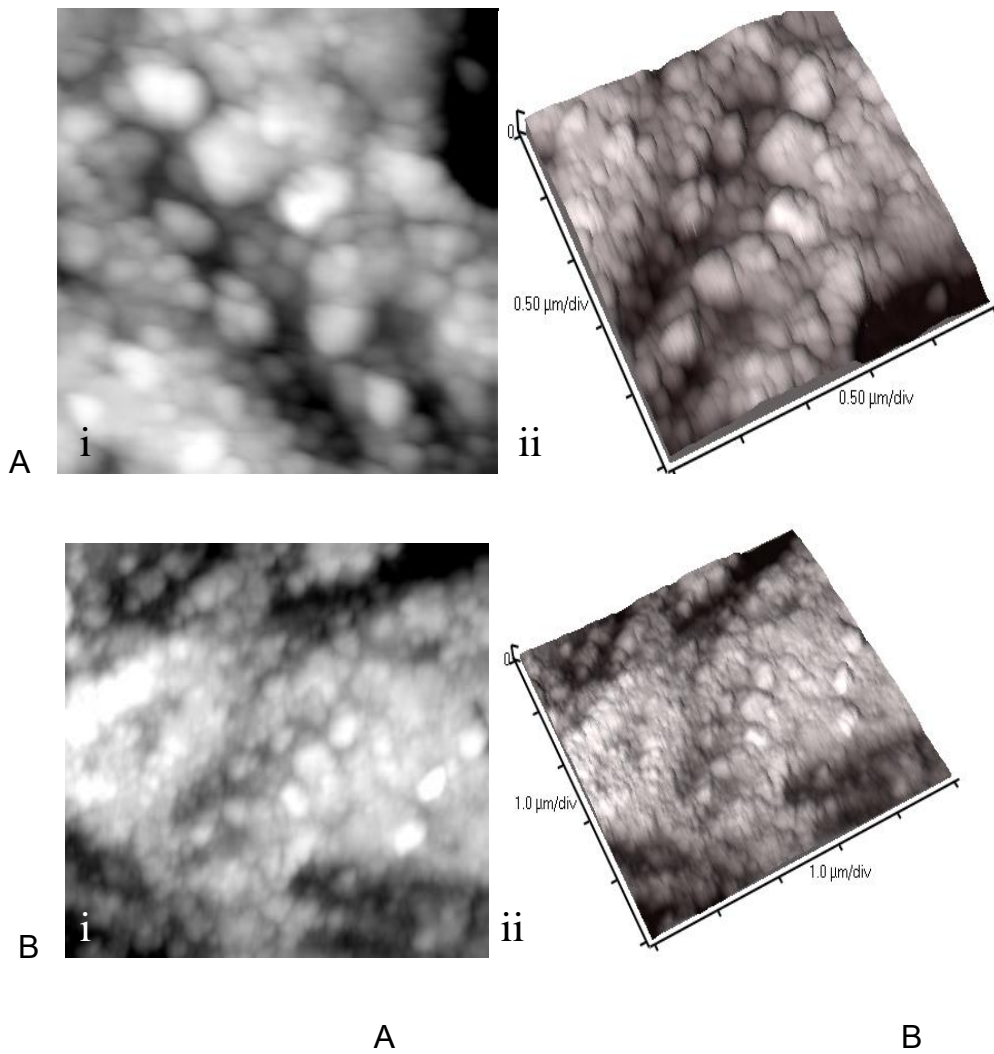
Figure 4.3 Representative AFM topography images of 72 hours *Listeria monocytogenes* (LM21) (A) biofilm in a range of 5 µm X5 µm. (B) f 2.5 µm. X 2.5 µm. (i) height data with lighter points being higher, (ii)3-D representation of height data
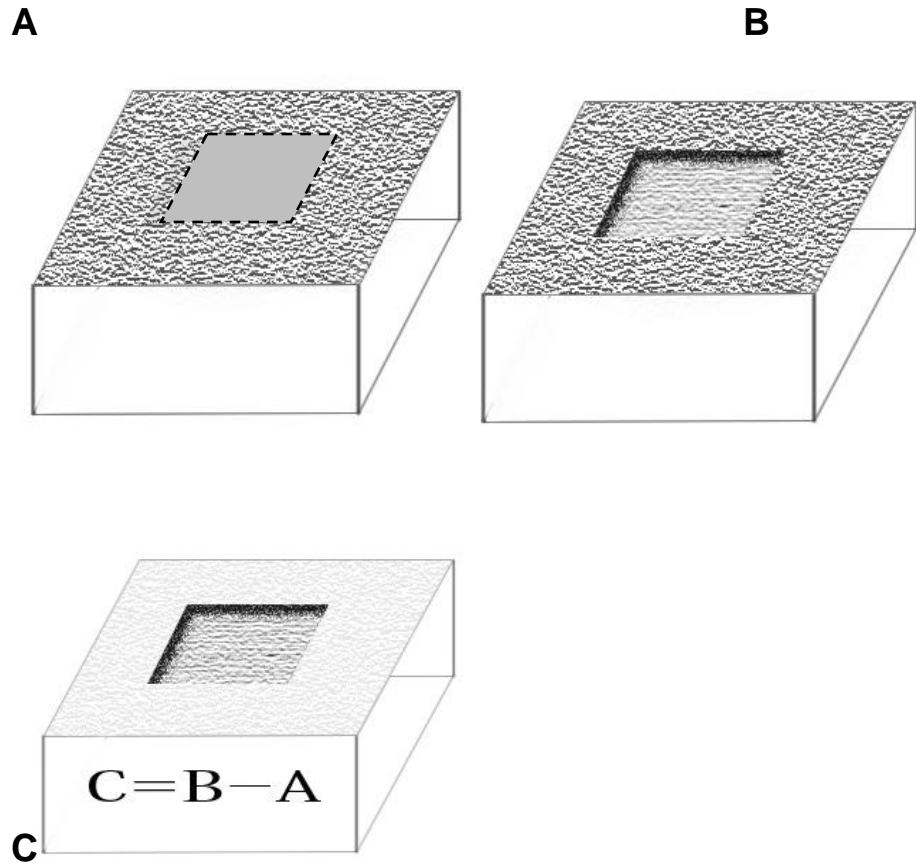
Figure 4.4   Nonabraded biofilm surface topography (A), abraded biofilm (B), and the resulting image after subtraction (C)

A

