ROLES OF TEMPERATURE AND IONIC STRENGTHS OF GROWTH IN BACTERIAL SURFACE BIOPOLYMER PROPERTIES, DLVO AND SPECIFIC INTERACTIONS GOVERNING THE BIOADHESION OF L. MONOCYTOGENES TO SILICON NITRIDE

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
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ROLES OF TEMPERATURE AND IONIC STRENGTHS OF GROWTH IN BACTERIAL SURFACE BIOPOLYMER PROPERTIES, DLVO AND SPECIFIC INTERACTIONS GOVERNING THE BIOADHESION OF L. MONOCYTOGENES TO SILICON NITRIDE

Abstract

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Chair: Nehal I. Abu-Lail, Ph.D.

The effects of surface biopolymers’ properties of L. monocytogenes EGDe on the electrostatic, Lifshitz-van der Waals and steric interactions to a model surface of silicon nitride AFM tip were investigated for the bacterial cells grown at five different temperatures (10, 20, 30, 37 and 40°C) and ionic strengths (0.003, 0.05, 0.1, 0.3, and 0.5M). The higher adhesion was always associated with lower negative cell surface potential and longer surface biopolymer brushes. Transitions in the adhesion affinities, physicochemical properties and the structure of bacterial surface brushes were observed for the cells grown at 30°C and IS of 0.1M. Our results suggested that the lowest long-range electrostatic repulsion which was partially balanced by the
Lifshitz-van der Waals attraction was responsible for the lowest energy barrier to adhesion as predicted by soft-particle analysis of the DLVO theory and the lower adhesion measured by AFM. For the cells grown at various temperatures, the contribution of “specific” forces to the overall adhesion force at the closest surface proximities was investigated. Adhesion forces were decoupled into specific (hydrogen bonding) and nonspecific force components using Poisson statistical analysis. The strongest specific and nonspecific attraction were observed for cells grown at 30°C, compared to those observed for cells grown at higher or lower temperatures, respectively. Our results showed that, irrespective of the temperature of growth investigated, hydrogen bonding forces were always stronger than the nonspecific forces.

Finally, we have investigated the factors that affect the molecular-scale bacterial adhesion process from a thermodynamic perspective at a fundamental level using statistical mechanics. By applying Boltzmann distribution to the probability histograms of bacterial adhesion affinities measured between the cells grown at various ionic strengths and silicon nitride by AFM, the total number of molecules involved in the adhesion process and the entropy of the bacterial surface molecules were estimated. Our results provided a thermodynamic evidence of the direct relationship between the strength of bacterial adhesion and the number of bacterial surface molecules available for interactions and the length of bacterial surface biopolymer brush.
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Dedication

This dissertation is dedicated to my parents:

to my father, Ali Gordesli, and to my mother, Ayse Gordesli.
Chapter 1

Introduction

1.1 Problem Statement and Research Objectives

Understanding the fundamentals of the adhesion processes that take place between bacteria and surfaces is important for many environmental and medical applications ranging from soil remediation\(^1\), marine fouling\(^2\), food and drinking water processing\(^3,4\) to medicine and dentistry\(^5\). Exploring the mechanisms by which a bacterial cell attaches to a surface can improve our fundamental understanding of bacterial adhesion phenomenon which can lead to the development of better strategies to avoid the problems associated with bacterial adhesion. While many researchers focused on studying the ability of bacteria to attach to surfaces and to further develop into biofilms, little has been understood in the critical initial step seen in all biofilm development processes, the initial bacterial cell attachment to a surface. This is in part because most of the studies performed to investigate bacterial adhesion process were carried using macro-scale systems, which are limited in their resolution to disclose the mechanisms that govern the bacterial adhesion process.

The structure of the bacterial surface biopolymer brush layer\(^6,7\), as well as steric interactions\(^8,9\) and nonspecific Lifshitz-van der Waals and electrostatic interactions\(^10-12\), and “specific” molecular interactions\(^13,14\) such as ligand-receptor and hydrogen bonds are important factors involved in the initial cell attachment to a surface. The mechanism by which a bacterium attaches to a surface is mainly influenced by these factors as well as by the chemical and physical natures of the environment and the interacting surfaces\(^15,16\).
The factors involved in the initial bacterial attachment to a surface can be unraveled by using nano-scale methods in combination with a physiochemical approach. Characterization of the bacterial cell and substratum surfaces in terms of their surface potentials quantified from measured electrophoretic mobilities and surface tensions calculated from measured contact angles with liquids of variable wettabilities offers the possibility to calculate the electrostatic and Lifshitz-van der Waals interactions between two entities by using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability. However, probing the steric interactions and the structure of bacterial surface biopolymer brush layer, as well as the “specific” factors which are associated with the molecular recognition phenomena requires high resolution tools. With the introduction of the atomic force microscope (AFM), it has become possible to identify and quantitatively evaluate the molecular characteristics of the bacterial adhesion process at the nanometer length scale.

The recent advances in studying bacterial adhesion by AFM can be summarized mainly in the measurement and identification of the mechanics and thermodynamic characteristics of bacterial surface biopolymers, probing molecular recognition events on bacterial surfaces, and quantification of bacterial interactions with different surfaces including cell-cell interactions. Despite these advances, few studies have attempted to combine experimental and appropriate theoretical and statistical tools in describing bacterial physiochemical surface properties and interactions with surfaces. This avenue of inquiry should provide new insights toward a better understanding of the fundamentals of the adhesion processes taking place between bacteria and surfaces.

The overall goal of my dissertation is to provide an in-depth understanding of the physiochemical and molecular mechanisms of the initial bacterial cell attachment to a surface by
using a model bacterium-substratum system in a well defined environment. For all the papers in this dissertation, pathogenic *Listeria monocytogenes* EGDe was chosen as our model bacterium due to three main reasons. First, *L. monocytogenes* are widely distributed around us and known for their propensity to attach to many different surfaces at different environmental conditions\textsuperscript{16,30-33}. This is in part because *L. monocytogenes* have unique adaptation strategies to environmental stresses such as pH, ionic strength and temperature\textsuperscript{34-53}. *L. monocytogenes* were observed to survive and initiate growth at low pH (pH 4)\textsuperscript{34,46,51} and high ionic concentration (10% v/w NaCl)\textsuperscript{34,39,40,52} in a wide range of temperatures (1-45 °C)\textsuperscript{32-36,38,43}. Second, *L. monocytogenes* are associated with food-borne disease outbreaks that are characterized by widespread distribution and relatively high hospitalization and fatality rates\textsuperscript{32,54}. The economic impacts due to big food-product recalls\textsuperscript{55,56} and the severity of the diseases and the treatment costs\textsuperscript{32,54} caused by *L. monocytogenes* have made this pathogen a major concern for the food industry and the public health. Third, although there have been ongoing efforts by the academia and food industries to reduce the risks of contamination of food processing surfaces by *L. monocytogenes*\textsuperscript{32}, an effective prevention strategy has yet to be developed. Understanding the mechanisms of the interactions between *L. monocytogenes* and the surfaces they attach to will improve the strategies aiming at preventing the attachment of *L. monocytogenes* to food-processing surfaces.

Silicon nitride was chosen as our model inert substratum because it is characterized by similar surface potentials to that of soil and glass\textsuperscript{57}, substrates to which *L. monocytogenes* frequently attach to in nature\textsuperscript{58} and in food industry\textsuperscript{59}. *L. monocytogenes* are primarily soil microorganisms and frequent contaminants of the raw materials and inert surfaces in the food industry environments\textsuperscript{33,38}. Since water is the main solvent used in the food processing industries and the main solvent used in preparing foods\textsuperscript{60}, the mechanisms of the interactions between *L. 
monocytogenes EGDe and silicon nitride were investigated under water. With the use of this model system and atomic force microscopy (AFM) as the primary investigation tool, the first goal of my research (Chapter 2 and Chapter 3) was to understand how physiochemical properties and structure of surface biopolymers of *L. monocytogenes* affect the initial attachment of this microbe to silicon nitride surface in water. The bacterial surface biopolymers were considered as polyelectrolyte brushes since they are charged. Our investigation was carried for pathogenic *L. monocytogenes* EGDe grown at five different temperatures (10, 20, 30, 37 and 40 °C) and five different ionic strengths (0.003M, 0.05M, 0.1M, 0.3M and 0.5M) to understand the adaptation mechanisms of this pathogen to the environmental conditions in which they grow (Chapter 2 and Chapter 3, respectively). This is important because the ability of *L. monocytogenes* to survive and grow over a wide range of environmental conditions such as temperature and ionic strength allows the pathogen to overcome food preservation and safety barriers, and pose a potential risk to human health\textsuperscript{32,33}. The temperature and ionic strength ranges investigated were chosen to bracket the conditions in which *L. monocytogenes* observed to survive and grow in the food processing environments\textsuperscript{3,33,53}.

While the ability of *L. monocytogenes* to attach to a wide variety of surfaces and to survive irrespective of the surrounding conditions has been well documented in the literature\textsuperscript{34-53}, the molecular mechanisms by which bacterial surface biopolymers control the bacterial adhesion to surfaces under various environmental conditions are largely missing. In this respect, our studies presented in Chapter 2 and Chapter 3 provide unique insights toward a better fundamental understanding of the adhesion of *L. monocytogenes* to inert surfaces under different growth stresses of temperature and ionic strength. Our results in Chapter 2 and 3 indicated that the structure and physiochemical properties of surface biopolymer polyelectrolyte brushes of *L.
*monocytogenes* EGDe had strong influences on the initial adhesion of this microbe to silicon nitride surface in water, and were dependent on the temperature and ionic strength (IS) used to grow the bacterial cells. In both of the studies presented in Chapter 2 and Chapter 3, we have observed that higher bacterial adhesion was associated with longer bacterial surface biopolymer brushes and lower negative cell surface potential. Transitions in the strengths of bacterial adhesion, and in the physiochemical and mechanical properties of bacterial surface biopolymers were observed for the cells grown at 30 °C (Chapter 2) and IS of 0.1M (Chapter 3) due to mainly the electrostatic effects as predicted by the soft-particle analysis of DLVO theory. Our results suggested that *L. monocytogenes* grown at various temperatures and ionic strengths should possess different array and/or different amounts of surface molecules which can contribute to the charging of the bacterial surface and the adherence of the bacteria to silicon nitride in water.

To understand the contributions of the fundamental components of the overall interaction force measured as the *adhesion force* between the bacterium and silicon nitride by AFM, we carried the study presented in Chapter 4. The measured adhesion forces between *L. monocytogenes* EGDe grown at five different temperatures (10, 20, 30, 37 and 40 °C) and silicon nitride were decoupled into specific (hydrogen bonding) and nonspecific (electrostatic and Lifshitz-van der Waals) force components using Poisson statistical analysis\(^{61,62}\). Our results showed that, irrespective of the temperature used to grow the bacterial cells, the nonspecific forces were always weaker than the hydrogen bonding forces. This finding has revealed the importance of specific interactions such as hydrogen bonding in predicting the overall adhesion mechanism of *L. monocytogenes* to inert surfaces. Our results suggested that disrupting the formation of the hydrogen bonds between the bacteria and the inert surfaces could serve as an
effective method for controlling the adhesion of \textit{L. monocytogenes} to inert surfaces in the food processing facilities.

To describe the favorability of bacterial adhesion to surfaces at the molecular level, we carried the study presented in Chapter 5. Thermodynamic analysis of bacterial adhesion to surfaces can be used to explain the favorability of bacterial adhesion process in terms of energy and entropy\textsuperscript{12,63,64}. However, this approach has always been questioned due to its macroscopic nature\textsuperscript{65-68}. In Chapter 5, we have proposed a new approach based on statistical mechanics\textsuperscript{69-71} to describe the molecular mechanism of bacterial adhesion from a thermodynamic perspective at a fundamental level. By applying the Boltzmann distribution of energies\textsuperscript{69} to the probability histograms of bacterial adhesion energies measured by AFM, the total number of molecules involved in the adhesion process and the entropy and/or the disorder of the bacterial surface molecules were estimated. Our results provided a thermodynamic evidence of the direct relationship between the strength of bacterial adhesion measured at a molecular level and the number of bacterial surface molecules available for interactions and the length of the bacterial surface biopolymer brush layer.

1.2 Literature Review

This dissertation aims at 1) describing the relationship between the physiochemical and mechanical properties of the surface biopolymers of \textit{L. monocytogenes} EGDe and their initial attachment to inert surfaces in water, and 2) the roles of temperature and ionic strength of growth in the biophysical mechanisms of the bacterial adhesion process. To achieve our goal, AFM was mainly used to measure the interaction forces and energies between the surface biopolymers of bacterial cells grown at different environmental conditions of temperature and ionic strength and a model surface of silicon nitride in water. In addition to quantifying the adhesion strengths
between bacteria and silicon nitride using AFM, soft-particle Derjaguin-Landau-Verwey-Overbeek (DLVO) theory\textsuperscript{10-12} of energy barriers to adhesion were predicted. Parameters to include in the soft-particle analysis of the DLVO theory were calculated from contact angle and electrophoresis measurements performed on \textit{L. monocytogenes} EGDe lawns and cells. The mechanical properties of bacterial surface biopolymers were estimated from modeling approach curves of the AFM force measurements.

Statistical methods were used to quantify the contributions of specific and nonspecific forces to the overall interaction forces measured by AFM, and whether certain parameters such as adhesion strength and mechanical properties were different among various treatments investigated. Basic definitions of terms used in the thesis, background on model bacteria used and basis of theoretical models applied are given below.

\textbf{1.2.1 \textit{Listeria monocytogenes}}

\textit{Listeria monocytogenes} are Gram-positive, facultatively anaerobic, food-borne bacterial pathogens that are considered among the most resilient food-borne pathogens present in the environment\textsuperscript{30-33}. \textit{L. monocytogenes} are primarily soil microorganisms and thus are frequent contaminants of raw materials used in the food processing plants\textsuperscript{33,38}. Consumption of contaminated food by \textit{L. monocytogenes} can cause severe and fatal infections such as listeriosis, encephalitis, meningitis, septicemia and intrauterine\textsuperscript{72-74}.

\textit{L. monocytogenes} are associated with food-borne disease outbreaks that are characterized by widespread distribution and relatively high hospitalization and fatality rates\textsuperscript{32,54}. As a result of the associated high fatality rate, the U.S. Department of Agriculture (USDA) and the U.S. Food and Drug Administration (FDA) established zero tolerance policies for the \textit{L. monocytogenes} in food products (Ready-to-eat food products)\textsuperscript{3,54}. The widespread outbreaks caused by \textit{L.}}
monocytogenes could be due to an increased virulence, a better adaptation to survival in foods, a broader distribution in the environment, or an increased ability to survive in food processing environments\textsuperscript{33}. For example, \textit{L. monocytogenes} were observed to survive in commercial cheese brine (23.8 w/v \% NaCl, pH 4.9) stored at 4°C for 259 days\textsuperscript{75}.

Unlike most other human food-borne pathogens, \textit{L. monocytogenes} have the ability to survive and grow over a wide temperature range (1 to 45 °C)\textsuperscript{76}. The efficacy of \textit{L. monocytogenes} in causing widespread outbreaks can be mostly attributed to their ability to survive and grow in such a wide temperature range on many surfaces. In addition, FDA’s investigation into the recently occurred \textit{L. monocytogenes outbreak reported in cantaloupes pointed to} the importance of controlling the temperature of the environment in which food is stored or processed and the sanitization of the surfaces in contact with food in preventing the contamination of food by \textit{L. monocytogenes} and fatal multistate \textit{Listeria} outbreaks\textsuperscript{56}.

1.2.1.1 Adaptation Mechanisms of \textit{L. monocytogenes} to Environmental Conditions

Studies indicated that \textit{L. monocytogenes} is a dynamic, highly changing bacterium, continually sensing and adapting in response to changes in the environmental conditions. One of the mechanisms used by \textit{L. monocytogenes} to adapt to its environment is the change in their physiochemical cell surface properties such as cell surface charge and hydrophobicity\textsuperscript{77-79}. For example, \textit{L. monocytogenes} ScottA incubated at low temperatures (15 and 20 °C) demonstrated a higher negative charge and hydrophobicity compared to when incubated at 37 °C at pH 7\textsuperscript{77}.

Another adaptation mechanism of \textit{L. monocytogenes} to the environmental changes is the regulation in the gene expression level leading to an increased or decreased synthesis of surface molecules, especially virulence proteins\textsuperscript{35,37,42,45,47}. For example, several cell wall-anchored virulence proteins were shown to be optimally expressed in \textit{L. monocytogenes} at \textit{in vivo}
temperatures\textsuperscript{45}. Similarly, environmental ionic strength\textsuperscript{42} and pH\textsuperscript{35} was shown to regulate the expression and activity of several virulence and adhesive factors in \textit{L. monocytogenes} such as hemolysin, listeriolysin-O (LLO) and flagellin.

Understanding the role of these surface molecules in controlling bacterial physiochemical properties, such as mechanical properties or charge distributions on the bacterial surface in relation to their interactions with surfaces could lead to a better understanding of the adaptation mechanisms and control of the contamination and spread of this pathogen.

\textbf{1.2.2 Describing Bacterial Adhesion Phenomenon}

In general, a two-step mechanism mediates bacterial attachment to a surface. Both mechanisms are influenced by the chemical nature of the interacting surfaces and the electrolytic environment\textsuperscript{16,80}. The first step involves the bacteria being transported close enough to allow initial attachment to take place\textsuperscript{81,82}. As the bacterial cell approaches to a surface of interest, the entire cell will be exposed to nonspecific physiochemical forces such as Lifshitz-van der Waals and electrostatic forces\textsuperscript{10-12}. The next crucial step in the attachment process is the irreversible attachment of cells to the surface, as bacteria locking on to the surface by the production of exopolysaccharides and or specific ligands, such as pili or fimbriae that may complex with the surface\textsuperscript{13}. In the transition from reversible attachment to irreversible attachment, various short range forces are involved, including covalent, ionic and hydrogen bonding forces\textsuperscript{13,83}.

Owing to their dimensions\textsuperscript{84}, bacterial cells in solution can be approached as colloidal particles, and hence the process of bacterial adhesion can be described by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability\textsuperscript{10,11}. The DLVO theory estimates the interaction forces and/or energies between two surfaces as a sum of the Lifshitz-van der Waals (LW) and electrostatic interactions referred to generally as the electrostatic double-layer
interactions. The LW interactions, which are of relatively long-range are usually described as attractive and not very sensitive to solution pH and ionic strength. The most important parameter determining the LW interaction is the Hamaker constant, which is a material property often estimated from contact angle measurements.

Electrostatic forces, which are of long-range, may be repulsive or attractive, depending upon the charge of the two surfaces interacting. Thus, electrostatic forces are very sensitive to the ionic strength and pH of the liquid solution in which measurements are performed. In general, the charging of a surface in a liquid can come about in two ways:

i) By the ionization or dissociation of surface groups (e.g., the dissociation of protons from surface carboxylic groups \(-\text{COOH} \rightarrow \text{-COO}^- + \text{H}^+\)), which leaves behind a negatively charged surface.

ii) By the adsorption of ions from solution onto a previously uncharged surface, (e.g., the binding of \(\text{Ca}^{+2}\) onto zwitterionic headgroups of lipid bilayer surfaces, which charges the surface positively.) or by the adsorption of ions from solution onto oppositely charged surface sites, e.g., the adsorption of cationic \(\text{Ca}^{+2}\) to anionic \(\text{COO}^-\) sites vacated by \(\text{H}^+\) or \(\text{Na}^+\).

Whatever the charging mechanism is taking place, the final surface charge is balanced by an equal but oppositely charged region of counterions, some of which are usually bound transiently to the surface, while others form an atmosphere of ions in rapid thermal motion close to the surface, known as the diffuse electric double layer. Bacterial cell surface charge originates from the presence of carboxyl, phosphate and amino groups in either dissociated or protonated form and the surface charge consequently depends upon the pH and ionic concentration of the suspending medium. It was suggested that at physiological pH values,
i.e. between pH 5 and 7, bacterial cells are generally negatively charged due to the excess of carboxyl and phosphate groups over amino groups\textsuperscript{17}.

Calculation of electrostatic interactions requires knowledge of the electrostatic surface potential of the interacting surfaces, which is usually approximated by zeta potential or surface potential from electrophoretic mobility measurements\textsuperscript{82}.

**Figure 1.1** Schematics of the two-step mechanism of initial bacterial attachment to a surface.

1.2.2.1 **Soft-particle Analysis of Derjaguin-Landau-Verwey-Overbeek (DLVO) theory**

Many researchers have now realized that the DLVO theory does not take into account that the bacterial cell surface is not a model colloid particle but a highly complex surface. The classical DLVO theory assumes that interacting surfaces, of which one is a bacterium in our
case, are perfectly smooth, with no asperities or surface structures\textsuperscript{88}. However, bacterial cells can form specific extracellular structures, and their cell walls are more complex structurally and chemically than the surface of colloidal particles\textsuperscript{89}.

Earlier studies used to rely on Smoluchowski formula to calculate the electrostatic double-layer interactions. According to the Smoluchowski formula, the surface potential is expressed as zeta potential and depends only on the viscosity of the medium which the measurements took place in. The Smoluchowski formula explains the migration of a hard-particle that is impenetrable to electrolytes in an external electric field. However, the bacterial cell surface structure is much more complicated and diverse than the surface of an inert hard-particle, and covered by “soft” biopolymers which are penetrable to electrolytes such as proteins, peptidoglycans, lipopolysaccharides (LPS) and phospholipids. Research studies attempted to use the hard-particle model to estimate the bacterial surface potential usually reported overestimated bacterial surface potentials\textsuperscript{90,91}.

Ohshima therefore developed an electrophoretic model for soft-particles such as bacteria, which takes into account the effects of the particle softness and the penetration of the polyelectrolyte in the particle\textsuperscript{92}. Soft-particle electrophoresis model developed by Ohshima\textsuperscript{92} predicts the Donnan potential for the bacterial cells in addition to the bacterial surface potential\textsuperscript{92-94}. For soft particles, the charge potential deep inside the surface layer is practically equal to the Donnan potential, and plays an important role in describing the electrostatic interactions of soft particles with the surfaces\textsuperscript{93}.

\textbf{1.2.3 Bacterial Adhesion to Solid Surfaces in Contact with Liquids}

Bacteria can be transported to a solid surface by Brownian motion, sedimentation due to differences in specific gravity between the bacteria and the bulk liquid and convective mass
transport due to the movement of the bulk fluid or by the active transport mediated by the flagella activity\textsuperscript{95}. Irrespective of the factors involved in the bacterial transportation to a surface, an interaction known as attachment has to take place in order to bind the bacteria and the surface together.

Several parameters have been taken into account in studying bacterial attachment to solid surfaces, which depend on the bacterial cell surface, the substrate surface, and the solution environment\textsuperscript{83,95}. Although the dominating factors involved in the bacterial attachment process to surfaces has remained elusive, bacterial and solid surface and environmental properties such as surface charge\textsuperscript{77,79,96}, hydrophobicity\textsuperscript{77,79,97}, and ionic strength\textsuperscript{98-100} were shown as the important parameters affecting Lifshitz-van der Waals and electrostatic interactions mediating the first step of the initial attachment phase of bacteria.