| 0 scan | after 4th scan | after 8th scan |

after 12th scan   after 16th scan   after 20th scan
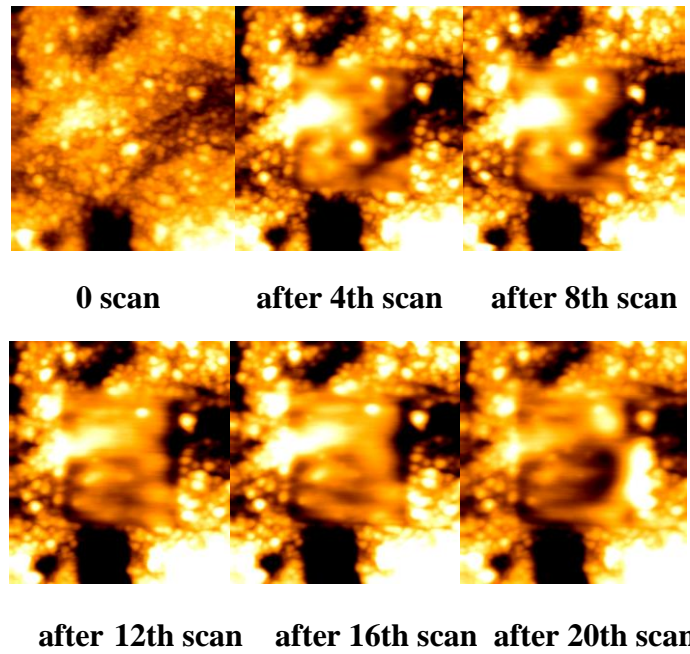
B
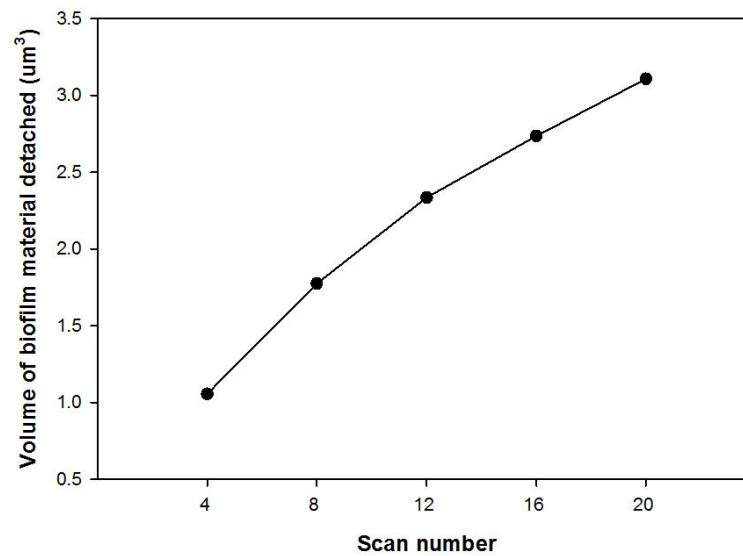
Figure 4.5 (A) Successive topographic images exhibiting a 2.5X2.5 μm abraded biofilm region via a raster-scanned cantilever of a biofilm equilibrated at 33%RH. Images were collected from left to right at an ~0nN loading force. (B) Cumulative volumes of biofilm material displaced as a function of scan number.