Nevertheless, the presence and properties of bacterial surface biopolymers are known to influence the steric interactions and “specific” molecular interactions involved in the initial cell attachment to a surface\textsuperscript{101,102}. However, although very important, the effects of the surface biopolymer properties on the bacterial attachment to surfaces are still largely unknown. Understanding how the biopolymer properties affect the bacterial adhesion to surfaces can detail the mechanisms mediating the bacterial adhesion process.

\subsection*{1.2.4 Steric Interactions between Bacterial Surface Biopolymers and AFM Tip}

Non-DLVO interactions such as steric interactions are an important type of interaction; their importance in bacterial adhesion has been recognized since AFM provided a way to quantify such interactions. Steric interactions arise from contact between the AFM tip and the surface polymeric chains extending from the bacterial surface into solution; as the AFM probe pushes down on the bacterial cell, the biopolymers are forced into a more compact spatial
arrangement. A model developed by Alexander\textsuperscript{103} and de Gennes\textsuperscript{104} for grafted polymers at relatively high surface coverage and further modified by Butt et al.\textsuperscript{105} can be used to describe the steric forces measured between a spherical AFM tip and a flat surface by integrating the force per unit area over the tip surface, to produce the interaction force ($F_{St}$, nN):

$$F_{St} = 50k_BTaL_o^{3/2}e^{-2\phi h/L_o}$$

(1.1)

where $k_B$ is the Boltzmann constant ($1.3807 \times 10^{-23}$ J/K), $T$ is the absolute temperature (298 K), $a$ is the tip radius (nm), $L_o$ is the equilibrium thickness of the polymer layer (nm), $\Gamma$ is the grafted polymer density in the brush layer (m$^{-2}$); reflecting how much of the surface is covered by polymers, and $h$ is the separation distance between the two surfaces (nm). In equation 1.1, $L_o$ and $\Gamma$ represent the fitting parameters.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_2.png}
\caption{Example of fitting AFM approach curve by the steric model given in equation 1.1, and the schematics of the evaluated fitting parameters, $L_o$; the equilibrium thickness of the polymer layer (nm), and $\Gamma$; the grafted polymer density in the brush layer (m$^{-2}$).}
\end{figure}
1.2.5 Statistical Analysis of AFM Bacterial Adhesion Measurements

The ability of AFM to quantify the molecular mechanisms of bacterial adhesion to surfaces in real time and in real environments gives it an edge over the traditional biological macroscopic techniques. In addition, with the statistical analysis of the AFM adhesion force and energy measurements by different methods, a wealth of information on the factors involved in the initial bacterial cell attachment to a surface can be unraveled. For example, decoupling of the overall interaction force into specific and nonspecific force components by using Poisson statistical analysis and the significance of different interaction forces in controlling bacterial adhesion can be determined\textsuperscript{106,107}. The Poisson statistical method does not require a large number of force measurements; thus minimizing the sample damage due to repetitive force measurements, and can allow for an accurate estimation of the magnitudes of specific and nonspecific force components, as described in the literature\textsuperscript{108-110}.

AFM studies of nano-scale bacterial adhesion to surfaces always demonstrate large variations as evident from the wide ranges and the large standard deviations in the quantified mean of the adhesion strength for any data\textsuperscript{114}. Heterogeneities in AFM data have been largely attributed to the wide array of molecules present on the bacterial surface\textsuperscript{23,111-114}. Large standard deviations are not unique to AFM analyses, but occur as well in macroscopic bond-strength evaluations\textsuperscript{115}. With statistical analysis of AFM data, one makes clever use of the large spread in data to quantify the most probable adhesion event in the distribution. For example, normal\textsuperscript{116}, log-normal\textsuperscript{57,117}, Weibull\textsuperscript{114}, and Poisson\textsuperscript{106,107} distributions have been used to describe the heterogeneity in the nano-scale bacterial adhesion measurements. Researchers have also used different statistical measures to represent a given data set of bacterial interactions with surfaces such as the mean, standard deviation of the mean, range, median and the standard error of the
mean. Statistical analysis can also be used to distinguish the differences in the AFM results such as the non-parametric group comparisons.

In Chapter 5, we have shown for the first time the use of Boltzmann distribution to explain the heterogeneity in the probability distributions of bacterial adhesion events measured by AFM.

1.3 Organization of the Dissertation

This dissertation represents manuscripts that I have written during the course of my Ph.D. Chapter 2 is a manuscript that has been published in *Langmuir* 2012, 28, 1360–1373 entitled “The Role of Growth Temperature in the Adhesion and Mechanics of Pathogenic *L. monocytogenes*: An AFM Study” by F. P. Gordesli and N. I. Abu-Lail. A copy right to reuse this published paper in the thesis has been obtained from American Chemical Society Publications and will be available upon request. My goal during this study was to improve our fundamental understanding of the role of the surface biopolymers of *L. monocytogenes* grown under various temperatures in controlling their interactions and biophysical properties. For this purpose, the mechanical properties and the adhesion strengths of the surface biopolymers of pathogenic *L. monocytogenes* EGDe were quantified using atomic force microscopy (AFM) in water for cells grown under five different temperatures (10, 20, 30, 37 and 40 °C). Our results indicated that adhesion force and energy quantified were at their maximum when the bacteria were grown at the optimum temperature of 30 °C. The higher adhesion observed at 30 °C was correlated with longer and denser biopolymers on the bacterial surface, higher proteins’ content, and the lower negative cell surface potential, compared to those observed when grown at 37, 40, 20 and 10 °C, respectively. In addition, theoretically predicted adhesion energies based on soft-particle analysis
of the DLVO theory linearly correlated with the adhesion energies measured by AFM. The manuscript was written by me and edited by Dr. Nehal I. Abu-Lail.

Chapter 3 is entitled “Impact of Ionic Strength of Growth on the Physiochemical Properties, Structure and Adhesion of L. monocytogenes Polyelectrolyte Brushes to a Silicon Nitride Surface in Water” by F. P. Gordesli and N. I. Abu-Lail. This chapter is ready to be submitted to Journal of Colloid and Interface Science. My first goal during this study was to detail the effects of physiochemical properties of the surface biopolymers of L. monocytogenes in their adhesion to silicon nitride surface in water by taking into account that these biopolymers were acting as polyelectrolyte brushes, as we have previously observed in Chapter 2. My second goal was to investigate the role of ionic strength of growth on the biophysical properties of the polyelectrolyte brushes of L. monocytogenes in relation to their interaction energies with the silicon nitride in water. The adhesion energies of pathogenic L. monocytogenes EGDe to silicon nitride surface were quantified using AFM in water for cells grown in media of five different ionic strengths (0.003M, 0.05M, 0.1M, 0.3M and 0.5M NaCl). Our results suggested that the structure and the electric properties of the polyelectrolyte brush layer of L. monocytogenes EGDe were dependent on the ionic strength of growth investigated. The transitions in the adhesion energies, surface and Donnan potentials, the free ion concentration in the polyelectrolyte brush layer, and in the heights of bacterial surface polyelectrolyte brushes occurred for the cells grown at IS of 0.1M.

Our results also indicated that, the adhesion affinity of L. monocytogenes to inert surfaces can change when the microbe is transferred to a different suspension due to the changes in the ionic strengths of the solutions. Therefore, predictive physiochemical strategies for the control of the adhesion of this microbe to inert surfaces in water should take into account the ionic strength
of the solutions in the transfer and growth route of *L. monocytogenes*. This manuscript was written by me and edited by Dr. Nehal I. Abu-Lail.

Chapter 4 is a manuscript that has been recently accepted by *Environmental Science and Technology* (2012) entitled “Combined Poisson and soft-particle DLVO analysis of the specific and nonspecific adhesion forces measured between *L. monocytogenes* grown at various temperatures and silicon nitride” by F. P. Gordesli and N. I. Abu-Lail. My goal during this study was to investigate the contributions of electrostatic and Lifshitz-van der Waals interactions and hydrogen bonds on the overall interaction between *L. monocytogenes* EGDe and silicon nitride at very close surface proximities such as at distances where “specific” interactions come into play (<1nm).

The adhesion forces measured between the bacteria grown at five different temperatures and silicon nitride were decoupled into “specific” hydrogen bonding and nonspecific forces using Poisson statistical analysis. By combining the results of Poisson analysis with the soft-particle analysis of the DLVO theory, the contributions of the Lifshitz-van der Waals and electrostatic forces to the overall nonspecific interaction forces were determined. The strongest specific and nonspecific attraction were observed for cells grown at 30 °C, compared to those observed for cells grown at higher or lower temperatures, respectively. Our results also showed that the Lifshitz-van der Waals attraction dominated the total nonspecific adhesion forces for all investigated thermal conditions at the closest separation distances. However, hydrogen bonding forces were always stronger than the nonspecific forces, suggesting a means to decrease the adhesion of *L. monocytogenes* to inert surfaces could be disrupting the formation of the hydrogen bonds between the bacteria and the inert surfaces. This manuscript was written by me and edited by Dr. Nehal I. Abu-Lail.
Chapter 5 is entitled “A New Thermodynamic Approach to Explaining the Molecular-Scale Bacterial Adhesion Measurements” by F. P. Gordesli and N. I. Abu-Lail. This manuscript is ready to be submitted to Colloids and Surfaces B: Biointerfaces as a short note. My goal during this study was to explain the initial bacterial cell attachment to a surface from a thermodynamic perspective. This study describes how statistical mechanics can be applied to bacterial adhesion phenomenon at a fundamental level. The concept of this study was based on our previous findings given in Chapter 3. By applying Boltzmann distribution to the probability histograms of bacterial adhesion energies measured by AFM, the total number of molecules involved in the adhesion process and the entropy of the bacterial surface molecules were estimated. To our knowledge this study is the first exposure of statistical mechanics to the bacterial adhesion phenomenon.

Finally, I summarized my work and the main conclusions in Chapter 6.
1.4 References


Chapter 2

The Role of Growth Temperature in the Adhesion and Mechanics of Pathogenic *L. monocytogenes*: An AFM Study

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Abstract

The adhesion strengths of pathogenic *L. monocytogenes* EGDe to a model surface of silicon nitride were quantified using atomic force microscopy (AFM) in water for cells grown under five different temperatures (10, 20, 30, 37 and 40 °C). The temperature range investigated was chosen to bracket the thermal conditions in which *L. monocytogenes* survive in the environment. Our results indicated that adhesion force and energy quantified were at their maximum when the bacteria were grown at 30 °C. The higher adhesion observed at 30 °C compared to the adhesion quantified for bacterial cells grown at 37, 40, 20 and 10 °C, respectively was associated with longer and denser bacterial surface biopolymer brushes as predicted from fitting a model of steric repulsion to the approach distance-force data as well from the results of protein colorimetric assays. Theoretically predicted adhesion energies based on soft-particle DLVO theory agreed well with the adhesion energies computed from AFM force-distance retraction data ($r^2 = 0.94$); showing a minimum energy barrier to adhesion at 30 °C.

**Keywords:** *L. monocytogenes*, soft-particle DLVO, AFM, adhesion force, adhesion energy, steric model, biopolymers and growth temperature.
2.1 Introduction

The Centers for Disease Control and Prevention (CDC) report that every year more than 5 million illnesses result in the United States from food-borne bacterial pathogens, costing up to $7 billion of medical expenses and industrial losses. Among those pathogens, *Listeria monocytogenes* are associated with food-borne disease outbreaks that are characterized by a widespread distribution and relatively high hospitalization and fatality rates. *L. monocytogenes* are Gram-positive, food-borne bacteria that are widely distributed in the environment. *L. monocytogenes* are the causative agents of potentially lethal infections, such as listeriosis and meningitis. Consumption of contaminated food by *L. monocytogenes* is believed to be the first key step in their route to infections. *L. monocytogenes* can contaminate food while growing in soil, during food processing as well as during food storage. *L. monocytogenes* can colonize, multiply and persist on many surfaces in the food industry environments, and survive in harsh conditions such as those often encountered during food processing. Unlike most other human food-borne pathogens, *L. monocytogenes* can survive and grow over a wide temperature range (1 to 45 °C), with optimal growth conditions between 30-37 °C. The efficacy of *L. monocytogenes* in causing widespread outbreaks is largely due to their ability to survive and grow in such a wide temperature range on many surfaces.

Previous studies have shown that the adaptation strategies of *L. monocytogenes* to varying temperatures in the environment are associated with induced variations in the bacterial synthesis of certain cell wall molecules. For example, when grown at *in vivo* temperature of 37 °C, *L. monocytogenes* increased the expression levels of cell wall anchored virulence proteins such as internalin A (inlA) and internalin B (inlB) compared to when grown at 16 °C or 20 °C. In addition, *L. monocytogenes* repress the expression of flagellin and motility genes at *in*
vivo temperatures, but optimally express flagella when grown at temperatures below 30 °C\textsuperscript{19,20}. Finally, it has been recently reported that the expression level of cell wall associated lipoteichoic acid in \textit{L. monocytogenes}, which confers resistance to antimicrobial cationic molecules\textsuperscript{22}, is also influenced by the growth temperature of the bacteria\textsuperscript{21}.

Although the impact of growth temperature on the synthesis of certain surface molecules of \textit{L. monocytogenes} is well studied, the role of these molecules and their properties in controlling the adhesion of pathogenic \textit{L. monocytogenes} to inert model surfaces \textit{in vitro} remains poorly understood. To improve our fundamental understanding of the role of the surface molecules of \textit{L. monocytogenes} grown under various temperatures in controlling their interactions and properties, we carried this study. The mechanical properties of the surface biopolymers of pathogenic \textit{L. monocytogenes} EGDe and the adhesion strengths of the bacterial cells to silicon nitride in water were measured using atomic force microscopy (AFM). The use of AFM to investigate the mechanical properties of bacterial surface biopolymers\textsuperscript{23-35} and bacterial interactions with surfaces\textsuperscript{23-42} has been well documented in the literature. In addition, predictions from soft-particle Derjaguin-Landau-Verwey-Overbeek (DLVO) theory\textsuperscript{43} of energy barriers to adhesion were compared to the adhesion energies computed from the AFM force measurements. Parameters to include in the soft-particle DLVO theory predictions were calculated from contact angle and electrophoresis measurements performed on \textit{L. monocytogenes} EGDe lawns and cells grown at various temperatures of interest. Finally, colorimetric assays of the bacterial composition of proteins were carried for cells grown at the various temperatures of growth.

\textbf{2.2. Materials and Methods}

\textbf{2.2.1 Bacterial cultures.} Pathogenic \textit{Listeria monocytogenes} EGDe was obtained from Prof. Mark Lawrence, an associate professor at the Department of Basic Sciences, the College of
Veterinary medicine at Mississippi State University. Among the highly pathogenic *L. monocytogenes* strains, EGDe was chosen as our model strain because it has a fully sequenced and annotated genome. The strain was activated by growing for twelve hours at 30 °C in a temperature controlled shaker rotating at a 150 rpm in brain heart infusion broth (BHIB). Following activation, cells were cultured in BHIB for 90 hours at 10 °C, 18 hours at 20 °C, 7 hours at 30 °C, 5.5 hours at 37 °C, and 4.5 hours at 40 °C, until cells reached late exponential phase of growth, the stage at which they were harvested. The growth of the bacterial cells was monitored by reading the optical density of the culture every hour at a wavelength of 600 nm. The 30 °C and 37 °C thermal conditions were chosen to represent the optimum environmental temperatures for *L. monocytogenes* growth and pathogenicity. Other higher and lower growth temperatures were chosen to bracket the environmental thermal stresses at which *L. monocytogenes* were observed to survive and grow in food processing environments.

2.2.2 Atomic force microscopy measurements. Prior to AFM force measurements, bacterial cells cultured at the temperature of interest were centrifuged twice at 5525 g for 10 minutes. After centrifugation, cells were attached to gelatin-coated mica disks according to the procedure detailed elsewhere. All AFM force measurements were performed with a PicoForce™ Scanning Probe Microscope with a Nanoscope IIIa controller and extender module (Bruker AXS Inc., Santa Barbara, CA) using silicon nitride cantilevers (DNP-S cantilevers with 0.06 N/m nominal spring constant, Bruker AXS Inc., Santa Barbara, CA). Silicon nitride cantilevers were chosen as our model inert surfaces because they are characterized by similar surface potentials to that of soil and glass, substrates to which *L. monocytogenes* frequently attach to in nature and in food industry environments. Since water is the main solvent used in the food processing industries and the main solvent used in preparing foods, all force measurements were...
performed under water. We have shown previously that attaching *L. monocytogenes* cells to gelatin coated mica and performing force measurements on bacterial cells under water do not affect the bacterial cell viability or integrity\textsuperscript{40}.

Prior to force measurements, the force constant of each cantilever was determined from the power spectral density of the thermal noise fluctuations in DI water\textsuperscript{51}. On average, the spring constant was found to be 0.060 ± 0.005 N/m (n = 6), very close to the nominal spring constant value reported by manufacturer in air. To locate the cells for force measurements and to ensure the cells population homogeneity and integrity, *L. monocytogenes* EGDe images were captured using TappingMode\textsuperscript{TM} under DI water at a scan speed of 1 Hz at a resolution of 256 pixels per line and 256 lines per image (Figure 2.1). Once a bacterial cell had been located via topographical scanning, the oscillation of the cantilever was stopped and the extending and retracting deflection-displacement curves measured between the bacterial surface biopolymers and the silicon nitride cantilever were captured using the AFM software. Force measurements were made on a bacteria-free area of the gelatin-coated mica disk before and after making a measurement on a bacterial cell. Equality of the measurements ensured that the tip properties had not been altered by contact with the bacterial surface biopolymers.

For each growth temperature investigated, at least thirteen cells from three different cultures were examined. On each cell, fifteen points were located to perform force measurements using the point and shoot feature of the AFM software (Figure 2.1). Given that the ratio of the bacterial cell area to that of the AFM tip is ~ 40, selecting 15 points on each cell surface ensured that the points were spaced apart to collect force curves from different locations on the bacterial surface. Retraction curves were measured at a rate of 580 nm/sec to minimize the hydrodynamic drag forces\textsuperscript{52}, and at a resolution of 4096 points.
Figure 2.1 TappingMode™ phase image of L. monocytogenes EGDe cells grown in BHIB at 30°C under water. The image is 20 × 20 μm with a tip oscillation amplitude of 500 mV. The white cross marks shown on the cell surfaces indicate locations where force measurements were made. Each cell had 15 marked locations.

2.2.3 Analysis of retraction curves. Retraction curves were considered individually because of the complex and heterogeneous nature of the interactions observed between the bacterial surface biopolymers and the AFM silicon nitride cantilever. Bacterial adhesion was quantified from the retraction curves in terms of adhesion force in nano-Newton (nN) and adhesion energy in atto-Joule (AJ). For each growth temperature investigated, at least 195 retraction curves were used to identify the adhesion force peaks and to compute the adhesion energies (Figure 2.2). Force and energy were correlated according to:

$$E_{adh}(AFM) = - \int_{h_i}^{h_f} Fdh$$

(2.1)

where $E_{adh}(AFM)$ is the adhesion energy quantified from the AFM retraction curves, $F$ is the pull-off force, and $h$ is the separation distance. The negative sign was used to convert pull-off
forces into adhesion forces. To quantify the adhesion energy, the integral in equation 2.1 was evaluated using the Trapezoidal rule (equation 2.2)\(^{53}\)

\[
E_{adh}(AFM) = \int_{h_i}^{h_f} Fdh \approx -\frac{h_f - h_i}{n} \left[ \frac{F(h_i) + F(h_f)}{2} + \sum_{k=1}^{n-1} F(h_i + k \frac{h_f - h_i}{n}) \right]
\] (2.2)

In equation 2.2, \(h_i\) and \(h_f\) were taken as the first and last distance points at which the retraction curve crosses the zero force axis (Figure 2.2), \(n\) was equal to the number of data points collected per retraction force curve in the integral interval and varied from one curve to another. In computing the integral in equation 2.2, a uniform grid was always used\(^{53}\).

![Graph](image)

**Figure 2.2** Example of an AFM retraction curve of *L. monocytogenes* EGDe grown in BHIB at 30°C. The curve shows the pull-off force peaks as indicated with circles. The gray area under the force-distance retraction curve is the adhesion energy. The arrows at the top of the curve indicate the bounds of integration where the adhesion energy was computed using equations 2.1 and 2.2.

### 2.2.4 Colorimetric proteins measurements

Bacterial cells grown at the temperature of interest were harvested at the late exponential growth phase as described above and washed twice by centrifugation at 5525g for 10 minutes each round. The collected bacterial pellet was then diluted
with 0.2 μm filtered DI water to make a suspension with an optical density of ~ 0.5 at a wavelength of 600 nm. The bacterial suspension was then assayed via colorimetric standard Lowry\textsuperscript{54} assay, to determine the amounts of bacterial proteins. Bovine serum albumin (BSA) was used as the standard.

2.2.5 Contact angle measurements. Prior to contact angle measurements, 20 ml of bacterial cells suspended in BHIB growth media and grown as described above at the temperature of interest were harvested and washed twice by centrifugation at 5525 g for 10 minutes each round. The collected bacterial pellet was then re-suspended in a 20 ml of 0.2 μm filtered DI water. The bacterial solution was then filtered on a cellulose acetate filter membrane (pore diameter, 0.45 μm, Sartorius, Aubagne, France) using negative pressure. Bacterial densities on the membrane filters were determined to be between $1 \times 10^7 - 5 \times 10^7$ cells/mm$^2$ corresponding to 40–200 layers of bacteria covering the filter membrane\textsuperscript{33}. To establish constant moisture content, the filters with bacteria on them were placed in a Petri-dish on the surface of 1% (wt/vol) agar prepared by dissolving Bacto agar (Difco, Detroit, Michigan) in filtered DI-water containing 10% (vol/vol) glycerol\textsuperscript{55}. Drying time for \textit{L. monocytogenes} lawns was determined to be ~40 min\textsuperscript{56}. Contact angles of \textit{L. monocytogenes} EGDe cells were quantified using two probing liquids characterized with different polarities. The liquids used were ultrapure water (18.2 MΩ·cm resistivity, Millipore Mili-Q Plus, Billerica, MA) and diiodomethane (99% pure, Alfa Aesar, Ward Hill, MA). The contact angles of the bacterial lawns were quantified using the sessile drop technique\textsuperscript{55} with a KRÜSS DSA100 drop shape analysis system (KRÜSS GmbH, Hamburg, Germany). For each measurement, a 2 μL droplet volume of the liquid of interest was used. We have previously showed that the volume of the liquid drop was large enough compared
to the roughness of the bacterial lawns\textsuperscript{33}. For each liquid used, at least 15 measurements were performed. All measurements were made at room temperature and at ambient humidity.

2.2.6 Electrophoretic mobility measurements. A Zeta potential analyzer (Zeta Sizer 3000 HAS, Malvern Instruments Ltd., Malvern, UK) was used to measure the electrophoretic mobilities of \textit{L. monocytogenes} EGDe cells grown at five different temperatures as described above. Prior to measurements, the bacterial cells grown at the temperature of interest were harvested at the late exponential growth phase as described above and washed twice by centrifugation at 5525g for 10 min each round. The collected bacterial pellet was then diluted with 0.2 μm filtered DI water and mixed with a NaCl solution to make a suspension with an optical density (λ = 600 nm) of ~0.05\textsuperscript{57}, pH 7 and final ionic strengths of 0.02, 0.04, 0.06, 0.1 and 0.15 M NaCl. All electrophoretic mobility measurements were performed five times at room temperature.