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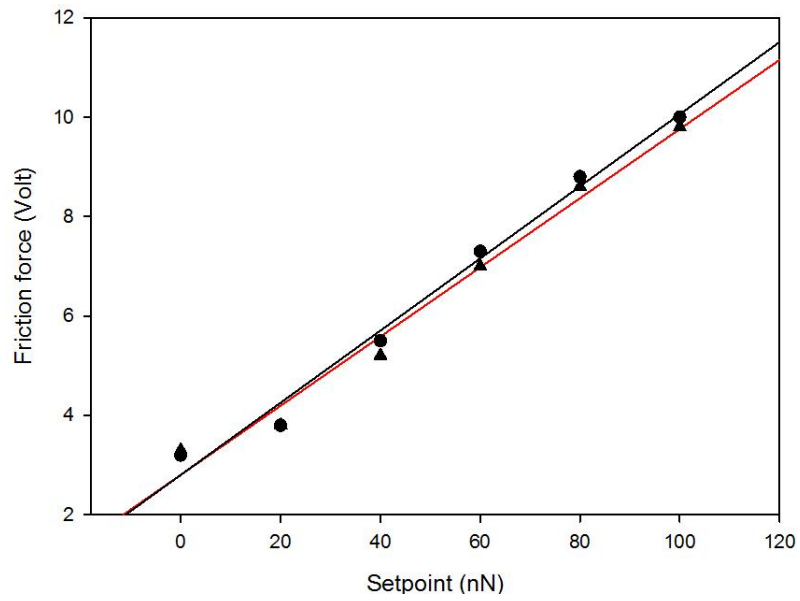
Figure 4.6 A typical friction force calibration curve as a function of applied load on silicon. Triangle symbols, calibration done prior to experiments on biofilm; circle symbols, results obtained after biofilm abrasion.

Trace　　　　　　　Retrace　　　　　½(Retrace-Trace)
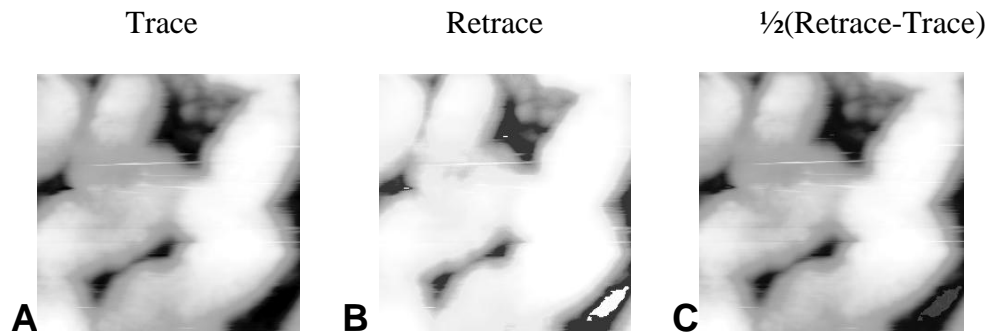
Figure 4.7 Frictional force images of nonabraded biofilm. (A) Image collected from scanning left to right (trace); (B) Image collected from scanning right to left (retrace); (C) LFM image after subtraction.