2.3 Mathematical Modeling

2.3.1 Calculation of biopolymer brush thickness and grafting density. To model the steric interactions measured between the AFM tip and \textit{L. monocytogenes} EGDe surface biopolymers, a steric model developed for a high density grafted polymer surface (polymer brush layer) was used. The interaction force between two surfaces ($F_{St}$), only one of which is coated with a polymer has been modeled following the work of Alexander\textsuperscript{58} and de Gennes\textsuperscript{59}. This model was modified by Butt et al.\textsuperscript{60} to describe the steric forces measured between a spherical AFM tip and a flat surface by integrating the force per unit area over the tip surface, to produce the interaction force ($F_{St}$, nN):

$$F_{St} = 50k_B T \alpha L_o \Gamma^{3/2} e^{-2\chi/L_o}$$

(2.3)
where $k_B$ is the Boltzmann constant ($1.3807 \times 10^{-23}$ J/K), $T$ is the absolute temperature (298 K), $a$ is the tip radius taken as reported by the manufacturer (40 nm), $L_o$ is the equilibrium thickness of the polymer layer (nm), $\Gamma$ is the grafted polymer density in the brush layer (m$^{-2}$); reflecting how much of the surface is covered by polymers, and $h$ is the separation distance between the two surfaces (nm). In equation 2.3, $L_o$ and $\Gamma$ represent the fitting parameters. For each growth temperature investigated, at least 131 approach curves were fitted individually by the steric model (Figure 2.5A, Table 2.2) and the evaluated fitting parameters ($L_o$ and $\Gamma$) were averaged (Figures 2.5B-C, Table 2.2).

2.3.2 Calculation of bacterial surface potential using soft-particle DLVO theory. Typically, bacterial adhesion to surfaces has been described by the classical DLVO theory of colloidal stability$^{61,62}$. This theory assumes that interacting surfaces, of which one is a bacterium in our case, are perfectly smooth, with no asperities or surface structures$^{63}$. However, the cell wall of bacterial cells is composed of complex biopolymers such as lipopolysaccharides (LPS), proteins, peptidoglycans, and phospholipids$^{64}$. Soft-particle DLVO theory developed by Ohshima$^{43}$ overcomes the limitations of the classical DLVO theory in describing bacterial adhesion to surfaces. The soft-particle DLVO theory accounts for the effect of biopolymers conformation and bacterial softness on bacterial adhesion by assuming the presence of ion-penetrable, charged polyelectrolyte layer around a rigid core$^{65}$. Therefore, soft-particle DLVO theory was used to quantify the bacterial surface potential of $L.\ monocytogenes$ EGDe cells grown at various temperatures and to explain the roles of electrostatic and Lifshitz-van der Waals interactions in governing the adhesion of $L.\ monocytogenes$ EGDe to silicon nitride in water. The theory approximates the electrophoretic mobility of soft particles as$^{25,43,65}$.
where $\mu$ is the electrophoretic mobility (m$^2$/V.s), $\varepsilon_o$ is the permittivity of vacuum ($8.85 \times 10^{-12}$ C$^2$/J.m), $\varepsilon_r$ is the relative permittivity of the solvent (78 for water), $\eta$ is the solvent viscosity (8.9 × 10$^{-4}$ Pa.s for water at room temperature), $\psi_o$ is the soft-particle surface potential (mV), $K_m$ is the Debye-Hückel parameter for the polymer layer (nm$^{-1}$), $\psi_{DON}$ is the Donnan Potential of the polymer layer (mV), $e$ is the electron charge (1.602 × 10$^{-19}$ C), $Z$ is the valence of the charged groups in the polymer brush, $N$ is the density of the charged groups (M), and $\frac{1}{\lambda_s}$ is the softness parameter (nm). The parameters $\psi_o$, $\psi_{DON}$ and $K_m$ are all functions of ionic strength of the solvent and can be written as$^{25,43,65}$:

$$\psi_o = \frac{k_BT}{ze} \ln \left\{ \frac{ZN}{2zn} + \left[ \left( \frac{ZN}{2zn} \right)^2 + 1 \right]^{1/2} \right\} + \frac{2zn}{ZN} \left\{ 1 - \left[ \left( \frac{ZN}{2zn} \right)^2 + 1 \right]^{1/2} \right\}$$  \hspace{1cm} (2.5)

$$\psi_{DON} = \frac{k_BT}{ze} \ln \left\{ \frac{ZN}{2zn} + \left( \frac{ZN}{2zn} \right)^2 + 1 \right\}^{1/2}$$  \hspace{1cm} (2.6)

$$K_m = \kappa \left[ 1 + \left( \frac{ZN}{2zn} \right)^2 \right]^{1/4}$$  \hspace{1cm} (2.7)

$$\kappa = \left( \frac{1000N_A}{\varepsilon_o \varepsilon_r k_BT} \sum_{i=1}^{N} z_i^2 e^2 n \right)^{1/2}$$  \hspace{1cm} (2.8)

where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, $z$ is the valence of bulk ions, $n$ is the concentration of bulk ions (M), $\kappa$ is the Debye-Hückel screening length (nm$^{-1}$), and $N_A$ is the Avogadro’s constant ($6.022 \times 10^{23}$ mol$^{-1}$). By fitting the soft-particle DLVO theory to the measured electrophoretic mobility data as a function of ionic strength (Figure 2.6A-
E), the fitting parameters; $ZN$ and $\frac{1}{\lambda_s}$ were determined for $L.\ monocytogenes$ EGDe cells grown at five different temperatures (Table 2.3). The parameter $ZN$ representing the spatial charge density in the polyelectrolyte region of the soft particle was then used to calculate the bacterial surface zeta potential ($\psi_o$, mV) in water (equation 2.5). An ionic strength of 0.0027 M was used for the ionic strength of ultrapure water$^{25,36}$.

2.3.3 Calculation of Hamaker constant. The effective Hamaker constant ($A_{132}$) for the interacting system (bacterium (1) / AFM tip (2) / water (3)) was calculated as:

$$A_{132} \approx (\sqrt{A_{11}} - \sqrt{A_{33}})(\sqrt{A_{22}} - \sqrt{A_{33}})$$  \hspace{1cm} (2.9)

The individual Hamaker constant for each of the interacting components (bacterium / AFM tip / water) was calculated as$^{66}$:

$$A_{ii} = 24\pi H_o^2 \gamma_{LW}^i$$  \hspace{1cm} (2.10)

where $H_o$ is the theoretical closest separation distance upon approach taken as 0.157 nm$^{67}$ and $\gamma_{LW}^i$ is the apolar (Lifshitz-van der Waals) surface tension component of the condensed material $(i)$ (mJ/m$^2$). Note that $\gamma_{LW}^i$ values for water and silicon nitride are known to be 21.8 and 32.6 mJ/m$^2$, respectively$^{33}$. The $\gamma_{LW}^i$ apolar surface tension component of the bacterial cells was obtained through contact angle measurements done on bacterial lawns using the thermodynamic-based harmonic-mean (HM) model; which was first proposed by Wu$^{68}$. The HM model has been used in the literature to approximate interactions of low energetic phases with apolar and polar surface tension components (equation 2.11)$^{33}$. This model is useful for hydrophilic polysaccharides$^{69}$ and proteins$^{70}$ which are the major components of bacterial surfaces$^{71}$. The contact angle ($\theta$) is related to $\gamma_{LW}^i$; the apolar part of surface tension of condensed material $(i)$
caused by dispersion energy between molecules, and to $\gamma_i^p$; the polar part of surface tension of condensed material ($i$) caused by dipole interaction included dipole moments and hydrogen bonds according to:

$$
(1 + \cos \theta)\gamma_L = 4 \left( \frac{\gamma_L^{LW} \gamma_S^{LW}}{\gamma_L^{LW} + \gamma_S^{LW}} + \frac{\gamma_L^p \gamma_S^p}{\gamma_L^p + \gamma_S^p} \right)
$$

(2.11)

where the subscripts “L” and “S” and in equation 11 refer to liquid and bacterial phases, respectively. Note that in equation 2.11, the liquid surface tensions are known ($\gamma_L^{LW}$ and $\gamma_L^p$ are 21.8 and 51 mJ/m$^2$ for water, and 48.5 and 2.3 mJ/m$^2$ for diiodomethane, respectively$^{33}$) while the bacterial (solid) surface tensions are unknown. Therefore, equation 2.11 has two unknowns ($\gamma_S^{LW}$ and $\gamma_S^p$). Using the two liquid contact angle measurements performed on bacterial lawns as described above, equation 2.11 was solved to estimate the magnitudes of the bacterial polar and apolar surface tension components. The bacterial apolar surface tension components were then used to calculate the Hamaker constants and eventually the Lifshitz-van der Waals interaction energies for the bacteria grown at different temperatures.

2.3.4 Calculation of soft-particle DLVO interaction energies. The total DLVO interaction energy ($E_{\text{total}}$) between the bacterium and the silicon nitride AFM tip was calculated as the sum of Lifshitz-van der Waals ($E_{LW}$) and electrostatic ($E_e$) interaction energies using a sphere-sphere geometry$^{72}$.

$$
E_{\text{total}} = E_{LW} + E_e
$$

(2.12)

The Lifshitz-van der Waals interaction energy between two dissimilar spheres was calculated using Hamaker expression, corrected for retardation effects as$^{73}$:
where $A_{132}$ is the effective Hamaker constant of the interacting system (I, equation 2.9), $a_1$ is the radius of the bacterium taken as 768 nm for *L. monocytogenes* EGDe$^{32}$, $a_2$ is the radius of the AFM tip taken as reported by the manufacturer (40 nm), $h$ is the separation distance between the AFM tip and the bacterial surface biopolymers (nm) and $\lambda_c$ is the “characteristic wavelength” of the interaction, often assumed to be 100 nm$^{25}$. The electrostatic interactions were calculated using the linearized version of the Poisson-Boltzmann expression as$^{72}$:

$$E_c = \frac{2\pi a_1 a_2 n k_B T}{(a_1 + a_2) \kappa^2} \left( \Phi_1^2 + \Phi_2^2 \right) \left[ \frac{2\Phi_1 \Phi_2}{\Phi_1^2 + \Phi_2^2} \ln \left\{ \frac{1 + \exp(-\kappa h)}{1 - \exp(-\kappa h)} \right\} + \ln(1 - \exp(-2\kappa h)) \right]$$

(2.14)

where $\Phi_1$ and $\Phi_2$ are the reduced potentials of the bacterium and the AFM tip respectively, which relate to their surface potentials $\psi_{o1}$ and $\psi_{o2}$ according to $\Phi_i = \frac{ze\psi_{oi}}{k_B T}$.$^{72}$ For bacterial cells, the soft-particle surface zeta potential ($\psi_{o1}$) calculated from equation 4, was used as the surface potential $\psi_{o1}$. For the silicon nitride tip, the zeta potential of -16 mV was used as $\psi_{o2}$.$^{74-76}$

**2.3.5 Distribution of adhesion affinities.** The log-normal probability distribution function with three fitting parameters ($a$, $b$, $x_o$) described by equation 2.15 was applied to the adhesion affinity data

$$y = a \exp \left[ -0.5 \left( \frac{\ln(x / x_o)}{b} \right)^2 \right]$$

(2.15)
In equation 2.15, $y$ is the probability of occurrence of an adhesion event, $a$ is the amplitude of the distribution and predicts the maximum probability of occurrences, $x$ is the adhesion affinity, $x_o$ is the adhesion affinity with the maximum probability of occurrence, and $b$ is a fitting parameter that indicates the width of the distribution function. The log-normal asymmetric probability peak distribution is described as the single-tailed probability distribution of any random variable whose logarithm is normally distributed\textsuperscript{77}. Sigma Plot version 11.0 (Systat software, Inc., Chicago, IL) was used to automatically estimate the adhesion affinities with the maximum probability of occurrences ($x_o$) for the bacterial cells grown at five different temperatures.

2.4 Statistical description of AFM data. Statistical tests were used to determine whether the adhesion strength of $L$. monocytogenes EGDe grown at various temperatures to silicon nitride quantified in terms of force or energy are different from each other. In addition, brush thicknesses and grafting densities of bacterial surface biopolymers were compared statistically as a function of the growth temperature. The nonparametric group comparisons Dunn’s ranks test available in Sigma Plot version 11.0 (Systat software, Inc., Chicago, IL) was applied to the data.

2.5 Results

2.5.1 Distribution of adhesion affinities as a function of temperature of growth. Our data presented in Figures 2.3 and 2.4 were heterogeneous and spanned a range of forces and energies for all growth temperatures investigated. To describe the heterogeneity in the distribution of adhesion force and adhesion energy values quantified between silicon nitride and $L$. monocytogenes cells grown at various temperatures, log-normal dynamic peak function was used to fit the data shown in the probability histograms of adhesion forces and energies (Figures 2.3 and 2.4). As can be seen from Figures 2.3 and 2.4, the distributions of adhesion affinities
quantified between the AFM tip and the bacterial surface biopolymers were wider for the cells grown at high temperatures (30, 37 and 40 °C) compared to those obtained for cells grown at lower temperatures (10 and 20 °C). In addition, the log-normal fits of the data indicated that the most probable adhesion force and energy quantified for the cells grown at 30 °C (0.468 nN and 141.0 AJ, respectively) were much higher than those of quantified at higher (37 and 40 °C) or lower (20 and 10 °C) temperatures, respectively (Table 2.1).

To further describe the data in the histograms of Figures 2.3A – E and 2.4A – E, the mean and median values of the distributions were compared (Figures 2.3F and 2.4F, Table 2.1). As can be seen from the trends of the means of adhesion values as functions of temperature of growth shown in Figures 2.3F and 2.4F, a transition in the adhesion affinity was observed for cells grown at 30 °C (0.494 nN, 147.6 AJ) in comparison to other lower or higher temperatures investigated. Pair-wise multiple comparison procedure (Dunn’s method) indicated that, adhesion force values identified for each growth temperature were statistically and significantly different from each other ($P < 0.05$). However, the computed energies for the 10 and 20 °C pair and for the 37 and 40 °C pair were not-statistically different in pairs but significantly different ($P < 0.05$) from the computed energies for cells grown at 30 °C. The mean values obtained for the adhesion force and energy were on average 23% and 29% higher than the median adhesion force and energy values obtained for the same data for all temperatures investigated, respectively (Table 2.1). The mean adhesion force and energy values were on average 18% and 21% higher than the most probable values obtained from the log-normal dynamic peak function fits, respectively. However, the trends of the median and the most probable values of adhesion forces and energies as functions of temperature of growth were very similar to the trends of adhesion data shown in Figures 2.3F and 2.4F.
2.5.2 Protein content of bacterial cells as a function of the temperature of growth. The amounts of proteins of the bacterial cells grown at five different temperatures were determined by colorimetric measurements. The amounts of proteins for the bacterial cells grown at 30 °C were found to be on average 65% higher than those obtained at other temperatures investigated (Table 2.1). The trend of the proteins’ content of the bacterial cells as a function of the temperature of growth was similar to the trends observed for adhesion affinities (Figures 2.3F and 2.4F).

Table 2.1: A summary of the most probable adhesion affinities ($x_o$) quantified by fitting log-normal dynamic peak function to the adhesion force (white filled rows) and adhesion energy (gray filled rows) data collected between L. monocytogenes EGDe individual cells grown at 10, 20, 30, 37 and 40 °C and silicon nitride under water. In addition, the quality of the fitting judged by the coefficient of correlation ($r^2$) values, and the mean, median, range and standard error of the mean (SEM) of all the data shown in Figures 2.3A-E and 2.4A-E, and the total protein contents of the bacteria are given below.

<table>
<thead>
<tr>
<th></th>
<th>10°C</th>
<th>20°C</th>
<th>30°C</th>
<th>37°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x_o$ (nN)</td>
<td>0.094</td>
<td>0.114</td>
<td>0.468</td>
<td>0.249</td>
<td>0.171</td>
</tr>
<tr>
<td>$r^2$</td>
<td>1.00</td>
<td>1.00</td>
<td>0.97</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td>Mean (nN)</td>
<td>0.110</td>
<td>0.160</td>
<td>0.494</td>
<td>0.335</td>
<td>0.199</td>
</tr>
<tr>
<td>SEM (nN)</td>
<td>0.004</td>
<td>0.007</td>
<td>0.006</td>
<td>0.008</td>
<td>0.004</td>
</tr>
<tr>
<td>Median (nN)</td>
<td>0.081</td>
<td>0.105</td>
<td>0.434</td>
<td>0.247</td>
<td>0.168</td>
</tr>
<tr>
<td>Range (nN)</td>
<td>0.996</td>
<td>0.987</td>
<td>2.158</td>
<td>1.724</td>
<td>1.592</td>
</tr>
<tr>
<td># of adhesion peaks</td>
<td>789</td>
<td>435</td>
<td>2228</td>
<td>1165</td>
<td>1749</td>
</tr>
<tr>
<td>Number of cells</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>10°C</th>
<th>20°C</th>
<th>30°C</th>
<th>37°C</th>
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<tr>
<td>$x_o$ (AJ)</td>
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<td>23.70</td>
<td>140.97</td>
<td>51.00</td>
<td>64.16</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.99</td>
<td>0.99</td>
<td>0.93</td>
<td>0.99</td>
<td>0.96</td>
</tr>
<tr>
<td>Mean (AJ)</td>
<td>25.04</td>
<td>26.32</td>
<td>147.61</td>
<td>80.96</td>
<td>73.76</td>
</tr>
<tr>
<td>SEM (AJ)</td>
<td>2.72</td>
<td>2.40</td>
<td>6.47</td>
<td>6.59</td>
<td>3.97</td>
</tr>
<tr>
<td>Median (AJ)</td>
<td>14.24</td>
<td>16.74</td>
<td>134.92</td>
<td>45.93</td>
<td>61.96</td>
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<tr>
<td>Range (AJ)</td>
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<td>220.70</td>
<td>454.24</td>
<td>474.93</td>
<td>309.28</td>
</tr>
<tr>
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<td>165</td>
<td>182</td>
<td>210</td>
<td>186</td>
<td>191</td>
</tr>
<tr>
<td>Proteins amount (g/L)</td>
<td>0.006</td>
<td>0.017</td>
<td>0.035</td>
<td>0.019</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Figure 2.3 A-E) Histograms that show the distribution of *adhesion force* (nN) values. Solid lines in the histograms indicate the log-normal probability distribution function (eq. 2.15) fits to the adhesion force data. The qualities of the fits described in terms of $r^2$ are given in Table 2.1. F) A bar-graph that shows the means of the distributions shown in Figures 2.3A-E as a function of the bacterial temperature of growth. Error bars indicate the values of the standard error of the mean.
Figure 2.4 A-E) Histograms that show the distribution of adhesion energy (AJ) values. Solid lines in the histograms indicate the log-normal probability distribution function (eq. 2.15) fits to the adhesion energy data. The qualities of the fits described in terms of $r^2$ are given in Table 2.1.

F) A bar-graph that shows the means of the distributions shown in Figures 2.4A-E as a function of the bacterial temperature of growth. Error bars indicate the values of the standard error of the mean.
2.5.3 Quantification of bacterial surface biopolymer brush thickness and grafting density as a function of temperature of growth. Steric repulsions measured between the bacterial surface biopolymers and the AFM tip were always observed in the approach data for all the growth temperatures investigated (Figure 2.5A). These continuous and monotonically increasing forces were modeled using equation 2.3, to estimate the bacterial surface biopolymer brush thickness ($L_0$) and grafting density ($\Gamma$) as a function of the temperature of growth (Table 2.2).

For bacterial cells grown at 30 °C, the height of the bacterial surface biopolymer brush ($176 \pm 13$ nm) was found to be on average 42% longer than that found at 20, 37 or 40 °C, and 60% longer than that found at 10 °C (Figure 2.5B, Table 2.2). Pair-wise multiple comparison procedure (Dunn’s method) indicated that, the computed values of bacterial surface biopolymer brush thickness for 20, 37 and 40 °C were not-statistically different from each other, but significantly different ($P < 0.05$) from those estimated for cells grown at 10 and 30 °C. In comparison, the grafting densities of the bacterial surface biopolymer brushes for cells grown at 30 °C ($2.4 \times 10^{16} \pm 0.2 \times 10^{16}$ biopolymers/m$^2$) and 37 °C ($2.2 \times 10^{16} \pm 0.2 \times 10^{16}$ biopolymers/m$^2$) were not-statistically different from each other, but significantly different ($P < 0.05$) and on average 38% larger than those found for cells grown at 10, 20 and 40 °C (Figure 2.5C, Table 2.2). Grafting densities of bacterial surface biopolymer brushes estimated for cells grown at 10, 20 and 40 °C were not statistically significantly different from each other. The longer and denser bacterial surface biopolymer brushes observed for cells grown at 30 °C compared to those observed at 37 °C, 20-40 °C pair, and 10 °C, respectively were correlated well with the higher bacterial adhesion strength to silicon nitride surface as can be seen from Figure 2.5D. The calculated values of $L_0\Gamma$ (# biopolymers/nm) for the cells grown at 30 °C were significantly
different \((P < 0.05)\) and 47\%, ~64\% and 77\% higher than those obtained at 37 °C, 20-40 °C pair, and 10 °C, respectively.

Table 2.2: A summary of the steric model fitting parameters, and the quality of the fits described in terms of \(r^2\) as a function of growth temperature. Errors are given in terms of the standard error of means. \(n\) values represent the number of individual approach curves that were fitted at each temperature investigated.

<table>
<thead>
<tr>
<th></th>
<th>10°C</th>
<th>20°C</th>
<th>30°C</th>
<th>37°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>(L_o) (nm)</td>
<td>70 ± 6</td>
<td>102 ± 7</td>
<td>176 ± 13</td>
<td>101 ± 10</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>(\Gamma \times 10^{-16}) (m(^2))</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>(L_o \times \Gamma) (nm(^4))</td>
<td>0.92 ± 0.06</td>
<td>1.5 ± 0.10</td>
<td>4.03 ± 0.12</td>
<td>2.13 ± 0.22</td>
<td>1.47 ± 0.08</td>
</tr>
<tr>
<td>(r^2)</td>
<td>0.96 ± 0.01</td>
<td>0.95 ± 0.01</td>
<td>0.99 ± 0.00</td>
<td>0.97 ± 0.01</td>
<td>0.98 ± 0.00</td>
</tr>
<tr>
<td>(n)</td>
<td>131</td>
<td>135</td>
<td>184</td>
<td>167</td>
<td>144</td>
</tr>
</tbody>
</table>
Figure 2.5 A) A comparison between the representative approach curves measured between *L. monocytogenes* cells and silicon nitride in water. Solid lines indicate the steric model fits to the data. B-C) Bar-graphs that show the means of the biopolymer brush thicknesses (*L*<sub>0</sub>, nm) and grafted densities (Γ, m<sup>-2</sup>) estimated for *L. monocytogenes* EGDe cells as a function of the bacterial growth temperature. Error bars indicate the standard error of the means. D) A correlation between the calculated values of *L*<sub>0</sub>Γ (# biopolymers/nm) and the most probable adhesion force values (nN) (data in circles), and the mean adhesion force values (nN) (data in squares) quantified between the AFM tip and the surface biopolymers of the bacteria grown at the five temperatures investigated. Solid and dashed lines represent the linear fits to the data in circles (r<sup>2</sup> = 0.98), and data in squares (r<sup>2</sup> = 0.95), respectively.