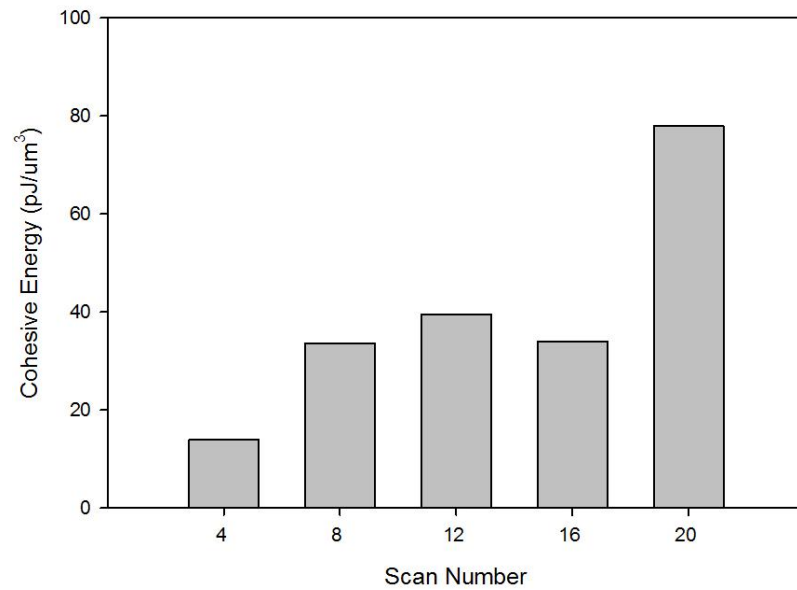
Figure 4.8 Cohesive energy (ε_{coh}) values plotted as a function of scan number.
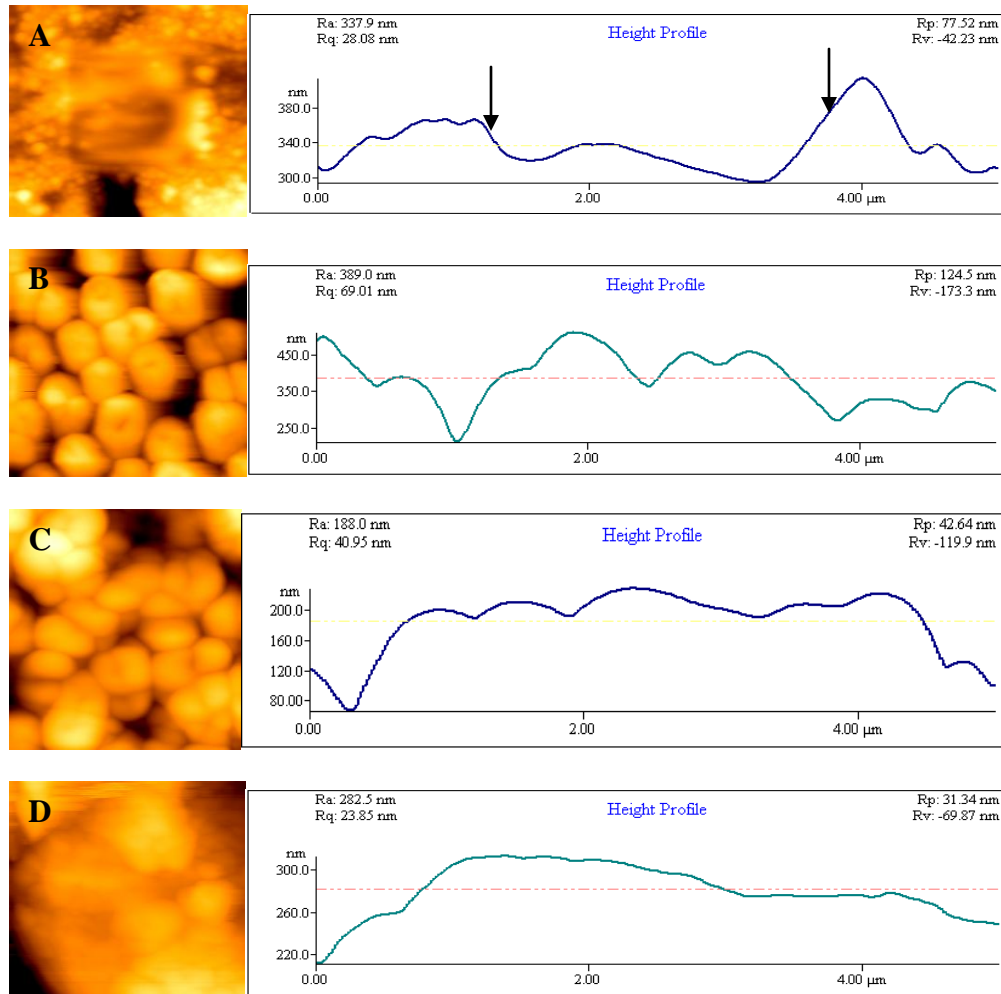
Figure 4.9 Topography of a 5 μm x 5 μm area after a total of 20 high pressure raster scans and linear analysis *of Listeria monocytogenes* biofilms. Each biofilm was equilibrated under 33% (A), 54% (B),   75% (C), or (D) 98% RH for 48 h prior to raster testing. Arrows represent the edge of the 2.5 x 2.5 μm raster scanning area, which was only visible in samples equilibrated at 33% RH (A).

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