### 2.5.4 Physiochemical properties of bacterial cells as a function of temperature of growth.

The electrophoretic mobility values of *L. monocytogenes* EGDe cells grown at five different temperatures, reached to non-zero asymptotic values as the salt concentration increased (Figures
which is characteristic of “soft” particles. By fitting the soft-particle DLVO theory to the measured electrophoretic mobility data quantified as a function of ionic strength (IS) (Figures 2.6A – E), the fitting parameters; $ZN$ and $\frac{1}{\lambda_s}$ were determined (Table 2.3). The parameter $ZN$ (M) representing the spatial charge density distribution in the polyelectrolyte region of the soft particle was the lowest for the cells grown at 30 °C (-0.008 M) followed by 37, 40, 20, and 10 °C, respectively. $ZN$ values quantified for 10-20 °C (-0.020 and -0.019 M, respectively) and 37-40 °C (-0.014 and -0.016, respectively) were very close to each other in pairs. In comparison, the softness parameter $\frac{1}{\lambda_s}$ (nm) was found to be the highest for cells grown at 30 °C (5.70 nm), followed by 37, 40, 20, and 10 °C, respectively. The $\frac{1}{\lambda_s}$ values were also very close to each other for the cells grown at the pairs of 10-20 °C (3.14 and 3.19 nm, respectively), and 37-40 °C (4.27 and 3.91 nm, respectively).

The bacterial surface zeta potentials ($\psi_o$, mV) under filtered DI water (IS of 0.0027 M) and salted water solutions (IS values of 0.02, 0.04, 0.06, 0.1 and 0.15 M NaCl) were calculated by using the $ZN$ value obtained for each temperature. The trends observed for the bacterial surface potentials calculated in salted water (Figure 2.6F, Table 2.3) and in DI water (Table 2.3) were similar to the trends of adhesion affinities (Figures 2.3F and 2.4F) of the cells grown at the five different temperatures investigated. The bacterial cells grown at 30 °C were on average 42% less electrostatically negative in DI water and all salted water solutions compared to cells grown at other lower or higher temperatures. The average contact angle values tested on L. monocytogenes EGDe grown at various temperatures ranged from 26.0 ° to 32.4 ° for water and from 39.5 ° to 50.0 ° for diiodomethane (Table 2.3).
The values of apolar surface tension component ($\gamma_{iLW}^i$) for the bacterial cells obtained through contact angle measurements ($33.3 \pm 1.9 \text{ mJ/m}^2$), and the values of individual Hamaker constant ($A_{i}$) calculated by using $\gamma_{iLW}^i$ for bacterial cells ($6.18 \times 10^{-20} \pm 0.35 \times 10^{-20} \text{ J}$) were relatively close to each other at all temperatures of growth investigated (Table 2.3). The effective Hamaker constant for the interacting system ($A_{132}$), calculated for cells grown at 30, 37 and 40 °C were similar in magnitudes ($2.35, 2.42$ and $2.24 \text{ J}$, respectively), and on average 32% higher than those values calculated for the cells grown at 20 and 10 °C ($1.93$ and $1.66 \text{ J}$, respectively).

**Table 2.3**: A summary of bacterial physiochemical properties as a function of temperature of growth. Errors in contact angles are in terms of the standard error of means.

<table>
<thead>
<tr>
<th></th>
<th>10°C</th>
<th>20°C</th>
<th>30°C</th>
<th>37°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Z_N$ (M)</td>
<td>-0.020</td>
<td>-0.019</td>
<td>-0.008</td>
<td>-0.014</td>
<td>-0.016</td>
</tr>
<tr>
<td>$\frac{1}{\lambda_s}$ (nm)</td>
<td>3.14</td>
<td>3.19</td>
<td>5.70</td>
<td>4.27</td>
<td>3.91</td>
</tr>
<tr>
<td>$\psi_o$ (mV) (water)</td>
<td>-32.22</td>
<td>-31.14</td>
<td>-16.77</td>
<td>-25.05</td>
<td>-28.19</td>
</tr>
<tr>
<td>$\psi_o$ (mV) (0.02M NaCl)</td>
<td>-6.29</td>
<td>-5.97</td>
<td>-2.56</td>
<td>-4.32</td>
<td>-5.13</td>
</tr>
<tr>
<td>$\psi_o$ (mV) (0.04M NaCl)</td>
<td>-3.19</td>
<td>-3.03</td>
<td>-1.28</td>
<td>-2.18</td>
<td>-2.59</td>
</tr>
<tr>
<td>$\psi_o$ (mV) (0.06M NaCl)</td>
<td>-2.14</td>
<td>-2.02</td>
<td>-0.86</td>
<td>-1.45</td>
<td>-1.73</td>
</tr>
<tr>
<td>$\psi_o$ (mV) (0.10M NaCl)</td>
<td>-1.28</td>
<td>-1.22</td>
<td>-0.51</td>
<td>-0.87</td>
<td>-1.04</td>
</tr>
<tr>
<td>$\psi_o$ (mV) (0.15M NaCl)</td>
<td>-0.86</td>
<td>-0.81</td>
<td>-0.34</td>
<td>-0.58</td>
<td>-0.69</td>
</tr>
<tr>
<td>$\Theta$ (water)</td>
<td>32.4 ± 0.6</td>
<td>29.3 ± 0.6</td>
<td>27.8 ± 0.4</td>
<td>26.0 ± 0.8</td>
<td>28.5 ± 0.7</td>
</tr>
<tr>
<td>$\Theta$ (diododethane)</td>
<td>49.8 ± 1.5</td>
<td>46.4 ± 0.7</td>
<td>40.4 ± 0.4</td>
<td>39.5 ± 0.4</td>
<td>42.0 ± 0.4</td>
</tr>
<tr>
<td>$\gamma_{iLW}^i$ (mJ/m$^2$)</td>
<td>30.54</td>
<td>32.09</td>
<td>34.64</td>
<td>35.03</td>
<td>33.97</td>
</tr>
<tr>
<td>$A_{11} (10^{-20})$ (J)</td>
<td>5.68</td>
<td>5.96</td>
<td>6.44</td>
<td>6.51</td>
<td>6.31</td>
</tr>
<tr>
<td>$A_{132} (10^{-21})$ (J)</td>
<td>1.66</td>
<td>1.93</td>
<td>2.35</td>
<td>2.42</td>
<td>2.24</td>
</tr>
</tbody>
</table>
Figure 2.6 Electrophoretic mobility of *L. monocytogenes* EGDe grown at (A) 10°C, (B) 20°C, (C) 30°C, (D) 37°C, and (E) 40°C as a function of ionic strength (M). Each point in Figures 2.6A-E represents an average of five experimental measurements with error bars representing the standard deviation. The solid lines represent the soft-particle DLVO theory fits to the data. The qualities of the fits were judged by the coefficient of correlation ($r^2$) values which were found to
be 0.86, 0.95, 0.91, 0.90 and 0.93 for data in Figures 2.6A-E, respectively. F) A scatter plot that shows the surface potential of the bacterial cells evaluated using equation 2.5 as a function of the bacterial temperature of growth and the ionic strength used in the electrophoretic measurements.

2.5.5 Soft-particle DLVO energy profiles as a function of temperature of growth. Results obtained from electrophoresis measurements of charge and contact angle measurements of bacterial surface tensions were used to predict the energy profiles between \textit{L. monocytogenes} EGDe cells and silicon nitride in water (Figure 2.7A) using soft-particle DLVO theory (equations 2.12-2.14). As can be seen from Figure 2.7A, all soft-particle DLVO energy profiles displayed a peak at approximately 1.6 nm separation distance, which is close to the theoretical closest separation distance upon approach$^{67}$. The maximum soft-particle DLVO energy barrier needed for the bacteria to overcome in order to adhere to the silicon nitride cantilever was on average \(~37\%\) lower for cells grown at 30 °C (10.3 $k_B T$) compared to that obtained for cells grown at 37, 40, 20 and 10 °C, respectively (Figure 2.7B, Table 2.4), which agreed well with the bacterial adhesion energy profile computed using AFM force-distance data as a function of temperature of growth (Figure 2.4F, Table 2.1). Figure 2.7C shows that the predicted maximum energy barriers to adhesion to silicon nitride calculated based on soft-particle DLVO theory and the computed bacterial adhesion energies to silicon nitride $E_{adh}(AFM)$ estimated based on AFM measurements as a function of temperature of growth investigated are linearly correlated ($r^2=0.94$).
Table 2.4: Maximum and total energy barriers to bacterial adhesion to silicon nitride in water, observed at approximately 1.60 nm separation distance upon approach.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$E_{\text{max}}(k_B T)$</th>
<th>$E_{\text{LW}}(k_B T)$</th>
<th>$E_e(k_B T)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10°C</td>
<td>17.52</td>
<td>-1.23</td>
<td>18.75</td>
</tr>
<tr>
<td>20°C</td>
<td>17.10</td>
<td>-1.43</td>
<td>18.53</td>
</tr>
<tr>
<td>30°C</td>
<td>10.29</td>
<td>-2.02</td>
<td>12.31</td>
</tr>
<tr>
<td>37°C</td>
<td>14.84</td>
<td>-2.07</td>
<td>16.91</td>
</tr>
<tr>
<td>40°C</td>
<td>16.09</td>
<td>-1.78</td>
<td>17.87</td>
</tr>
</tbody>
</table>

Figure 2.7 A) Total energy profiles calculated between silicon nitride AFM tip and *L. monocytogenes* EGDe cells grown at 10, 20, 30, 37, and 40°C using soft-particle DLVO theory (equations 2.12-2.14) B) A scatter graph that shows the maximum energy barrier $E_{\text{max}}(k_B T)$ to adhesion at approximately 1.60 nm as a function of temperature of growth. The quantified values for $E_{\text{max}}(k_B T)$ are given in Table 2.4. C) A correlation between the maximum energy barriers to adhesion to silicon nitride $E_{\text{max}}$ (AJ) based on soft-particle DLVO theory and the most probable adhesion energies (AJ) (data in circles), and the mean adhesion energies (data in squares) quantified between the silicon nitride AFM tip and the bacteria grown at the five temperatures investigated. Solid and dashed lines represent the linear fits to the data in circles ($r^2 = 0.94$), and data in squares ($r^2 = 0.94$), respectively.
2.6 Discussion

2.6.1 Effects of temperature of growth on bacterial adhesion forces and energies. Adhesion forces and energies measured between the biopolymers of L. monocytogenes and silicon nitride in water as a function of the bacterial growth temperature revealed similar trends (Figures 2.3 and 2.4). However, when adhesion forces and adhesion energies were compared for their abilities to resolve differences in the adhesion strengths of L. monocytogenes to silicon nitride in water as a function of growth temperature, the use of adhesion forces was preferred. This was attributed to the fact that statistically significant differences were obtained among adhesion forces quantified at the five temperatures of growth investigated (P<0.05, Dunn’s method). In comparison, when energy was used to quantify bacterial adhesion, the computed energies for the 10 and 20 °C pair and for the 37 and 40 °C pair were not-statistically different in pairs but significantly different from the computed energies for cells grown at 30 °C.

As can be seen from Figures 2.3A – E and 2.4A – E, the distributions of adhesion affinities were wider for cells grown at high temperatures (30, 37 and 40 °C) compared to those of cells grown at low temperatures (10 and 20 °C) (see Table 2.1 for the range of adhesion data). The heterogeneities in the AFM measurements of bacterial adhesion23-42, and the use of dynamic peak functions to describe the heterogeneities in AFM data24,27,41 are commonly reported in the literature. Heterogeneities in AFM data have been largely attributed to the wide array of molecules present on the bacterial surface26,27,37,38,42. The cell wall of L. monocytogenes is primarily composed of peptidoglycans and proteins44. The secondary cell-wall components are polyanionic polymers such as teichoic acids (TAs) and lipoteichoic acids (LTAs)78. The wider distributions in the AFM adhesion affinity data observed for cells grown at the favorable temperatures of growth (30 and 37 °C) can be attributed to the presence of higher amount of
proteins on the bacterial surface as was shown from our colorimetric measurements of protein content (Table 2.1).

The variations in the adhesion affinities observed for cells grown at the temperatures investigated can be correlated with the variations in the composition of bacterial surface molecules expressed at different temperatures of growth. Our colorimetric measurements of bacterial content of proteins indicated that cells grown at 30 °C were characterized by higher protein content compared to cells grown at respectively lower and higher temperatures (Table 2.1). Literature studies as well indicate that *L. monocytogenes* adjust the expression of their surface virulence proteins at various temperatures of growth\(^{17,79}\). Most of the virulence proteins that have been identified in *L. monocytogenes* such as *inlA*, *inlB* and *inlC* are under the control of positive regulatory factor A (PrfA)\(^ {46}\). PrfA, is a thermo-responsive virulence regulator\(^ {18,46,79-81}\). PrfA-mediated virulence gene transcription is activated at temperatures above 30 °C and is shut off at around 20 °C or below\(^ {81}\). Previously, it was reported that PrfA-mediated internalin genes (*inlA*, *inlB*, and *inlC*) showed lower levels of transcripts in *L. monocytogenes* EGDe grown at 16 °C than those grown at 30, 37 or 42 °C, while the levels of transcripts were not significantly different when the bacteria were grown at 30, 37 and 42 °C\(^ {17}\). In addition, other internalin-like virulence genes (*inlG*, *inlJ*, lmo0514, and lmo1290) also encoding peptidoglycan-bound proteins showed the highest levels of transcripts at 30 °C and/or 37 °C followed by 42 and 16 °C, respectively\(^ {17}\). Nevertheless, more recently a positive correlation between the adhesion strength of *L. monocytogenes* to inert glass surface and the relative mRNA levels of *inlA* and *inlB* was established\(^ {82}\). As it was reported, the relative mRNA levels of *inlA* and *inlB* genes of wild-type strains of *L. monocytogenes* isolated from catfish processing plants, in addition to one ATCC
7644 strain and *L. monocytogenes* EGDe, all of which were grown at 30 °C were inversely correlated with the ease of cell removal from a glass surface after attachment.

Interestingly, our molecular-scale adhesion results agree well with the previous studies which qualitatively investigated the adhesion strength of *L. monocytogenes* to model surfaces in terms of macro-scale biofilms and individual cells as functions of temperature of growth. As it was found, the strength of biofilm formation of *L. monocytogenes* EGDe onto the polystyrene and cellulose membranes (OD595) were superior at the incubation temperature of 30 °C, while the biofilm strength was not significantly different at the incubation temperatures of 18, 25 or 37 °C. The observed trend in the biofilm strength was attributed to the amount of polysaccharide in the extracellular polymeric matrix (EPS) of the biofilms which as well showed the same temperature dependency as the strength of the biofilm, both with a maximum at 30 °C. The number of adherent *L. monocytogenes* NCTC 11994 cells to polycarbonate membranes was as well higher at 30 °C compared to that measured at 37 °C or 25 °C.

### 2.6.2 Effects of temperature of growth on the mechanical properties (*L*₀ and Φ) of bacterial surface biopolymers

The biopolymers of the surface of *L. monocytogenes* EGDe changed their structure depending upon the temperature used to grow the bacterial cells. Biopolymers were the most extended and most crowded when the bacterial cells were grown at optimum growth temperature of 30 °C (*L*₀ =176 ± 13 nm, and 2.4×10¹⁶ ± 0.2×10¹⁶ biopolymers/m²). Previously, we have shown that *L. monocytogenes* brushes are charged; acting as polyelectrolyte brushes. Polyelectrolyte brushes are known to stretch in the direction normal to the grafting density. The length to which chains are extended is largely controlled by the free energy of the chains. The free energy of a biopolymer chain is the summation of the entropic interactions at that given temperature and the energy caused by excluded volume interactions and incorporates both...
electrostatic and non-electrostatic effects\textsuperscript{86}. Since entropy (energy dispersal) is directly proportional to temperature as well as to the biopolymer density on the bacterial surface, entropy of the biopolymer chains at 30 °C is expected to be lower than that of chains at 37 °C and higher than that at 40, 20 and 10 °C, respectively, as can be seen from the values of apolar surface tension components for the bacterial cells ($\gamma_i^{L,W}$); caused by dispersion energy between molecules\textsuperscript{68} (Table 2.3). The surface potential of the cells is minimal at 30 °C compared to that at lower and higher temperatures investigated (Table 2.3); resulting in lower electrostatic interactions at 30 °C. Therefore, at 30 °C, the entropy will most likely counter-balance the electrostatic interactions in the chain, resulting in more neutral-like biopolymer chains. For neutral biopolymer brushes on a solid surface, short-range intermolecular repulsion causes the chains to partially stretch in the direction normal to the grafting surface\textsuperscript{58,59}. In comparison, at lower and higher temperatures investigated, the electrostatic effects observed for the cells at these temperatures (Table 2.3) will most likely dominate and thus held responsible for extending the biopolymers chains. Because electrostatic interactions are long-range in nature, the chains can become stretched even at lower grafting densities\textsuperscript{74}.

When compared to other studies in the literature, the mechanical properties of \textit{L. monocytogenes} EGDe biopolymers were in reasonably good agreement with the mechanical properties reported for the surface biopolymers of other microorganisms such as \textit{Escherichia coli} JM109 grown at 37 °C\textsuperscript{24}, \textit{Pseudomonas putida} KT2442\textsuperscript{25}, \textit{Pseudomonas aeruginosa} (ATCC 10145)\textsuperscript{31}, and \textit{Acidithiobacillus ferrooxidans} (ATCC 23270)\textsuperscript{34}.

\textbf{2.6.3 Effect of temperature of growth on bacterial surface potential.} The calculated values of \textit{L. monocytogenes} surface potentials indicated that the bacterial cells grown at 10 °C were the most electrostatically negative followed by the cells grown at 20, 40, 37 and 30 °C, respectively.
Our results of bacterial surface potentials are consistent with those previously reported. For example, *L. monocytogenes* ScottA incubated at low temperatures (15 and 20 °C) demonstrated a higher negative charge compared to when incubated at 37 °C at pH 7. The high negative charge of *L. monocytogenes* ScottA grown at 15 and 20 °C was linked to the presence of temperature-dependent production of flagella by the bacteria. *L. monocytogenes* were observed to be motile via flagella below 30 °C, but at temperatures above 30 °C such as those present in vivo, they lost their motility. It was noted that the rich protein (and protein-associated COOH/COO²) content of flagella could explain the high specific electric properties of cells cultivated at 15 or 20 °C. Motility-associated highly charged proteins may account for the higher negative surface potentials observed for *L. monocytogenes* EGDe cells grown below 30 °C.

Previously, it was reported that *L. monocytogenes* and *L. innocua*, regardless of the temperature or the stage of growth, lacked an isoelectric point over the pH range of 2 to 7. This was linked to the presence of phosphate groups with a very low pKa, suggesting a very high extent of dissociation in the phosphodiester bridges as in teichoic acids. Most interestingly, growth temperature was observed to have a strong influence on the expressions levels of lipoteichoic acids (LTA) in the cell wall of *L. monocytogenes* EGDe. By using nuclear magnetic resonance (NMR) and mass spectrometry (MS) technologies, it has been revealed that there are two structural variants of LTA in the cell wall of *L. monocytogenes* EGDe, one is (LTA1) similar to that observed in other bacteria like *Staphylococcus aureus* or *Streptococcus pneumonia*, whereas the other one (LTA2) has emerged for the first time. It was found that the expression level of LTA1 was comparable for the cells grown at room temperature and 37 °C, while the expression of LTA2 was quite higher for the cells grown at 37 °C. Since the high expression of LTA in the bacterial cell has been related to the high content of phosphate...
groups, this may explain the high negative surface potential of \textit{L. monocytogenes} EGDe grown at 37 °C. For the cells grown at 30 °C, the low negative surface potential may reflect mixed contributions of protein- or peptidoglycan-associated COO⁻ and NH₃⁺ groups, resulting in a more neutral but non-uniform charge distribution on the cell surface.

\textbf{2.6.4 Effect of temperature of growth on bacterial surface hydrophilicity.} The average contact angle values tested on \textit{L. monocytogenes} EGDe grown at various temperatures ranged from 26.0° to 32.4° for water (Table 2.3). Since hydrophobic surfaces exhibit water contact angles higher than 65°, the obtained contact angles of water indicated that \textit{L. monocytogenes} EGDe were hydrophilic when grown at all temperatures investigated. This finding is consistent with previous reports; as \textit{L. monocytogenes} always possessed hydrophilic properties irrespective of the temperature of growth or the pH of the growth media.

Since \textit{L. monocytogenes} EGDe were mostly hydrophilic when grown at 37 °C, compared to when grown at 30, 40, 20 and 10 °C, respectively (see Table 2.3 for water contact angles), adhesion to the hydrophilic silicon nitride AFM tip was expected to be the highest for the cells grown at 37 °C. However, the negative surface potential of the cells was minimal when grown at 30 °C (Table 2.3), which resulted in a higher adhesion to the negatively charged silicon nitride surface compared to that at 37 °C and other temperatures investigated (Figures 2.3F and 2.4F), suggesting that repulsive electrostatic interactions were dominant compared to the attractive van der Waals interactions between the bacterial cells and silicon nitride. This was confirmed based on the soft-particle DLVO predictions of energy barriers given in Table 2.4.

\textbf{2.6.5 Relationship between bacterial adhesion strength and predicted energy barriers based on soft-particle DLVO theory calculations.} Soft-particle DLVO theory predicted a lower value of the maximum energy barrier to the adhesion of the bacteria grown at 30 °C compared to the
energy barriers predicted at 37, 40, 20 and 10°C, respectively (Table 2.4). The soft-particle DLVO predictions correlated linearly with the AFM results of adhesion strength of bacteria to the silicon nitride surface in water at all investigated (Figure 2.7C, $r^2 = 0.94$). Our results suggest that the reduced electrostatic forces which were partially balanced by attractive van der Waals forces at 30 °C are likely responsible for the lower barriers to adhesion predicted for these cells and thus the higher adhesion measured by AFM.

2.7 Summary

The biopolymers on the surface of L. monocytogenes EGDe were shown to undergo a temperature-induced structural change from extended and dense biopolymers at 30 °C to shorter and less crowded at lower (10 and 20 °C) and higher (37 and 40 °C) temperatures of growth (Table 2.2). Accompanying this structural change, greater adhesion forces and energies were quantified between the bacterial surface biopolymers and silicon nitride in water at 30 °C compared to other lower and higher temperatures of growth investigated. In addition, soft-particle DLVO theory predicted lower values of the maximum energy barriers to adhesion at 30 °C. Our results suggest that the optimum temperature of growth of L. monocytogenes EGDe was in fact 30 °C. The enhanced adhesive capacity for the cells when grown especially at 30 °C was related to both their longer and denser biopolymers on their surfaces, furnishing much more binding arms for the bacteria to attach to silicon nitride; higher proteins’ content, providing more adhesive components to attach to the surface; and their lower negative surface potential, resulting in a weaker electrostatic repulsion between the cell surface and silicon nitride compared to those observed when grown at 37, 40, 20 and 10 °C, respectively.

The lack of genetic studies that detail the biopolymer composition of L. monocytogenes EGDe cells at temperatures relevant to food processing environments was prohibitive to
thorough explanations of the adhesion trends observed at 10, 20 and 40 °C. Irrespective of that, we were able to display the strong influence of the bacterial temperature of growth on the mechanical properties of bacterial surface biopolymers, the physiochemical properties of bacterial cells, and total proteins’ content on the bacterial initial attachment to silicon nitride.

2.8 Acknowledgements

We would like to thank Prof. Markus Flury; a professor of Soil Physics/Vadose Zone Hydrology in the Department of Crop and Soil Sciences at Washington State University for allowing us to use his goniometer and Zeta potential analyzer to do the contact angle and electrophoresis measurements, his PhD student Nirmalya Chatterjee, and our former co-workers Dr. Bong-Jae Park and Corina Chilibeck for helping us to do the contact angle and electrophoresis measurements reported in this study. We would like to thank as well the National Science Foundation grant EEC-0823901 for financial support of this work.
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Chapter 3

Impact of Ionic Strength of Growth on the Physiochemical Properties, Structure and Adhesion of *L. monocytogenes* Polyelectrolyte Brushes to a Silicon Nitride Surface in Water

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Abstract

The adhesion energies between pathogenic *L. monocytogenes* EGDe to a model surface of silicon nitride were quantified using atomic force microscopy (AFM) in water for cells grown in media of five different ionic strengths of NaCl (IS of 0.003M, 0.05M, 0.1M, 0.3M and 0.5M NaCl). The physiochemical properties of *L. monocytogenes* EGDe surface brushes were shown to have a strong influence on the adhesion of the microbe to the silicon nitride surface. The transitions in the adhesion energies, physiochemical properties and the structure of bacterial surface polyelectrolyte brushes were observed for the cells grown in the media of 0.1M added NaCl. Our results suggested that the highest long-range electrostatic repulsion which was partially balanced by the Lifshitz-van der Waals attraction for the cells grown at 0.1M was responsible for the highest energy barrier to adhesion for these cells as predicted by the soft-particle DLVO theory and the lower adhesion measured by AFM.

**Keywords:** *L. monocytogenes*, soft-particle DLVO, AFM, adhesion energy, surface potential, Donnan potential, steric model, polyelectrolyte brush layer, and ionic strength of growth.
3.1 Introduction

Although the food industry has made progress in reducing the prevalence of food-borne pathogens in the food processing environments, large multistate outbreaks due to the consumption of contaminated food by *Listeria monocytogenes* continue to occur\(^1\). This is in part because *L. monocytogenes* is primarily a soil microorganism and ubiquitously distributed in the environment\(^2,3\), and thus is a frequent contaminant of raw materials used in the food processing plants\(^2\). In addition, *L. monocytogenes* is a particularly difficult pathogen to eliminate from the food processing plants because of its propensity to adhere to food contact surfaces and to form biofilms\(^4-7\). The high degree of its adaptability to the environmental stresses\(^8-17\) is another reason for the difficulty in controlling the persistence and the spread of this pathogen in the food processing environments, since treatments used in food processing and preservation often utilize stressing agents to which *L. monocytogenes* is resistant\(^4,5,7,9\). For example, table food salt (Sodium Chloride, NaCl) is one of the most commonly employed agent for food preservation and has been used in various steps of food preparation to inhibit the growth of spoilage and pathogenic bacteria by increasing the ionic strength of the solution\(^2,4,7\). However, *L. monocytogenes* is highly resistant to osmotic stress and is able to survive even at ionic strength (IS) of 2M NaCl solution (10% w/v NaCl)\(^2,7\).

During food processing, *L. monocytogenes* can be exposed routinely to environments under different ionic strengths\(^2,4\). When transferred to a more favorable environment such as an environment under IS similar to that of water, *L. monocytogenes* can rapidly grow, colonize and persist on food contact surfaces\(^2,7\). Since, water is the main solvent used in the food processing industries and the main solvent used in preparing foods\(^18\), the bacteria exposed to different ionic
strengths at different food processing facilities will likely to be transferred to the environments under IS similar to that of water.

Osmo-adaptation in *L. monocytogenes* can involve both regulation at the gene expression level\(^7,13,19\), and physicochemical changes\(^20-23\) at the bacterial surface structural level which in turn can affect its adhesion affinity to surfaces. Understanding the adaptation mechanisms of *L. monocytogenes* to the changes in the environmental stresses, in relation to its interactions with the surfaces could lead to a better understanding and control of the contamination and spread of this pathogen in the food processing plants.

The current study provides a detailed investigation for the impact of five different ionic strength of growth (IS of growth media, and IS of 0.05M, 0.1M, 0.3M and 0.5M added NaCl in growth media) on the physiochemical properties of *L. monocytogenes* EGDe surface brushes and their adhesion to the model inert surface of silicon nitride in water. The physiochemical properties of the bacterial surface polyelectrolyte brush layer represented by surface charge and surface tension were quantified respectively from *electrophoresis* and *contact angle* measurements by using Ohshima’s soft-particle electrophoresis model\(^24\) and thermodynamic-based harmonic-mean (HM) model\(^25\). A steric model for bacterial surface polyelectrolyte brushes\(^26-28\) was applied to probe the changing structure of the brush layer as a function of ionic strength of growth.

The adhesion energies of the bacterial cells grown at different ionic strengths to the model inert surface of silicon nitride in water were measured using atomic force microscopy (AFM). Direct adhesion energy measurements were compared with the energy predictions based on soft-particle DLVO theory\(^24,29,30\) calculations. Finally, the effects of physiochemical
properties and the structure of bacterial surface polyelectrolyte brushes on the adhesion of the microbe to silicon nitride surface were investigated.

3.2 Materials and Methods

3.2.2 Bacterial cultures. Pathogenic *L. monocytogenes* EGDe was obtained from Prof. Mark Lawrence, an associate professor at the Department of Basic Sciences, the College of Veterinary medicine at Mississippi State University. Among the highly pathogenic *L. monocytogenes* strains, EGDe was chosen as our model strain because it has a fully sequenced and annotated genome. The strain was activated by growing for twelve hours at 30 °C in a temperature controlled shaker rotating at a 150 rpm in Difco™ brain heart Infusion broth (BHIB without dextrose). Following activation, cells were cultured in BHIB (dissolved in water with IS of 0.003 M), and in BHIB with added NaCl (IS of 0.05M, 0.1M, 0.3M and 0.5M added NaCl) at 30°C fixed temperature.

The Difco™ brain heart infusion broth (measured as pH 6.8 at 30 °C) contains 5g/L sodium chloride (NaCl) and 2.5g/L disodium phosphate (Na₂HPO₄) as buffers to prevent drastic shifts in pH during the growth of the bacterial cells, as the bacterial cells frequently produce acids as waste products during their growth. Since the bacterial cells were grown in the same media, the contribution of the buffers to the ionic strength of BHIB media were normalized for all conditions investigated. Therefore, the impact of ionic strength of growth on the physiochemical properties, structure and adhesion of *L. monocytogenes* polyelectrolyte brushes to silicon nitride surface in water was interpreted by the solvent concentrations (DI water, IS of 0.003M, and IS of 0.05M, 0.1M, 0.3M and 0.5M added NaCl).

Previously, the growth of *L. monocytogenes* in BioV LCGM™ BHIB media was monitored by the conductivity and pH measurements (initial pH 6.8 ± 0.2 at 37 °C). The pH of
the medium did not show a significant change during the first 6 hours of bacterial growth. However, it decreased from 6.7 to approximately 5.6 during the next 4 hours of growth with a 16.4% decrease of the initial pH. The same trend was as well observed for the conductivity measurements. Since *L. monocytogenes* cells metabolize the substrates in the media that are usually non-charged or weakly charged molecules into small and highly charged molecules, an increase in the ionic strength of the media can increase the conductivity. However it should be noted that the conductivity measurements monitored the total ionic composition change in the media, which included the release of H⁺ and other ions, whereas the pH measurement detected only the release of H⁺ ions.

In our study, the growth of *L. monocytogenes* cells at the five different ionic strengths of BHIB was monitored by reading the optical density of the culture every hour at a wavelength of 600 nm, until cells reached late exponential phase of growth, the stage at which they were harvested. The bacterial cells reached the late exponential phase after approximately ~ 5.5 hours for all conditions investigated, indicating that the pH shift during the bacterial growth was not significant based on the results of the study discussed above.

### 3.2.3 Atomic force microscopy measurements.

Prior to AFM force measurements, cultured bacterial cells in BHIB media adjusted to the ionic strength of interest were centrifuged twice at 5525g for 10 minutes. After centrifugation, cells were attached to gelatin-coated mica disks according to the procedure detailed elsewhere. All AFM force measurements were performed in TappingMode™ using a PicoForce™ Scanning Probe Microscope with a Nanoscope IIIa controller and extender module (Bruker AXS Inc., Santa Barbara, CA). Forces were measured under DI water using silicon nitride cantilevers (DNP-S cantilevers with 0.06 N/m nominal spring constant (Bruker AXS Inc., Santa Barbara, CA). We have shown previously that attaching
*L. monocytogenes* cells to gelatin-coated mica and performing force measurements on bacterial cells under water do not affect bacterial cell viability or integrity\(^{37}\).

The force constant of each cantilever was determined from the power spectral density of the thermal noise fluctuations in DI water\(^{38}\). On average, the spring constant was found to be 0.056 ± 0.004 N/m (n=7), very close to the nominal spring constant value reported by manufacturer in air. Once a bacterial cell had been located via topographical scanning, the oscillation of the cantilever was stopped and the extending and retracting deflection displacement curves measured between the silicon nitride tip and bacterial surface biopolymers were captured using the AFM software. Force measurements were made on a bacteria-free area of the gelatin-coated mica disk before and after making a measurement on a bacterial cell. Equality of the measurements ensured that the tip properties had not been altered by contact with the bacterial surface biopolymers.

For each ionic strength of growth investigated, 14 bacterial cells were examined from three different cultures. On each cell, 15 points were located on the cell surface to perform force measurements using the point and shoot feature of the AFM software. Retraction curves were measured at a rate of 580 nm/sec to minimize the hydrodynamic drag forces\(^{39}\), and at a resolution of 4096 points.

**3.2.4 Analysis of retraction curves.** Retraction curves were considered individually because of the complex and heterogeneous nature of the interactions observed between the bacterial surface biopolymers and the AFM silicon nitride cantilever\(^{34-37,40-43}\). Bacterial adhesion was quantified from the retraction curves in terms of *adhesion energy* in atto-Joule (AJ). For each retraction curve, the *adhesion energy* was computed as the area under the retraction force-distance curve.
using the Trapezoidal rule approximation of integrals\textsuperscript{43}, as we have detailed previously\textsuperscript{44} (Chapter 2).

3.2.5 Contact angle measurements. Prior to contact angle measurements, 20 ml of bacterial cells suspended in BHIB and grown as described above in the BHIB media of different ionic strengths were harvested and washed twice by centrifugation at 5525g for 10 minutes each round. The collected bacterial pellet was then re-suspended in a 20 ml of 0.2 μm filtered DI water. The bacterial solution was then filtered on a cellulose acetate filter membrane (pore diameter, 0.45 μm, Sartorius, Aubagne, France) using negative pressure. Bacterial densities on the membrane filters were determined to be between $1 \times 10^7 – 5 \times 10^7$ cells/mm\textsuperscript{2} corresponding to 40~200 layers of bacteria covering the filter membrane\textsuperscript{44}. To establish constant moisture content, the filters with bacteria on them were placed in a Petri-dish on the surface of 1% (wt/vol) agar prepared by dissolving Bacto agar (Difco, Detroit, Michigan) in filtered DI-water containing 10% (vol/vol) glycerol\textsuperscript{45}. Drying time for \textit{L. monocytogenes} lawns was determined to be \textasciitilde 40 min\textsuperscript{46}. Contact angles of \textit{L. monocytogenes} EGDe cells were quantified using two probing liquids characterized with different polarities. The liquids used were ultrapure water (18.2 MΩ·cm resistivity, Millipore Mili-Q Plus, Billerica, MA) and diiodomethane (99% pure, Alfa Aesar, Ward Hill, MA). The contact angles of the bacterial lawns were quantified using the sessile drop technique\textsuperscript{45} with a KRÜSS DSA100 drop shape analysis system (KRÜSS GmbH, Hamburg, Germany). For each measurement, 2 μL droplet volume of the liquid of interest was used. We have previously showed that the volume of the liquid drop was large enough compared to the roughness of the bacterial lawns\textsuperscript{44}. For each liquid used, at least 20 measurements were performed. All measurements were made at room temperature and at ambient humidity.
3.2.6 Electrophoretic Mobility Measurements. The electrophoretic mobilities of *L. monocytogenes* cells grown in BHIB media of different ionic strengths were measured by injecting 10 ml of bacterial cells using a syringe into a Zetasizer 3000 HSA (Malvern Instruments, Malvern, UK) at room temperature. Prior to measurements, the bacterial cells were harvested at the late exponential growth phase as described above and washed twice by centrifugation at 5525g for 10 min each round. The collected bacterial pellet was then diluted with 0.2 μm filtered DI water and mixed with a NaCl solution to make a suspension with an optical density (λ = 600 nm) of ~ 0.05, pH 7 and final ionic strengths of 0.02, 0.04, 0.06, 0.1 and 0.15 M NaCl. All electrophoretic mobility measurements were performed five times at room temperature.

3.3 Mathematical Modeling

3.3.1 Calculation of bacterial surface and Donnan potentials using the Ohshima’s soft-particle electrophoresis model. The electric properties of a particle can be characterized by the zeta potential, which is the electrical potential of the interface between the aqueous solution and the stationary layer of such a fluid attached to the particle surface. In general, the zeta potential of a particle (ζ, mV) is calculated from electrophoretic mobility measurements by using the Smoluchowski formula described by:

\[
\mu = \frac{\varepsilon_0 \varepsilon_r \zeta}{\eta}
\]  

(3.1)

where \(\mu\) is the electrophoretic mobility (m²/V.s), \(\varepsilon_0\) is the permittivity of vacuum (8.85 \times 10^{-12} C²/J.m), \(\varepsilon_r\) is the relative permittivity of the solvent (78 for water), \(\eta\) is the solvent viscosity (8.9 \times 10^{-4} Pa.s for water at room temperature). The Smoluchowski formula explains the migration of a hard-particle that is impenetrable to electrolytes in an external electric field."
However, the bacterial cell surface structure is much more complicated and diverse than the surface of an inert hard-particle, and covered by “soft” biopolymers which are penetrable to electrolytes such as proteins, peptidoglycans, lipopolysaccharides (LPS) and phospholipids. Ohshima therefore developed an electrophoretic model for soft-particles such as bacteria, which takes into account the effects of the particle softness and the penetration of the polyelectrolyte in the particle. The approximate electrophoretic mobility formula for soft particles is expressed as:

$$
\mu = \frac{e_0 e_r \psi_o/K_m + \psi_{DON}/\lambda_s}{\eta (1/K_m + 1/\lambda_s)} + eZN/\eta \lambda_s^2
$$

(3.2)

where $\psi_o$ is the soft-particle surface potential (mV), $K_m$ is the Debye-Hückel parameter for the polymer layer (nm$^{-1}$), $\psi_{DON}$ is the Donnan Potential of the polymer layer (mV), $e$ is the electron charge ($1.602 \times 10^{-19}$ C), $Z$ is the valence of the charged groups in the polymer brush, $N$ is the density of the charged groups in the polymer brush (M), and $1/\lambda_s$ is the softness parameter (nm).

The soft-particle surface potential ($\psi_o$, mV) and the Debye-Hückel parameter ($K_m$, nm$^{-1}$) can be written as:

$$
\psi_o = k_B T/z e \left( \ln \left( \frac{Z N}{2 \pi n^e} \right) + \left( \frac{Z N}{2 \pi n^e} \right)^2 + 1 \right)^{1/2} + \frac{2 \pi n^e}{Z N} \left( 1 - \left( \frac{Z N}{2 \pi n^e} \right)^2 + 1 \right)^{1/2}
$$

(3.3)

$$
K_m = \kappa \left[ 1 + \left( \frac{Z N}{2 \pi n^e} \right)^2 \right]^{1/4}
$$

(3.4)

where $k_B$ is the Boltzmann constant ($1.3807 \times 10^{-23}$ J/K), $T$ is the absolute temperature (298 K), $z$ is the valence of bulk ions, $n^e$ is the concentration of bulk ions (M), and $\kappa$ is the Debye-
Hückel screening length (nm\(^{-1}\)) given as \(\kappa = \left(\frac{1000N_A}{\varepsilon e_kT} \sum_{i=1}^{N} z_i^2 e^2 n_i^\infty \right)^{1/2} \) where \(N_A\) is the Avogadro’s constant (6.022 × 10\(^{23}\) mol\(^{-1}\)).

For soft particles, one has to consider not only the charge distribution outside the polyelectrolyte layer but also that inside the polyelectrolyte layer\(^{48}\). In addition to the surface potential which is defined as the potential at the boundary between the polyelectrolyte layer and the surrounding electrolyte solution, the Donnan potential, which is related to the volume density of fixed charges in the polyelectrolyte layer, plays an important role in describing the electric properties of soft particles\(^{24,48,49}\) (Figure 3.1). For particles covered by a thick polyelectrolyte layer, the charge potential deep inside the surface layer is practically equal to the Donnan potential\(^{48}\).

\[
\psi_{DON} = \frac{k_BT}{ze} \ln\left\{\frac{ZN}{2zn^\infty} + \left(\frac{ZN}{2zn^\infty}\right)^2 + 1\right\}^{1/2}
\]

As can be seen from equations 3.3, 3.4, and 3.5, the parameters \(\psi_o\), \(K_m\) and \(\psi_{DON}\) are all functions of ionic strength \((n^\infty, M)\) of the solvent. By fitting the soft-particle electrophoresis model (eq. 3.2) to the measured electrophoretic mobility data as a function of ionic strength of the solvent where the mobility measurements were performed (Figures 3.3A-E), the fitting parameters; \(ZN\) and \(1/\lambda_s\) were determined for \(L.\ monocytogenes\) EGDe cells grown in media of different ionic strengths. The parameter \(ZN\) representing the spatial charge density in the polyelectrolyte region of the soft particle was then used to calculate the bacterial surface potential \((\psi_o, eq. 3.3)\) and Donnan potential \((\psi_{DON}, eq. 3.5)\) in water with IS of 0.003 M\(^{32-34}\).
**Figure 3.1** Representative schematic for the surface potential distribution (mV) and the free ion concentration distribution in the polyelectrolyte brush layer of a soft-particle (bacterial cell in our case) suspended in an electrolyte solution. This schematic was adapted from Ohshima, H (2008) 48.

### 3.3.2 Calculation of “effective” ionic strength in the polyelectrolyte brush layer and on the surface of bacterial cells.

The concentrations of free ions $n_i$ of charge $e \varepsilon_i$ inside the polyelectrolyte brush layer, and on the surface of bacteria can be related to that in the bulk $n_i^\infty$ using the Boltzmann distribution$^{51-53}$:

$$n_i = n_i^\infty \exp\left(-\frac{e \varepsilon_i \psi}{k_B T}\right)$$  \hspace{1cm} (3.6)

where $\psi$ is described as the mean electrostatic potential (mV), and $e \varepsilon_i \psi$ is the electrical potential energy (J)$^{51}$. For the bacterial cells, the Donnan potential $\psi_{DON}$, and the surface potential $\psi_o$ can be used as the mean electrostatic potential inside the polyelectrolyte brush layer and on the surface of bacterial cell, respectively.
In a pure liquid such as water (IS of 0.003M), the free ions in the solution are $\text{H}_3\text{O}^+$ and $\text{OH}^-$ ions, with valences +1 and -1, respectively. Thereby, the “effective” ionic strength in the brush ($C_s$, M) and on the bacterial surface ($C_o$, M) shown in Figure 3.1 were related to the number density of ions by equation 3.7 for all investigated (Table 3.1).

$$C = \frac{1}{2} \sum_i n_i z_i^2$$  \hspace{1cm} (3.7)

### 3.3.3 Calculation of polyelectrolyte brush layer thickness and the grafted biopolymer density.

To quantify the equilibrium thickness of the polyelectrolyte brush layer ($L_o$, nm) (Figure 3.1), and the grafted biopolymer density in the brush layer ($\Gamma$, m$^{-2}$); reflecting how much of the surface is covered by biopolymers, steric interactions measured between the AFM tip and $L.\text{ monocytogenes}$ EGDe surface biopolymers were modeled by following the work of Alexander and de Gennes

$$F_{St} = 50k_BT aL_o \Gamma^{3/2} e^{-2\pi a h/L_o}$$  \hspace{1cm} (3.8)

where $F_{St}$ is the interaction force between two surfaces (nN), $a$ is the tip radius taken as reported by the manufacturer (40 nm), and $h$ is the separation distance between the two surfaces (nm). In equation 3.8, $L_o$ and $\Gamma$ represent the fitting parameters. For each ionic strength of growth investigated, at least 132 approach curves were fitted individually by the steric model, and the evaluated fitting parameters ($L_o$ and $\Gamma$) were averaged (Table 3.1).

### 3.3.4 Calculation of soft-particle DLVO interaction energies.

The total soft-particle DLVO interaction energy ($E_{total}$) between the bacterium and the silicon nitride AFM tip was calculated as the sum of Lifshitz-van der Waals ($E_{LW}$ ) and electrostatic ($E_e$) interaction energies

$$E_{total} = E_{LW} + E_e$$  \hspace{1cm} (3.9)
The electrostatic interactions were calculated using the linearized version of the Poisson-Boltzmann expression using a sphere-sphere geometry\(^{56}\)

\[
E_e = \frac{2\pi a_1 a_2 n^2 k_B T}{(a_1 + a_2)^2} \left( \Phi_1^2 + \Phi_2^2 \right) \left[ \frac{2\Phi_1 \Phi_2}{\Phi_1^2 + \Phi_2^2} \ln \left\{ \frac{1 + \exp(-k \hbar)}{1 - \exp(-k \hbar)} \right\} + \ln(1 - \exp(-2k \hbar)) \right]
\]  
(3.10)

where \(a_1\) is the radius of the bacterium taken as 768 nm for \(L. \) monocytogenes \(\) EGDe\(^{57}\), \(a_2\) is the radius of the AFM tip taken as reported by the manufacturer (40 nm), \(\Phi_1\) and \(\Phi_2\) are the reduced potentials of the bacterium and the AFM tip respectively, which relate to their surface potentials \(\varphi_{\alpha_1}\) and \(\varphi_{\alpha_2}\) according to \(\Phi_i = \frac{ze\varphi_{\alpha_i}}{k_B T}\) \(^{56}\), and \(h\) is the separation distance between the AFM tip and the bacterial surface biopolymers (nm). For bacterial cells, the soft-particle surface potential \(\varphi_{\alpha})\) calculated from equation 3, was used as the surface potential \(\varphi_{\alpha_1}\). For the silicon nitride tip, the zeta potential of -16 mV was used as \(\varphi_{\alpha_2}\) \(^{58-60}\).

The Lifshitz-van der Waals interaction energy between two dissimilar spheres was calculated using Hamaker expression, corrected for retardation effects as\(^{61}\)

\[
E_{LW} = \frac{-A_{132} a_1 a_2}{6h(a_1 + a_2)(1 + 11.12 \frac{h}{\lambda_c})}
\]  
(3.11)

where \(A_{132}\) is the “effective” Hamaker constant of the interacting system (J), and \(\lambda_c\) is the “characteristic wavelength” of the interaction, often assumed to be 100 nm\(^{32,33}\).

3.3.5 Calculation of Hamaker constants. The “effective” Hamaker constant \(A_{132}\) for the interacting system: bacterium (1) / AFM tip (2) / water (3) was calculated as

\[
A_{132} \approx (\sqrt{A_{11}} - \sqrt{A_{33}})(\sqrt{A_{22}} - \sqrt{A_{33}})
\]  
(3.12)

The individual Hamaker constant for each of the interacting components was calculated as\(^{54}\):
\[ A_{ii} = 24\pi H_o^2 \gamma_i^{LW} \] (3.13)

where \( H_o \) is the theoretical closest separation distance upon approach taken as 0.157 nm\(^2\) and \( \gamma_i^{LW} \) is the apolar (Lifshitz-van der Waals) surface tension component of the condensed material \((i)\) (mJ/m\(^2\)). The \( \gamma_i^{LW} \) apolar surface tension component of the bacterium (1) was obtained through contact angle measurements done on bacterial lawns using the thermodynamic-based harmonic-mean (HM) model\(^\text{ef}\). Note that \( \gamma_i^{LW} \) values for silicon nitride (2) and water (3) are known to be 32.6 and 21.8 mJ/m\(^2\), respectively\(^4^4\).

### 3.3.6 Calculation of apolar surface tension component of bacterial cells using the thermodynamic-based harmonic-mean (HM) model.

The HM model has been used in the literature to approximate interactions of low energetic phases with apolar and polar surface tension components (eq. 3.14)\(^\text{25,34,44}\). This model is useful for hydrophilic polysaccharides\(^\text{ef}\) and proteins\(^6^3\) which are the major components of bacterial surfaces\(^6^4\). The HM model relates contact angle (\( \theta \)) to surface tension (mJ/m\(^2\)) of condensed material \((i)\) by

\[
(1 + \cos \theta)\gamma_L = 4 \left[ \frac{\gamma_i^{LW} \gamma_S^{LW}}{\gamma_i^{LW} + \gamma_S^{LW}} + \frac{\gamma_i^{P} \gamma_S^{P}}{\gamma_L^{P} + \gamma_S^{P}} \right] \tag{3.14}
\]

where \( \gamma_i^{LW} \) is the apolar part of surface tension of the condensed material \((i)\) caused by dispersion energy between molecules (mJ/m\(^2\)), and \( \gamma_i^{P} \) is the polar part of surface tension of the condensed material \((i)\) caused by dipole interaction included dipole moments and hydrogen bonds (mJ/m\(^2\)), and the subscripts “\( L \)” and “\( S \)” refer to liquid and bacterial phases, respectively. Note that in equation 3.14, the liquid surface tensions are known (\( \gamma_L^{LW} \) and \( \gamma_L^{P} \) are 21.8 and 51 mJ/m\(^2\) for water, and 48.5 and 2.3 mJ/m\(^2\) for diiodomethane, respectively\(^4^4\)) while the bacterial (solid) surface tensions are unknown.
Therefore, equation 3.14 has two unknowns (\( \gamma_{3}^{LW} \) and \( \gamma_{3}^{P} \)). Using the two liquid contact angle measurements performed on bacterial lawns as described above, equation 14 was solved to estimate the magnitudes of the bacterial polar and apolar surface tension components. The bacterial apolar surface tension components were then used to calculate the individual Hamaker constants (eq. 3.13) and eventually the “effective” Hamaker constants (eq. 3.12) and the Lifshitz-van der Waals interaction energies (eq. 3.11) for the bacteria grown in media of different ionic strengths.

3.4 Statistical description of AFM data. The experimental approach force-distance data were fit to steric model using an in-house Matlab written code that minimizes the difference between experimental measured forces and theoretical predictions of forces. Statistical tests were used to determine whether the adhesion energy of \( L. \) monocytogenes EGDe grown in media of different ionic strengths to silicon nitride are different from each other. In addition, the thicknesses and grafting densities of bacterial surface polyelectrolyte brushes were compared statistically as a function of ionic strength of growth. The nonparametric group comparisons Dunn’s ranks test available in Sigma Plot version 11.0 (Systat software, Inc., Chicago, IL) was applied to the data.

3.5 Results and Discussion

3.5.1 Distribution of AFM adhesion energies as a function of ionic strength of growth. Adhesion energies between silicon nitride and bacterial cells grown in media of different ionic strengths were computed individually from AFM retraction curves. The use of AFM to investigate the bacterial interactions with surfaces\(^{36,37,40-42}\) and the procedure we used to quantify AFM adhesion energies\(^{34,44}\) have been well documented in the literature.

As can be seen from Figures 3.2A-E, our adhesion data were heterogeneous and spanned a range of energies at all investigated IS conditions. The heterogeneities in the AFM
measurements of bacterial adhesion have been commonly reported in the literature\textsuperscript{37,41,42,65,66}, and largely attributed to the wide array of molecules present on the bacterial surface\textsuperscript{65,67,68}. For instance, \textit{L. monocytogenes} is a Gram-positive bacterium and the cell wall of \textit{L. monocytogenes} is composed of peptidoglycans, proteins, and polyanionic polymers such as teichoic acids and lipoteichoic acids\textsuperscript{31}.

Our results show that the adhesion affinity of \textit{L. monocytogenes} to silicon nitride surface in water was affected by the ionic strength of the media in which the cells were grown. As can be seen from Figure 3.2F and Table 3.1, the mean and the median values of bacterial adhesion energies computed from AFM retraction curves were the lowest for the cells grown at IS of 0.1M added NaCl, followed by 0.3M, 0.05M, 0.5M added NaCl, and IS of 0.003M, respectively. In comparison, the mean energy values were on average 28% higher than the median energy values for all investigated conditions (Figure 3.2F, Table 3.1). Pair-wise multiple comparison procedure (Dunn’s method) indicated that, adhesion energies computed for each condition were statistically and significantly different from each other ($P < 0.05$), except for those computed for IS of 0.5M added NaCl and IS of 0.003M.

The observed differences in the adhesion affinities can be attributed to the different surface characteristics of the bacteria when grown in media of different ionic strengths as will be discussed later. Osmo-adaptation in \textit{L. monocytogenes} can involve both regulation at the gene expression level\textsuperscript{7,13,19}, and physicochemical changes\textsuperscript{20-23}, which may also affect the structure and the composition of the surface molecules of \textit{L. monocytogenes} contributing to the adherence of the bacterium to silicon nitride surface.
Table 3.1: Summary of the results for all ionic strength of growth investigated.

<table>
<thead>
<tr>
<th>Ionic strength (NaCl, M)</th>
<th>0.003</th>
<th>0.05</th>
<th>0.1</th>
<th>0.3</th>
<th>0.5</th>
</tr>
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<tr>
<td><strong>AFM Adhesion Energy Results</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean adhesion energy (AJ)</td>
<td>147.6</td>
<td>103.5</td>
<td>43.5</td>
<td>51.2</td>
<td>133.5</td>
</tr>
<tr>
<td>Standard error of mean (AJ)</td>
<td>6.4</td>
<td>6.3</td>
<td>3.2</td>
<td>5.5</td>
<td>9.5</td>
</tr>
<tr>
<td>Median adhesion energy (AJ)</td>
<td>135.0</td>
<td>71.1</td>
<td>28.0</td>
<td>29.5</td>
<td>85.8</td>
</tr>
<tr>
<td># of adhesion energies</td>
<td>200</td>
<td>179</td>
<td>195</td>
<td>173</td>
<td>170</td>
</tr>
</tbody>
</table>

| Physiochemical Properties of *L. monocytogenes* EGDe |
|------------------|------------------|------------------|------------------|
| $\psi_o$ (mV)    | -16.8            | -26.8            | -33.7            | -30.2            | -20.0            |
| $\psi_{DON}$ (mV) | -30.4            | -44.8            | -53.7            | -49.3            | -35.3            |
| $C_0$ (bulk, M)  | 0.003            | 0.004            | 0.005            | 0.005            | 0.004            |
| $C_s$ (biopolymer brush, M) | 0.005 | 0.008 | 0.011 | 0.009 | 0.006 |
| $\Theta$ (water) | 28° ± 2°         | 30° ± 2°         | 35° ± 3°         | 34° ± 2°         | 25° ± 3°         |
| $\Theta$ (diiodomethane) | 40° ± 2° | 41° ± 1° | 42° ± 1° | 42° ± 1° | 39° ± 1° |
| $\gamma_i^{LW}$ (mJ/m²) | 34.6 | 33.1 | 29.1 | 30.2 | 35.0 |
| $A_{11}$ (10⁻²⁰) (J) | 6.4  | 6.1  | 5.4  | 5.6  | 6.5  |
| $A_{132}$ (10⁻²¹) (J) | 2.4  | 2.1  | 1.4  | 1.6  | 2.4  |

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<td>$E_{max} (k_B T)$</td>
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<td>$E_LW (k_B T)$</td>
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<td>$E_e (k_B T)$</td>
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**Figure 3.2** A-E) Histograms that show the distribution of *adhesion energy* (AJ) values quantified between the surface biopolymers of *L. monocytogenes* cells grown at A) IS of 0.003M, and at B-E) IS of added NaCl (0.05M, 0.1M, 0.3M and 0.5M NaCl, respectively) and silicon nitride in water. F) A bar-graph that shows the mean and the median of the distributions shown in Figures 3.2A-E as a function of the ionic strength of growth media.
3.5.2 Physiochemical properties of bacterial cells as a function of ionic strength of growth.

By fitting the soft-particle electrophoresis model (eq. 3.2) to the measured electrophoretic mobility data as a function of ionic strength of the media of mobility measurements, the fitting parameters; $ZN$ and $1/\lambda_s$ were determined (Figures 3.3A –E). The bacterial surface potentials ($\psi_s$, mV) and Donnan potentials ($\psi_{DON}$, mV) under filtered DI water were calculated by using the $ZN$ parameter obtained for each ionic strength of growth investigated.

The $ZN$ parameter representing the spatial charge density of the charged groups in the polyelectrolyte brush layer of bacterial cells\textsuperscript{48} was dependent upon the ionic strength used to grow the bacteria (Figures 3.3A-E), which in turn led to the variations in the values of bacterial surface potentials (eq. 3.3) and Donnan potentials (eq. 3.5) in DI water when the bacteria were grown at different ionic strengths (Figure 3.3F, Table 3.1). The calculated values of \textit{L. monocytogenes} surface and Donnan potentials indicated that the bacterial cells grown at IS of 0.1M were the most electrostatically negative (-33.7 mV and -53.7 mV, respectively), followed by the cells grown at IS of 0.3M, 0.05M, 0.5M and 0.003M, respectively. In a pure liquid such as water, the variations in the bacterial surface and Donnan potentials can originate from the variations in the dissociation of surface groups such as carboxyl, phosphate and amino groups present on the bacterial surface. These results suggest that \textit{L. monocytogenes} grown in the media of five different ionic strengths should possess different array and/or different amounts of surface molecules which can contribute to the charging of the bacterial surface. Irrespective of the ionic strength of the growth media investigated, Donnan potentials were always higher than the surface potentials (Figure 3.3F, Table 3.1), which is characteristics of the potential distribution in the polyelectrolyte region of the soft particles\textsuperscript{24,48,49} (Figure 3.1). This phenomenon can be simply explained by the fact that in the vicinity of a charged surface, the concentration of
counter-ions in the solution is always higher than that in the bulk solution\textsuperscript{53,54} (Figure 3.1). By using the Boltzmann distribution (equations 3.6 and 3.7), the “effective” ionic strength in the polyelectrolyte brush layer \((C_r, M)\) and on the bacterial surface \((C_o, M)\) shown in Figure 3.1 were quantified. The “effective” ionic strengths were found to be 45\% lower on the surface of the bacteria compared to those found in the polyelectrolyte brush layer for all investigated conditions (Table 3.1). The highest free ion \((\text{H}_3\text{O}^+ \text{ and } \text{OH}^-)\) concentration in the polyelectrolyte brush layer and on the bacterial surface was found for the cells grown at IS of 0.1M (Table 3.1), as expected. Since the migration of the counter-ions is driven by the electrical potential energy \((ez_i\nu_iJ)\textsuperscript{51}\), the highest “effective” ionic strength on the bacterial surface was found for the cells which were the most electrostatically negative, (bacterial cells grown at IS of 0.1M).

The average contact angle values tested on \textit{L. monocytogenes} EGDe indicated that bacterial cells were hydrophilic at all ionic strengths of growth investigated. This finding is consistent with previous reports; as \textit{L. monocytogenes} always possessed hydrophilic properties irrespective of the ionic strength of growth\textsuperscript{20,22}, temperature of growth\textsuperscript{20,23,34} or the pH of the growth media\textsuperscript{21,44}. The values of apolar surface tension component \((\gamma^LW_i)\) for the bacterial cells obtained through contact angle measurements \((32.4 \pm 2.6 \text{ mJ/m}^2)\), and the values of individual Hamaker constant \((A_{i1})\) calculated by using \(\gamma^LW_i\) for bacterial cells \((6.0 \times 10^{-20} \pm 0.4 \times 10^{-20} \text{ J})\) were relatively close to each other at all investigated conditions (Table 3.1). The “effective” Hamaker constant for the interacting system \((A_{i32})\) calculated for cells grown at IS of 0.1M and 0.3M \((1.5 \times 10^{-21} \pm 0.1 \times 10^{-21} \text{ J})\) was on average 35\% lower than those values calculated for the cells grown at IS of water \((0.003\text{M}), 0.05\text{M} \text{ and } 0.5\text{M}, \text{ which indicates a lower affinity for the interaction between silicon nitride and bacterial cells grown at IS of 0.1M and 0.3M in water.}
Figure 3.3 Electrophoretic mobility of *L. monocytogenes* EGDe grown at A) IS of 0.003M and B) IS of added NaCl (0.05M, 0.1M, 0.3M and 0.5M NaCl, respectively) as a function of ionic strength of the media of mobility measurements (M). Each point in Figures 3.3A-E represents an average of five experimental measurements with error bars representing the standard deviation. The solid lines represent the soft-particle electrophoresis model (eq. 3.2) fits to the data. The qualities of the fits were judged by the coefficient of correlation ($r^2$) values which were found to be 0.91, 0.83, 0.82, 0.90 and 0.90 for data in Figures 3.3A-E, respectively. F) A scatter plot that shows the surface potentials (eq. 3.3) and Donnan potentials (eq. 3.5) of the bacterial cells as a function of ionic strength of growth.
3.5.3 Bacterial surface polyelectrolyte brush thickness and grafting density as functions of ionic strength of growth. The polyelectrolyte brushes on the surface of *L. monocytogenes* EGDe changed their structure depending upon the ionic strength used to grow the bacterial cells. As can be seen from Figure 3.4A and Table 3.1, for the cells grown at IS of 0.1M (28 ± 1 nm), the length to which polyelectrolyte brushes was extended was on average 53% lower than those found for IS of 0.05M and 0.3M, and 81% lower than those for IS of 0.003M and 0.5M. Pair-wise multiple comparison procedure (Dunn’s method) indicated that, the computed values of bacterial surface brush layer height for 0.05M and 0.3M pair, and for 0.003M and 0.5M pair were not-statistically different from each other, but significantly different (*P* < 0.05) from those estimated for cells grown at IS of 0.1M. On the other hand, the grafting densities of bacterial surface brushes estimated for cells grown at IS of 0.1M and 0.3M (5.1 × 10^{-16} ± 0.2 × 10^{-16} m^{-2}) were not-statistically different from each other, and on average 55% higher than those found for IS of 0.003M, 0.05M, and 0.5M (Figure 3.4B, Table 3.1).

The length to which polyelectrolyte brushes are extended correlates with the “effective” ionic strength in the polyelectrolyte brush layer^{51,52}, which is usually described by the general power law relationship \( L_o \sim C_s^{-m} \). The exponent that best represented our data was found to be \( m \sim 0.5 \) (Figure 3.4C), which was in agreement with the electrostatic wormlike chain (WLC) model prediction \( (L_o \sim C_s^{-1/2})^{51,52} \), as previously observed for the polyelectrolyte brush layer height of *Pseudomonas putida* KT2442^{32}. As can be seen from Table 3.1, the strongest intermolecular electrostatic repulsion in the polyelectrolyte brush layer of the cells grown at IS of 0.1M, which was evident from the highest Donnan (\( \psi_{DON} \), mV) potential as well from the highest “effective” ionic strength in the brush layer \( (C_s, M) \) compared to those of cells grown at
other ionic strengths investigated, could be held responsible for the lowest length and the highest grafting density of the brushes observed at IS of 0.1M.

Figure 3.4 A-B) Bar-graphs that show the means of the polyelectrolyte brush thicknesses ($L_0$, nm) and grafted densities ($\Gamma$, m$^{-2}$) estimated for $L.\text{monocytogenes}$ EGDe cells as a function of the ionic strength of growth. Error bars indicate the standard errors of the means. C) The scaling relationship between the “effective” ionic strength in the polyelectrolyte brush layer ($C_s$) and the brush layer height $L_0$ (nm) describing the model for the polyelectrolyte brushes of $L.\text{monocytogenes}$ EGDe cells was in good agreement ($r^2 = 0.99$), with the electrostatic wormlike chain (WLC) model ($L_0 \sim C_s^{-1/2}$)$^{51,52}$. 

$C_s^{-1/2}$
3.5.4 Predicted energy barriers based on soft-particle DLVO theory calculations. Results obtained from electrophoresis measurements of charge and contact angle measurements of bacterial surface tensions were used to predict the energy profiles between *L. monocytogenes* EGDe cells and silicon nitride in water (as in our case) (Figure 3.5A) and in salted water with IS of 0.05M, 0.1M, 0.3M and 0.5M (for comparison purposes) using soft-particle DLVO theory (equations 3.9-3.11).

For highly charged surfaces in dilute electrolyte solution such as in water, a strong long-range repulsion peaks at some distance, usually between 1 and 4 nm\(^5\). As can be seen from Figure 3.5A, all soft-particle DLVO energy profiles displayed a peak at approximately 2 nm separation distance, as we have previously observed as well for the interaction between *L. monocytogenes* EGDe grown at various temperatures and silicon nitride in water\(^3\). The maximum soft-particle DLVO energy barrier needed for the bacteria to overcome in order to adhere to the silicon nitride in water was on average 23% higher for cells grown at IS of 0.1M (18 \(k_B T\)) compared to those obtained for cells grown at IS of 0.3M, 0.05M, 0.5M and 0.003M, respectively (Figure 3.5A, Table 3.1), which agreed well with the bacterial adhesion energy profile computed using AFM force-distance data as a function of ionic strength of growth (Figure 3.5B, Table 3.1).

According to the soft-particle DLVO theory, bacterial adhesion is driven by the sum of Lifshitz–van der Waals interactions, usually described as attractive, and also electrostatic interactions, which may be repulsive or attractive, depending upon the charge of the two surfaces interacting. Since both silicon nitride and *L. monocytogenes* EGDe are negatively charged in water at all investigated conditions, the long-range electrostatic interactions were repulsive for our system (Table 3.1) and played a significant role in the adhesion of the microbe to silicon
nitride surface in water. The Lifshitz-van der Waals interactions which depend on the Hamaker constant of the interacting system (eq. 3.11) were found to be attractive for all investigated conditions (Table 3.1). Since, the “effective” Hamaker constant for the interacting system ($A_{132}$) calculated for cells grown at IS of 0.1M and 0.3M was lower than those values calculated for the cells grown at IS of water (0.003M), 0.05M and 0.5M, the Lifshitz-van der Waals attraction was the lowest for cells grown at IS of 0.1M and 0.3M (Table 3.1).

One critical aspect to the soft-particle DLVO theory is the ionic strength of the suspending medium which can indicate the primary factor mediating the bacterial adhesion$^{54}$. Since the bacterial cell surface charge and hence the surface potential (eq. 3.3) strongly depends upon the ionic strength of the suspending medium, it is no surprise to observe a different energy profile for the interaction between $L. monocytogenes$ and silicon nitride in salted water (Figure 3.5C) compared to that observed in pure water (Figure 3.5A). As can be seen from Figure 3.5C, for the case when the ionic strength of the suspending medium (water) is increased, the soft-particle DLVO interaction curve approaches zero, which indicates a strong attraction between the two surfaces at all separations. This prediction is in well agreement with the previous studies; as the ionic strength of the suspending medium increased, the adhesion efficiency of $L. monocytogenes$ to various inert surfaces as well as to stainless steel increased$^{20,69}$. This phenomenon can be explained by the Boltzmann distribution of free ions in the suspending medium; the higher the ionic strength of the suspending solution the more counter-ions can be available to shield and thus neutralize the negative charge of the cell surface. Thereby, the electrostatic repulsion between $L. monocytogenes$ and the silicon nitride in salted water will be suppressed as the ionic strength of the suspending medium increases.
Figure 3.5 A) Total energy profiles calculated between *L. monocytogenes* EGDe cells and silicon nitride AFM tip in pure water using soft-particle DLVO theory (equations 3.9-3.11). B) A linear correlation ($r^2 = 0.91$) between the maximum energy barriers to the bacterial adhesion to silicon nitride $E_{max} (AJ)$ estimated based on soft-particle DLVO theory and the mean of adhesion energies (AJ) quantified between the silicon nitride AFM tip and the bacteria grown in media of five different ionic strengths. C) Total energy profiles between the bacterial cells and silicon nitride AFM tip in pure water (IS of 0.003M) and in salted water with IS of 0.003M, 0.05M, 0.1M, 0.3M and 0.5M, for the case when the bacterial cells were both grown and then interact with silicon nitride at the same ionic strength of interest.

3.6 Conclusions

Our results suggest that the ionic strength of the growth media has a strong influence on the surface physiochemical properties of *L. monocytogenes* which in turn affects the adhesion affinity of the microbe to inert surfaces. The structure and the electric properties of the surface
The polyelectrolyte brush layer of *L. monocytogenes* EGDe were shown to be dependent on the ionic strength of growth, suggesting variations in the array and/or amount of the surface molecules contributing both to charging of the bacterial surface and adherence of the microbe to inert surfaces. The transitions in the adhesion energies (AJ), surface and Donnan potentials (mV), the “effective” ionic strengths (M) and in the heights of bacterial surface polyelectrolyte brushes (nm) occurred for the cells grown at IS of 0.1M. The highest electrostatic repulsion which was partially balanced by the Lifshitz-van der Waals attraction for the bacterial cells grown at IS of 0.1M was held responsible for the highest energy barrier to adhesion predicted for these cells and thus the lower adhesion measured by AFM.

Our results also showed that, the adhesion affinity of *L. monocytogenes* to inert surfaces can change when the microbe is transferred to a different suspension due to the changes in the ionic strengths of the solutions. Therefore, predictive physiochemical strategies for the control of the adhesion of this microbe to inert surfaces in water should take into account the ionic strength of the solutions in the transfer and growth route of *L. monocytogenes*.

### 3.7 Acknowledgement

We would like to thank Prof. Markus Flury; a professor of Soil Physics/Vadose Zone Hydrology in the Department of Crop and Soil Sciences at Washington State University for allowing us to use his goniometer and Zeta potential analyzer to do the contact angle and electrophoresis measurements, and our co-workers Asma Eskhan and Dr. Bong-Jae Park for helping us in performance of the electrophoresis and contact angle measurements. We would like to thank Josue Orellana and Dr. Amer Hamdan for their MATLAB programs used in the analysis of the AFM adhesion energies and steric properties of the biopolymer brushes reported in this study. We would like to thank as well the National Science Foundation grant EEC-0823901 for financial support of this study.
3.8 References


Chapter 4

Combined Poisson and soft-particle DLVO analysis of the specific and nonspecific adhesion forces measured between $L.\ monocytogenes$ grown at various temperatures and silicon nitride

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Abstract

Adhesion forces between pathogenic *L. monocytogenes* EGDe and silicon nitride (Si$_3$N$_4$) were measured using atomic force microscopy (AFM) under water and at room temperature for cells grown at five different temperatures (10, 20, 30, 37 and 40°C). Adhesion forces were then decoupled into specific (hydrogen bonding) and nonspecific (electrostatic and Lifshitz-van der Waals) force components using Poisson statistical analysis. The strongest specific and nonspecific attraction forces were observed for cells grown at 30°C, compared to those observed for cells grown at higher or lower temperatures, respectively. By combining the results of Poisson analysis with the results obtained through soft-particle Derjaguin-Landau-Verwey-Overbeek (DLVO) analysis, the contributions of the Lifshitz-van der Waals and electrostatic forces to the overall nonspecific interaction forces were determined. Our results showed that the Lifshitz-van der Waals attraction forces dominated the total nonspecific adhesion forces for all investigated thermal conditions. However, irrespective of the temperature of growth investigated, hydrogen bonding forces were always stronger than the nonspecific forces. Finally, by combining Poisson analysis with soft-particle analysis of DLVO forces, the closest separation distances where the irreversible bacterial adhesion takes place can be determined relatively easily. For all investigated thermal conditions, the closest separation distances were < 1nm.

**Keywords:** *L. monocytogenes*, adhesion, soft-particle DLVO, Poisson, AFM, Hydrogen bonds, nonspecific forces.
4.1 Introduction

*Listeria monocytogenes* are food-borne bacterial pathogens associated with lethal food-borne disease outbreaks that are characterized by a widespread distribution\(^1,2\). The efficacy of *L. monocytogenes* in causing outbreaks is largely due to their ability to survive and grow in a wide temperature range (1 to 45°C)\(^3\) on many surfaces\(^4,5\). The results of the Food and Drug Administration investigation of the recently occurred *L. monocytogenes* outbreak reported in cantaloupes pointed to the importance of controlling the temperature of the environment in which food is stored or processed and the sanitization of the surfaces in contact with food in preventing the contamination of food by *L. monocytogenes*\(^6\). *L. monocytogenes* can contaminate food while growing in soil\(^7,8\), during food processing as well as during food storage\(^1,5,8\). Understanding the mechanisms by which *L. monocytogenes* attach to surfaces and the effect of the temperature of growth on the strength of such interactions are crucial to developing tools that can be implemented to reduce the risks of food contamination by *L. monocytogenes*.

In general, a two-step mechanism mediates bacterial attachment to a surface. Both mechanisms are influenced by the chemical nature of the interacting surfaces and the electrolytic environment\(^5,9\). The first step involves long-range nonspecific interactions. These interactions determine whether the two surfaces are able to get close enough to molecular contact. As the bacterial cell approaches to a surface of interest, the entire cell will be exposed to nonspecific physiochemical forces such as Lifshitz-van der Waals and electrostatic forces, as usually described by DLVO theory\(^10,11\). Close to molecular contact (< 1 nm)\(^12\), specific short-range molecular interactions come into play. At such distances, the bacterial cell can lock to a surface of interest by forming hydrogen, ionic or covalent bonds between its surface molecules and/or extracellular structures and the surface of interest\(^13,14\). This crucial step is described as the
irreversible bacterial attachment to the surface\textsuperscript{13}, in part because at the end of this step much stronger physical or chemical forces (e.g. scraping, scrubbing or chemical cleaners) are required to remove the bacteria from the surface.

Although the dominating factors involved in the bacterial attachment process to surfaces remain elusive, quantitative information on the overall interaction force between the bacterium and the surface can be directly obtained with high resolution under physiological conditions using AFM\textsuperscript{15,16}. The use of AFM to investigate bacterial interaction forces with surfaces has been well documented in the literature\textsuperscript{17-24}. However, decoupling of the overall interaction force into specific and nonspecific force components requires statistical analysis of the AFM data\textsuperscript{25-27} such as Poisson statistical analysis method\textsuperscript{25,28}. The Poisson statistical method does not require a large number of force measurements; thus minimizing the sample damage due to repetitive force measurements\textsuperscript{28}, and can allow an accurate estimation of the magnitudes of specific and nonspecific force components, as described in the literature\textsuperscript{29-31}.

The current study provides a detailed investigation of the fundamental components of the overall interaction forces mediating the adhesion of pathogenic \textit{L. monocytogenes} EGDe grown at five different temperatures (10, 20, 30, 37 and 40 °C) to a model surface of Si\textsubscript{3}N\textsubscript{4} under water. Si\textsubscript{3}N\textsubscript{4} AFM tips were chosen as our model inert surfaces because they are characterized by similar surface potentials to those of soil and glass\textsuperscript{18}, substrates to which \textit{L. monocytogenes} frequently attach to in nature\textsuperscript{32} and in food industry environments\textsuperscript{33}. The methodology used in this study can be applied to a wide array of inert surfaces of interest to food industry such as stainless steel. The temperature range investigated was chosen to bracket the thermal conditions in which \textit{L. monocytogenes} survive in the environment\textsuperscript{1,34-35}. Since water is the main solvent...
used in the food processing industries and the main solvent used in preparing foods\textsuperscript{36}, all force measurements were performed under water.

The overall interaction forces measured as adhesion forces between \textit{L. monocytogenes} EGDe grown at various temperatures and Si\textsubscript{3}N\textsubscript{4} under water by AFM were decoupled into specific and nonspecific force components using Poisson statistical method. Since there are no ligand/receptor bonds to be expected between Si\textsubscript{3}N\textsubscript{4} and the bacterial surface in water\textsuperscript{37}, the specific forces arise from hydrogen bonding in our system\textsuperscript{12}. The nonspecific force obtained through Poisson analysis for each temperature of growth investigated was assumed to be the sum of Lifshitz-van der Waals and electrostatic forces as described by DLVO\textsuperscript{10-12}. The electrostatic forces obtained for each condition investigated were modeled using the bacterial surface potentials previously derived from Ohshima’s soft-particle electrophoresis modeling\textsuperscript{38}. The contribution of the Lifshitz-van der Waals and electrostatic forces to the total nonspecific force was quantified at the closest separation distances using the soft-particle analysis of the DLVO forces\textsuperscript{38-39} for each condition investigated.

\textbf{4.2 Materials and Methods}

\textbf{4.2.1 Bacterial cultures.} Among the highly pathogenic \textit{L. monocytogenes} strains, EGDe was chosen as our model bacterium because it has a fully sequenced and annotated genome\textsuperscript{40}. The strain was cultured at various temperatures of growth that ranged from 10 \textdegree C to 40 \textdegree C as detailed in Chapter 2.

\textbf{4.2.2 AFM measurements.} Prior to AFM force measurements, cultured bacterial cells were attached to gelatin-coated mica disks according to the procedure detailed elsewhere\textsuperscript{41}. All AFM force measurements were performed under DI water at room temperature using Si\textsubscript{3}N\textsubscript{4} cantilevers (0.06 N/m DNP-S, Bruker AXS Inc., Santa Barbara, CA) in TappingMode\textsuperscript{TM} with a PicoForce\textsuperscript{TM}
Scanning Probe Microscope with a Nanoscope IIIa controller and extender module (Bruker AXS Inc.). We have shown previously that attaching *L. monocytogenes* cells to gelatin-coated mica and performing force measurements on bacterial cells under water at room temperature do not affect bacterial cell viability or integrity\(^2\).

We have chosen to perform our experiments at room temperature and not at the temperature of growth due to three main reasons: 1) *L. monocytogenes* can get exposed to different environmental temperatures before their entry into food processing plants, whereas the first and foremost contamination of surfaces in the food industry environments mainly occurs at room temperatures\(^3\), 2) the possible “hot spots” in the food processing plants where *L. monocytogenes* colonized such surfaces showed that, these “hot spots” were usually the environments under room-temperatures such as floors, drain floors, sponges and brushes, food contact surfaces, condensate, and even walls and ceilings\(^4\), and 3) regardless of the route of contamination of food products, understanding the adaptation mechanisms of *L. monocytogenes* to the changes in the environmental temperature, in relation to their interactions with inert surfaces can lead to a better understanding and control of the contamination and spread of this pathogen in the environment.

The force constant of each cantilever was determined from the power spectral density of the thermal noise fluctuations in DI water\(^5\). On average, the spring constant was found to be 0.060 ± 0.005 N/m (n = 6). Force measurements were made on a bacteria-free area of the gelatin-coated mica disk before and after making a measurement on a bacterial cell. For each condition investigated, 10 bacterial cells were examined from three different cultures. On each cell, 15 points were located to perform force measurements using the point and shoot feature of
the AFM software. Retraction curves were measured at a rate of 580 nm/sec to minimize the hydrodynamic drag forces\textsuperscript{46}, and at a resolution of 4096 points.

4.2.3 Analysis of retraction curves. Bacterial adhesion was quantified from the retraction curves in terms of adhesion force in nano-Newton (nN). In general, a retraction curve measured between the AFM tip and the biopolymers of a bacterial surface encompasses single or multiple adhesion peaks. Adhesion peaks are the results of specific and nonspecific interactions between the AFM tip and a single or several biopolymers chains on the bacterial surface (Figure 4.1).

![AFM retraction curves](image)

**Figure 4.1** Examples of AFM retraction curves measured between a silicon nitride AFM tip and *L. monocytogenes* EGDe surface biopolymers in water for each temperature of growth investigated. The arrows represent the locations of the adhesion peaks for 30 °C. Each adhesion peak in the retraction curve has two coordinates (pull-off distance and pull-off force). The pull-off distance indicates the location at which the AFM tip contacted the bacterial surface biopolymer chain while the pull-off force is equivalent to the adhesion force and represents the sum of all interaction forces (specific and nonspecific forces) measured between the bacterial surface biopolymers and the AFM tip.
4.3 Modeling

4.3.1 Poisson analysis of adhesion forces quantified between bacterial surface biopolymers and silicon nitride (Si$_3$N$_4$). The application of Poisson analysis of adhesion forces measured between bacterial surface biopolymers and Si$_3$N$_4$ requires two assumptions$^{25, 28, 31}$: the adhesion force ($F$) develops as the sum of discrete bond interactions and these bonds form randomly and all have similar forces ($F_i$). The adhesion force ($F$) is related to the number of bonds ruptured during the pull-off event by:

$$ F = nF_i $$

(4.1)

where $F_i$ represents the individual hydrogen bond force in our system. A sampling of many of these events produces a mean measured adhesion force ($\mu_F$). The Poisson probability function that was used to describe the adhesion forces can be described by:

$$ P(n; \mu_F) = (\mu_F)^n \frac{\exp(-\mu_F)}{n!} $$

(4.2)

where $P(n; \mu_F)$ is the probability of observing a certain magnitude of adhesion forces, and $n$ represents the various magnitudes of adhesion forces and/or various numbers of bonds formed; the probability of which is given by equation 4.2.

If the Poisson probability function is able to fit the distribution of adhesion forces measured between bacterial surface biopolymers and Si$_3$N$_4$ (Figure 4.2), then the mean and the variance of adhesion forces will be the same ($\mu_F = \sigma_F^2$). When nonspecific interaction forces such as Lifshitz-van der Waals and electrostatic forces are taken into account with parameter $F_o$, one can derive the following equations:

$$ \mu_F = \mu_o F_i + F_o $$

(4.3)
\[ \sigma_F^2 = \sigma_n^2 F_i^2 = \mu_F F_i - F_i F_o \] (4.4)
a linear regression through the plot of the variance of the adhesion forces \( (\sigma_F^2) \) against the mean of the adhesion forces \( (\mu_F) \) for a given data set (equation 4.4) will have a slope of \( F_i \) and the intercept of \(-F_i F_o\). These values can thus be used to quantify the magnitudes of the specific forces \( (F_i) \) and the nonspecific forces \( (F_o) \).

For each condition investigated; the distribution of all bacterial adhesion AFM force data was shown to follow the Poisson distribution (Figure 4.2). The mean and the variance of the adhesion forces collected between an individual cell and Si3N4 under water were used to obtain a single data point (Table 4.1), and 10 data points were used to quantify the specific \( (F_i) \) and nonspecific \( (F_o) \) forces (Figures 4.3A-E, Table 4.2).

**Table 4.1:** Summary of the mean \( (\mu_F) \), variance \( (\sigma_F^2) \) and the number of adhesion forces \( (n) \) measured between *L. monocytogenes* EGDe cells grown at five different temperatures and Si3N4 in water.

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<td>-0.090</td>
<td>0.003</td>
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-0.110   --   533  -0.160 --   399  -0.494 --   1669  -0.335 --   872  -0.200 --   1087
Figure 4.2 Distribution of the pull-off forces measured between ten *L. monocytogenes* EGDe cells and silicon nitride in water for each temperature of growth investigated. The solid lines represent the theoretical Poisson distributions fits of the distributions of adhesion forces.
4.3.2 Soft-particle analysis of DLVO forces governing the nonspecific interactions between bacteria and silicon nitride (Si₃N₄). The nonspecific Lifshitz-van der Waals ($F_{LW}$) and electrostatic ($F_e$) forces measured between the bacterium and Si₃N₄ are the components of the total DLVO force ($F_{DLVO}$)\(^{10,12}\).

\[
F_{DLVO} = F_{LW} + F_e
\]  

(4.5)

DLVO forces can be quantified by considering the bacterium and the AFM tip as spheres\(^{48}\). The Lifshitz-van der Waals force was calculated using Hamaker expression, corrected for retardation effects as\(^ {49}\)

\[
F_{LW} = \frac{-A_{132}a_1a_2}{6h(a_1 + a_2)(1 + 11.12 h/\lambda_c)} \left[ \frac{1}{h} + \frac{11.12}{\lambda_c(1 + 11.12 h/\lambda_c)} \right]
\]  

(4.6)

where $a_1$ is the radius of *L. monocytogenes* EGDe taken as 768 nm\(^ {50}\), $a_2$ is the radius of the AFM tip taken as reported by the manufacturer (40 nm), $A_{132}$ is the effective Hamaker constant for the bacteria-Si₃N₄-water system and was previously determined\(^ {22}\), $h$ is the separation distance between AFM tip and bacteria, and $\lambda_c$ is the “characteristic wavelength” of the interaction, often assumed to be 100 nm\(^ {51}\).

The electrostatic interaction force was calculated using the linearized version of the Poisson-Boltzmann expression as\(^ {48}\)

\[
F_e = \frac{4\pi a_1a_2n k_BT}{(a_1 + a_2)\kappa} \left( \Phi_1^2 + \Phi_2^2 \right) \left\{ \frac{2\Phi_1\Phi_2}{\Phi_1^2 + \Phi_2^2} \exp(-\kappa h) - \exp(-2\kappa h) \right\} \left\{ \frac{1}{1 - \exp(-2\kappa h)} \right\}
\]  

(4.7)
where $\kappa$ is the inverse Debye length defined as: $\kappa = (\frac{1}{\varepsilon_0 \varepsilon_r k_B T} \sum_{i=1}^{N} z_i^2 e^2 n_i)^{\frac{1}{2}} \text{ (m}^{-1}\text{)}, n$ is the molar concentration of the medium (mol/m$^3$) multiplied by Avogadro’s number (#/mol), $\varepsilon_0$ the permittivity of vacuum, $\varepsilon_r$ the relative permittivity of the solvent (78 for water), $k_B$ the Boltzmann constant, $T$ absolute room temperature (298 K), $e$ the electron charge, $z$ the valance of the ion species in the medium (1 for Na$^+$ and -1 for Cl$^-$), $\Phi_1$ and $\Phi_2$ are the normalized dimensionless surface potentials of the tip and bacterium, respectively and defined as: $\Phi_i = ze\psi_i / k_B T$ where $\psi_1$ and $\psi_2$ are the tip and bacterium surface potentials (V). The surface potential for Si$_3$N$_4$ was taken as -16.4 mV$^{52,53}$.

Note that the surface potentials of the bacteria grown at all conditions investigated were previously determined using Ohshima’s soft-particle modeling$^{38}$ of electrophoretic mobility measurements done on a given bacterial strain in a range of electrolyte concentrations$^{22,38-39}$. This model takes into account the effects of the particle softness ($1/\lambda$) and the density of the charged groups present within the soft layer (ZN) by considering them as two fitting parameters in the analysis$^{38-39}$. Both parameters are important in describing bacterial adhesion to surfaces$^{54-55}$, as the cell wall of bacteria is composed of “soft” biopolymers such as proteins, peptidoglycans, lipopolysaccharides and phospholipids, which are generally characterized by variable charges$^{56}$. In addition, Ohshima’s model considers not only the charge density outside the soft layer but also that inside the layer by the Donnan potential, which is a function of the fitting parameter ZN. Previously, we have estimated the two fitting parameters mentioned above for L. monocytogenes EGDe grown at the various temperatures (Table 4.2). Our estimated fitting parameters are in good agreement with previously published values in the literature$^{54,57}$. For example, in our study, the ZN (M) and softness $1/\lambda$ (nm) parameters were ranging from -0.020M
to -0.008M, and 3.14 nm to 5.70 nm, respectively\(^2\). In comparison, for two _oral Streptococcal_ strains, _S. salivarius_ HB and HBC12, the ZN parameters were -0.013M and -0.015M, respectively\(^5\), and for _Pseudomonas syringae_ strains the softness values were ranging from 1.50 nm to 3.84 nm\(^5\).

Although the soft-particle analysis of DLVO forces introduces a significant improvement over the classical approach, it suffers from few shortcomings. These are associated with restrictions in solving the Ohshima’s model with regards to bacterial size, charge distribution within the soft layer and Debye Hückel thickness that stems from the environmental conditions used in the bacterial adhesion experiment\(^5\). Quantitatively, the model is valid only when the following four constrains on the experimental conditions are met; 1) the Donnan potential is sufficiently low at large electrolyte concentrations, 2) the multiplication of the Debye Hückel thickness (\(\kappa\)) by the thickness of the brush layer (L) is >> 1, 3) the multiplication of the Debye Hückel thickness (\(\kappa\)) by the hard core size of the particle (the bacterial size without the brush layer surrounding it in our case) (a) is >>1, and 4) the multiplication of the inverse of the softness parameter (1/\(\lambda\)) by (L) is >>1.

When the Donnan potential was calculated at a sufficiently large electrolyte concentration of 0.1 M, we found that it ranges between -1.0 mV for bacteria grown at 30 °C, and -2.56 mV for bacteria grown at 10 °C (Table 4.2). These Donnan potentials are very similar to those reported in the literature and ranged between -1.6 to -3.1 mV in pH range of 5-8 and ionic strength concentration of 0.1 M for fibrillated and non-fibrillated oral streptococcal strains\(^5\). Second, the \(\lambda L\), \(\kappa L\) and \(\kappa a\) numbers were always larger than 1 for all bacterial conditions of growth tested (Table 4.2). Therefore, although Ohshima’s model has shortcomings, the soft particle analysis of the DLVO forces is valid for our experimental conditions.
According to the sign convention of the colloids and interface science\textsuperscript{12}; a negative force indicates attraction, while a positive value stands for repulsion. The nature of the forces we obtained through the Poisson analysis and the DLVO calculations, as well as the nature of the adhesion forces measured by AFM were interpreted in accordance to this sign convention.

4.4 Results

4.4.1 Specific and nonspecific forces at varying temperatures of growth. Poisson probability function (equation 4.2) was able to fit the distribution of all adhesion forces (Figure 4.2) collected for each thermal condition investigated ($r^2 = 0.95 \pm 0.03$). Through the use of Poisson statistical analysis, specific and nonspecific components of the overall adhesion force were quantified by applying linear regressions to the scatter plots of mean versus variance of adhesion forces (Figures 4.3A-E). The coordinates of the data points used in Figures 4.3A-E are listed in Table 4.1. The quality of a given linear regression to fit the AFM data was judged by the coefficient of correlation ($r^2$) value. On average, $r^2$ values were $0.92 \pm 0.03$.

The nature of specific (hydrogen bonding) and nonspecific forces for all conditions investigated were attractive, and on average, the specific forces were 33\% stronger than the nonspecific forces (Table 4.2). A transition in the specific and nonspecific forces was observed at 30\textdegree C (Figure 4.3F). Specific and nonspecific forces at 30\textdegree C were on average 60\% higher than those obtained at lower or higher temperatures investigated (Table 4.2). When summed, the total of specific and nonspecific forces estimated by Poisson model was approximately, on average, 18\% higher than the mean of adhesion forces measured by AFM (Table 4.2).
Figure 4.3 A-E) The relationships between the means and the variances of the pull-off forces measured between *L. monocytogenes* cells grown at five temperatures of growth and silicon nitride in water. The error bars represent the standard errors of the means. Solid lines represent linear regression fits to the data and were used to calculate the specific and nonspecific components of the adhesion (Table 4.2). F) A scatter-graph that shows the specific and nonspecific force components predicted using Poisson statistical analysis as a function of the bacterial temperature of growth.
4.4.2 Nonspecific Lifshitz-van der Waals and electrostatic forces at varying temperatures of growth. Since the nonspecific forces are primarily due to Lifshitz-van der Waals and electrostatic forces as described by DLVO theory\textsuperscript{10-12}, the nonspecific force obtained through Poisson analysis for each condition investigated was assumed as the sum of Lifshitz-van der Waals and electrostatic forces (eq. 4.5) at a very close surface proximity. By using DLVO theory (eqs. 4.5-4.7), one can easily calculate the closest separation distance where the total nonspecific force estimated by Poisson model was observed. With the knowledge of this distance, Lifshitz-van der Waals and electrostatic force components of the total nonspecific force can be calculated using equations 4.6 and 4.7, respectively. By following the methodology described above, the minimum closest separation distance was calculated as 0.24 nm for 30°C in comparison to that averaged (~0.44 nm) for other temperatures investigated (Table 4.2).

Lifshitz-van der Waals forces were attractive for all conditions investigated and on average 73\% higher than the electrostatic forces calculated at the same distances (Table 4.2). The attractive Lifshitz-van der Waals force obtained for cells grown at 30°C was on average 68\% higher than those obtained at other lower or higher temperatures of growth investigated. The electrostatic forces calculated for 10, 20, 37 and 40°C were also attractive and similar in magnitudes (~ -0.02 nN), whereas for 30°C, the electrostatic force had almost no contribution to nonspecific forces (~0.00 nN) (Table 4.2).
Table 4.2: Summary of the adhesion forces measured by AFM and their specific and nonspecific force components as estimated using the Poisson statistical analysis of the adhesion forces. In addition, the results of the soft-particle analysis of the electrophoretic mobilities and the bacterial surface potentials using the Ohshima’s model are provided. Finally, a comparison between the Poisson approach estimates of the nonspecific forces and the nonspecific forces calculated using the DLVO theory was used to estimate the closest separation distance at which adhesion takes place.

<table>
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<tr>
<th></th>
<th>10°C</th>
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<th>30°C</th>
<th>37°C</th>
<th>40°C</th>
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<td>-0.14</td>
<td>-0.38</td>
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<td>Lifshitz-van der Waals forces ($F_{LW}$) (nN)</td>
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<td>-0.05</td>
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<tr>
<td>Total forces ($F_t = F_i + F_a$) (nN)</td>
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<td>-0.21</td>
<td>-0.64</td>
<td>-0.42</td>
<td>-0.28</td>
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<td>-0.16</td>
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<td>a* (nm) (in water)</td>
<td>698</td>
<td>665</td>
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*data obtained from AFM imaging and/or force measurements, 5data obtained from steric model fitting to AFM approach curves. Debye Hückel thickness ($\kappa$) in water was estimated as $1.71 \times 10^8$ m$^{-1}$. 


4.5 Discussion

4.5.1 The strengths of nonspecific interactions at close surface proximities. The nonspecific force contribution derived from Poisson analysis was found to act within a very close surface proximity similar to distances where specific forces are generally reported. The total nonspecific forces derived from Poisson analysis were attractive for all conditions investigated (Table 4.2). This finding is consistent with previous results of Poisson analysis decoupling of bacterial interaction forces with variable inert surfaces\textsuperscript{37, 58-59}, except for the study investigating the interactions between streptococci and stainless steel, in which the total nonspecific force was repulsive due to additional electrostatic interactions between bacteria and the conducting surface\textsuperscript{60}.

Given that both Si\textsubscript{3}N\textsubscript{4} and \textit{L. monocytogenes} EGDe grown at the temperatures investigated are negatively charged in water\textsuperscript{22}, one can expect that electrostatic force acting between the two will be repulsive. Here, the electrostatic interactions were attractive and/or had no effect at the separation distances close to molecular contact (~0.3 nm)\textsuperscript{12, 61} (Table 4.2). At very close surface proximities where atoms take place, the electrostatic force is averaged out to a large extent, because the molecules thermally rotate and hence display both repulsive and attractive interactions\textsuperscript{10, 12}. Thus, depending on the mutual orientation of the interacting molecules, the electrostatic force can be attractive, repulsive or neutral. For example, the neutral nature of the electrostatic interactions observed between Si\textsubscript{3}N\textsubscript{4} and the bacteria grown at 30°C may reflect the mixed contributions of proteins’ or peptidoglycans’ associated COO\textsuperscript{-} and NH\textsubscript{3}\textsuperscript{+} groups on the bacterial surface. However, it should be noted that the nature of nonspecific forces are versatile in the way the same force can have very different effects at short and long ranges\textsuperscript{12}. 

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The strongest attractive nonspecific force between the bacteria and Si$_3$N$_4$ in water was observed for cells grown at 30°C, compared to those grown at higher and lower temperatures investigated (Figure 4.3F, Table 4.2). Lifshitz-van der Waals attraction was found to be the dominant interaction force to the total nonspecific forces for all investigated conditions (Table 4.2). The strength of the Lifshitz-van der Waals force depends on the Hamaker constant of the interacting system, which is directly proportional to the dispersion energy between molecules$^{62-63}$ and hence to the biopolymer density on the bacterial surface. Thereby, the highest Lifshitz-van der Waals attractions observed for 30°C can be attributed to a higher density of biopolymers on the cells’ surfaces at 30 °C compared to when cells are grown at 37, 40, 20 and 10°C, respectively, as we have previously observed$^{22}$.

### 4.5.2 The strengths of hydrogen bonds derived from Poisson analysis.

A hydrogen bond can be understood as the attractive interactions observed between an electronegative proton donor (X–H) and an electronegative proton acceptor (Y) in which the directionality of the interacting atoms usually tends towards linearity (X–H…Y)$^{64}$. Linear networks of hydrogen bonds increase the dipole moment of the charges in the interacting atoms and lead to stronger hydrogen bonds$^{65-66}$. A linear directionality is the hallmark of hydrogen bonding$^{64}$. According to the recently proposed description of “hydrogen bond” by IUPAC$^{64}$, electrostatic forces play a significant role in the directionality of the interacting molecules and hence the strength of hydrogen bonding. However, there is no single physical force that can be ascribed to hydrogen bonds$^{64}$. Although the strength of the hydrogen bond is very sensitive to the structure of the interacting molecules where the geometries and the directionality vary$^{67}$, the typical strength of a hydrogen bond lies between 0.1 nN and 0.4 nN$^{68}$, and there is not a lower limit for hydrogen bonds$^{64}$. The hydrogen
bond strengths we derived for all conditions investigated are comparable to those reported in the literature (Table 4.2).

Hydrogen bond length also gives an indication of the bond strength; the length of a strong bond will be shorter than the length of a weak bond^{12, 69}. Hydrogen bond length represents the distance between the donor atom (X) and the acceptor atom (Y). Since the separation distance for the rupture event of a chemical bond by AFM would be theoretically the bond length^{70}, the closest separation distances between the AFM tip and the bacterial surface, which were calculated by a methodology combining Poisson and soft-particle DLVO analyses as described earlier, can give an indication of the hydrogen bond strength. For cells grown at 30°C, the distance calculated (0.24 nm) is the shortest of all, thus it is realistic to expect the strongest hydrogen bond for cells grown at 30°C compared to cells grown at other temperatures. Indeed, this is what our Poisson analysis predicted for the strength of hydrogen bond (-0.38 nN) for cells grown at 30°C compared to those predicted for cells grown at other temperatures (Figure 4.3F, Table 4.2). The extremely good agreement between the closest separation distances estimated by soft-particle analysis of DLVO forces based on experimental AFM measurements of nonspecific forces (Table 4.2) and those reported theoretically in the literature (0.157 nm^{71} and/or 0.3 nm^{12, 61}) is a further evidence for the accuracy of our methodology and the validity of the soft particle analysis of DLVO forces at our experimental conditions.

The hydrogen bonding between L. monocytogenes and Si$_3$N$_4$ in water will predominantly involve oxygen (O) and/or nitrogen (N) as the electronegative atoms. The hydrogen bonding observed between these electronegative atoms (O···H−O, O···H···N, and/or N···H···O) can arise from the interactions between the surface silanols (SiH$_3$−OH)$^{28}$ of Si$_3$N$_4$ and the surface hydroxyl and/or amine groups of the outer membrane molecules of the bacterium. The cell wall
of *L. monocytogenes* is composed of peptidoglycans, proteins, and polyanionic polymers such as teichoic acids and lipoteichoic acids, all of which can engage in hydrogen bonds formation with hydrated Si₃N₄ in water by their functional groups.

Consequently, one can expect that the strengths of hydrogen bonds derived for all thermal conditions investigated will be close to each other. However, the strength of the hydrogen bonding was dependent on the temperature used to grow the bacteria (Figure 4.3F, Table 4.2). This can be attributed to the structural changes in the surface biopolymers of *L. monocytogenes* grown at different temperatures as we have previously observed²². Biopolymers were the most extended when cells were grown at 30°C, compared to when grown at 37, 40, 20 and 10°C, respectively²² (Table 4.2), and acting as polyelectrolyte brushes which are known to stretch in the direction normal to the grafting surface. Since the linear directionality leads to stronger hydrogen bonds, the most extended biopolymers observed for the bacteria grown at 30°C will have the most favorable geometry for a linear directionality which can lead to the strongest hydrogen bond observed for 30°C. In addition, the specific force contribution from Poisson analysis may involve a discrete adhesive bacterial cell surface site rather than a single molecular force. The observed differences in the magnitudes of hydrogen bonding forces may reflect the involvement of multiple hydrogen bonds in the interactions.

**4.5.3 Re-coupling of adhesion forces.** Specific and nonspecific forces derived from Poisson analysis are the components of the overall adhesion force measured by AFM, which we have interpreted as the hydrogen bonding forces and DLVO forces (Lifshitz-van der Waals and electrostatic forces), respectively. When summed, the total of specific and nonspecific attractive forces was on average 18% higher than the mean overall adhesion forces for all conditions.
investigated (Table 4.2). This can be attributed to the potential lack of representation of other repulsive forces in the summation we have used.

When two interacting surfaces are less than few nanometers apart, other non-DLVO forces come into play\textsuperscript{12, 71, 74}. These short range interactions are referred to as *hydration forces* when the medium is water, and they are generally repulsive when the approaching surfaces are hydrophilic\textsuperscript{12}. Both Si\textsubscript{3}N\textsubscript{4} and *L. monocytogenes*, irrespective of the temperature of growth, have always possessed hydrophilic properties\textsuperscript{22}. Thereby, the additional repulsive interaction may arise from repulsive hydration forces, which exhibits oscillations with roughly a mean periodicity of the diameter of the water molecule (~0.25 nm) at separations <1.5 nm\textsuperscript{74}. The difference between $\overline{F}$ and $F_t$ was the highest at 0.24 nm compared to those at 0.32 and 0.40, 0.48, and 0.57 nm, respectively, suggesting the existence of the hydration forces in our system (Table 4.2). However, this possible repulsion had very low share in the overall adhesion in comparison to hydrogen bonding and total nonspecific forces (Table 4.2).

**4.5.4 Remarks on how to design new methodologies to control *L. monocytogenes* adhesion to inert surfaces.** The scenarios for conditions at which bacteria grow and then attach to surfaces in the food industry are endless. However, based on the sequence of steps in a food processing plant, the practices that aim at controlling bacterial contamination of surfaces may vary. We have observed that when cells were grown at different temperatures then transferred to room temperature, their adhesion strengths to Si\textsubscript{3}N\textsubscript{4} surface varied. The strongest specific and nonspecific attraction forces involved in the adhesion of *L. monocytogenes* EGDe to Si\textsubscript{3}N\textsubscript{4} in water were observed for the optimum growth temperature of 30°C, compared to those observed at 37, 40, 20 and 10°C, respectively, indicating the importance of the cooling systems in reducing the microbial adhesion to inert surfaces, even when they get transferred to environments at room
temperature. However, irrespective of the temperature of growth, hydrogen bonding forces were found to be stronger than nonspecific forces, suggesting a means to decrease the adhesion of *L. monocytogenes* to inert surfaces should focus on preventing hydrogen bonds’ formation between the microbe and surfaces. One strategy that can lead to a reduction in the number of binding sites for hydrogen bonds, electrostatic interactions as well for the hydrophobic interactions can rely on introducing ions to the environments where surfaces are contaminated with bacteria. Ions that can bind to electron donor groups of bacterial surface molecules can also reduce the adhesion of bacteria to surfaces. For example, using a low concentration of silver ions (50 ppb) was shown to be effective in destabilizing the biofilm matrix of *Staphylococcus epidermidis* by reducing the hydrogen binding sites, and hence the intermolecular forces within the extracellular polymeric substances of the biofilm\textsuperscript{75}. Another strategy can rely on the creation of polymer films on the surfaces that lack the functional groups capable of forming hydrogen bonds with bacteria. For example, coating of biomaterial surfaces with such polymer films was shown to be effective in reducing the adhesion of *Staphylococcus aureus* and *Staphylococcus epidermidis* to film surfaces\textsuperscript{76}.

**4.6 Acknowledgement.** We would like to thank Prof. Mark Lawrence; an associate professor at the Department of Basic Sciences, the College of Veterinary medicine at Mississippi State University for providing us with the *L. monocytogenes* strain EGDe, Prof. Markus Flurry; a professor of Soil Physics/Vadose Zone Hydrology in the Department of Crop and Soil Sciences at Washington State University for allowing us to use his goniometer and Zeta potential analyzer to do the contact angle and electrophoresis measurements, implicitly used in the current study, and National Science Foundation grant EEC-0823901 for financial support of this study.
4.7 References.


Chapter 5

A New Thermodynamic Approach to Explaining the Molecular-Scale Bacterial Adhesion Measurements

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Abstract

The current study describes a new thermodynamic approach to explaining the factors that affect the molecular-scale bacterial adhesion process at a fundamental level. By applying the Boltzmann distribution of energies to the probability histograms of bacterial adhesion energies measured by atomic force microscopy (AFM) between the surface biopolymers of bacterial cells and a model surface of silicon nitride in water, the total number of molecules involved in the adhesion process and the entropy or the disorder of the bacterial surface molecules were estimated. Our results provided a thermodynamic evidence of the direct relationship between the strength of bacterial adhesion measured at a molecular level and the number of bacterial surface molecules available for interactions and the length of the bacterial biopolymer brush.

**Keywords:** Bacterial adhesion, Boltzmann distribution, Adhesion energy, Entropy, AFM.
5.1 Introduction

The likelihood of a bacterium to establish contact with a potential surface of interest can be predicted on the basis of macroscopic laws of thermodynamics\textsuperscript{1,2}. However, thermodynamic analyses of bacterial adhesion to surfaces have always been questioned due to the macroscopic nature of the approach\textsuperscript{3-6}. For instance, thermodynamic analysis of the adhesion of a bacterium to a surface cannot account for specific molecular interactions, such as ligand-receptor interactions, hydrogen, ionic or covalent bonding between the bacterial surface molecules and the surface of interest. However, it is well accepted that such molecular interactions are critical for fundamental understanding of the bacterial adhesion process\textsuperscript{7,8}. Thereby, accredited information on the strength of the overall adhesion between a bacterium and a surface of interest relies heavily on direct measurements of such molecular interactions using various tools such as the atomic force microscopy (AFM) technique.

Statistical mechanics can provide an estimation of macroscopic thermodynamic quantities such as energy and entropy by applying the Boltzmann distribution to the molecular-level data measured for the large number of particles that constitute the macroscopic system of interest\textsuperscript{9,10}. The Boltzmann distribution has been used to explain how various types of energy act in a range of cellular processes where molecular details increase in importance such as the kinetics of enzyme-catalyzed reactions and the diffusion of ions across charged membranes\textsuperscript{10}.

Here, Boltzmann distribution was applied to the distribution of adhesion energies measured at the molecular level between the many biopolymers that compose the surface of \textit{Listeria monocytogenes} EGDe grown in media of five different ionic strengths (IS of 0.05M, 0.1M, 0.3M and 0.5M NaCl) and a model surface of silicon nitride in water using atomic force microscopy (AFM). Statistical thermodynamics was then used to predict the number of surface
molecules involved in the bacterial adhesion to the silicon nitride surface, and the entropy of the bacterial surface molecules. Finally, the relationship between the entropic interactions and the length to which the bacterial surface biopolymers were extended was investigated.

5.2 Materials and Methods

5.2.1 Bacterial cultures. *L. monocytogenes* EGDe was activated by growing for twelve hours at 30 °C in a temperature controlled shaker rotating at a 150 rpm in brain heart infusion broth (BHIB). Following activation, cells were cultured in BHIB (IS of water, 0.003 M\(^{11,12}\)), and in BHIB with added NaCl (IS of 0.05M, 0.1M, 0.3M and 0.5M) at 30°C fixed temperature until cells reached late exponential phase of growth, the stage at which they were harvested. The growth of the bacterial cells was monitored by reading the optical density of the culture every hour at a wavelength of 600 nm.

5.2.2 Atomic force microscopy measurements. Prior to AFM force measurements, cultured bacterial cells in BHIB were centrifuged twice at 5525g for 10 minutes. After centrifugation, cells were attached to gelatin-coated mica disks according to the procedure detailed elsewhere\(^{13,14}\). All AFM force measurements were performed in TappingMode™ using a PicoForce™ Scanning Probe Microscope with a Nanoscope IIIa controller and extender module (Bruker AXS Inc., Santa Barbara, CA). Forces were measured under DI water using silicon nitride cantilevers (DNP-S cantilevers with 0.06 N/m nominal spring constant (Bruker AXS Inc., Santa Barbara, CA). We have shown previously that attaching *L. monocytogenes* cells to gelatin-coated mica and performing force measurements on bacterial cells under water do not affect bacterial cell viability or integrity\(^{15}\).

The force constant of each cantilever was determined from the power spectral density of the thermal noise fluctuations in DI water\(^{16}\). On average, the spring constant was found to be
0.056 ± 0.004 N/m, very close to the nominal spring constant value reported by manufacturer in air. Once a bacterial cell had been located via topographical scanning, the oscillation of the cantilever was stopped and the extending and retracting deflection displacement curves measured between the silicon nitride tip and bacterial surface biopolymers were captured using the AFM software. Force measurements were made on a bacteria-free area of the gelatin-coated mica disk before and after making a measurement on a bacterial cell. Equality of the measurements ensured that the tip properties had not been altered by contact with the bacterial surface biopolymers.

For each ionic strength of growth media investigated, 14 bacterial cells were examined from three different cultures. On each cell, 15 points were located to perform force measurements using the point and shoot feature of the AFM software. Retraction curves were measured at a rate of 580 nm/sec to minimize the hydrodynamic drag forces, and at a resolution of 4096 points.

5.2.3 Analysis of retraction and approach curves. Retraction curves were considered individually because of the complex and heterogeneous nature of the interactions observed between the bacterial surface biopolymers and the AFM silicon nitride cantilever. Bacterial adhesion was quantified from the retraction curves in terms of adhesion energy in atto-Joule (AJ). For each retraction curve, the adhesion energy was computed as the area under the retraction force-distance curve using the Trapezoidal rule, as we have detailed previously (Chapter 2).

The experimental approach force-distance data were fit to steric model following the work of Alexander and de Gennes using an in-house Matlab written code that minimizes the difference between experimental measured forces and theoretical predictions of forces. Note that
the fitting parameters; the equilibrium thickness \( (L_o, \text{ nm}) \) and the grafting density \( (\Gamma, \text{ m}^{-2}) \) of the biopolymer layer on the bacterial surface were previously determined (Chapter 3).

5.3 Modeling

5.3.1 Application of Boltzmann distribution to bacterial adhesion energies. Statistical mechanics, which was first initiated by the work of Boltzmann on gas kinetics\textsuperscript{26}, provides a molecular-level interpretation of energy \( (J) \) and entropy \( (J/K) \) by applying the probability theory to the thermodynamic behavior of macroscopic systems\textsuperscript{9,10}. The second law of thermodynamics serves as a bridge between the macroscopic and molecular level understanding of thermodynamic quantities such as balancing the distribution of molecules between the maximum entropy and minimum energy states in a specified system at equilibrium. In statistical mechanics, Boltzmann’s definition of entropy \( (S, J/K) \) quantifies disorder at the molecular level by\textsuperscript{10}:

\[
S = k_B \ln w
\]

where \( k_B \) is the Boltzmann constant \( (1.3807 \times 10^{-23} \text{ J/K}) \), and \( w \) is the number of different possible configurations of the system’s molecules. In statistical mechanics, a specific configuration of molecules is called a “state” and each state is associated with a specific energy value\textsuperscript{9,10,26}. Since, molecules spread out to achieve a higher molecular disorder at a higher energy of state, the concentrations of molecules will decrease monotonically with the increasing energy of states as defined by the Boltzmann distribution\textsuperscript{9,10,26,27}

\[
\exp\left(-\frac{E_i}{k_B T}\right)
\]

where \( E_i \) is the energy per molecule in an individual state \( (J) \), and \( T \) is the temperature (298 K for our case). A probability distribution of the energies of possible states describes the probability of finding the system in a certain energy of state. The Boltzmann distribution for the
frequency with which that state having energy $E_i$ occurs, or the probability of its occurrence, is proportional to\textsuperscript{26}:

$$
P_i = \frac{W_i \exp\left(-\frac{E_i}{k_B T}\right)}{\sum_i W_i \exp\left(-\frac{E_i}{k_B T}\right)}
$$

(5.3)

where $W_i$ is the statistical weight, the degeneracy, or the number of states in a given energy state $E_i$, which increases with the increasing energy\textsuperscript{9,10,26}. Therefore, although the concentrations of molecules at the individual states decrease with increasing energy, there are many more states of a given energy at high energy values, and the product of this rising $W_i$ and the falling exponential function has a bulge at the most probable energy of the system\textsuperscript{26}.

By utilizing the fact that energy of various types is shared among molecules according to the Boltzmann’s distribution\textsuperscript{10} for our system (a bacterial cell), we have assumed that computed bacterial adhesion energies are associated to specific configurations or specific states of the molecules on the bacterial surface.

$$
P_i = \frac{W_i \exp\left(-\frac{E_{adh}/\alpha}{k_B T}\right)}{\sum_i W_i \exp\left(-\frac{E_{adh}/\alpha}{k_B T}\right)}
$$

(5.4)

where $E_{adh}/\alpha$ represents the adhesion energy per molecule in an individual state (AJ), and $W_i$ is used as an adjustable parameter indicating the increment of the states as the size of the adhesion energy increases.

By fitting the Boltzmann probability function given in equation 5.4 to the probability histograms of bacterial adhesion energies (Figures 5.1A-E), the fitting parameter $\alpha$ representing the total number of molecules involved in the adhesion of \textit{L. monocytogenes} EGDe to silicon
nitride surface in water was quantified for all ionic strength of growth investigated. The fitting parameter $\alpha$ was also used as an indicator of the number of different possible configurations of the system’s molecules $w$ given in equation 5.1 to quantify the entropy or the disorder at the molecular level (Table 5.1).

5.4 Results and Discussion

5.4.1 Boltzmann distribution of AFM adhesion energies as a function of ionic strength of growth. As can be seen from Figures 5.1A-E, the adhesion energies between silicon nitride and bacterial cells grown in media of different ionic strengths were heterogeneous at all investigated conditions, as commonly reported in the AFM measurements of bacterial adhesion\textsuperscript{18-21,23}. The observed heterogeneity in the distribution of adhesion energies was described well by the Boltzmann probability distribution (eq. 5.4). The ability of the Boltzmann distribution to fit the adhesion energy histograms was judged based on the estimated values of $r^2$ (the coefficient of determination, often used to judge the adequacy of a regression model) which was on average 0.96 for all investigated conditions.

The fitting parameter $\alpha$ representing the total number of molecules involved in the bacterial adhesion to silicon nitride surface in water was the lowest for the cells grown at IS of 0.1M, followed by 0.3M, 0.05M, 0.5M, and 0.003M, respectively. The very same trend was also observed for the mean values of bacterial adhesion energies computed from AFM retraction curves as a function of ionic strength of growth (Table 5.1), as can be seen from the linear correlation between the total number of molecules and the bacterial adhesion energies (Figure 5.1F).

Our results suggest that the adhesion of \textit{L. monocytogenes} to silicon nitride surface in water can be affected by the number of molecules involved in the bacterial adhesion process. The
heterogeneities in the AFM measurements of bacterial adhesion have been largely attributed to the wide array of molecules present on the bacterial surface. For instance, *L. monocytogenes* is a Gram-positive bacterium and the cell wall of *L. monocytogenes* is composed of peptidoglycans, proteins, and polyanionic polymers such as teichoic acids and lipoteichoic acids. The wider distributions in the AFM adhesion data observed for cells grown at 0.003M, 0.05M and 0.5M (Figures 5.1A, 5.1B and 5.1E) can also be attributed to the presence of higher number of molecules on the bacterial surface (Table 5.1).

**Table 5.1**: A summary of the total number of molecules quantified by fitting the Boltzmann distribution to the probability histograms of adhesion energies measured between *L. monocytogenes* EGDe individual cells grown at five different ionic strengths and silicon nitride under water by AFM. In addition, the quality of the fitting judged by the coefficient of correlation ($r^2$) values, the mean and the standard error of the mean of all the measured bacterial adhesion energies, and the entropy and the length of the surface molecules of the bacterial cells are given for all ionic strength of growth investigated.

<table>
<thead>
<tr>
<th>Ionic strength (NaCl, M)</th>
<th>0.003</th>
<th>0.05</th>
<th>0.1</th>
<th>0.3</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ (total number of molecules)</td>
<td>18275</td>
<td>11574</td>
<td>4191</td>
<td>4419</td>
<td>12153</td>
</tr>
<tr>
<td>$r^2$ (the quality of fitting)</td>
<td>0.93</td>
<td>0.93</td>
<td>0.98</td>
<td>0.99</td>
<td>0.96</td>
</tr>
<tr>
<td>Mean adhesion energy (AJ)</td>
<td>147.6</td>
<td>103.5</td>
<td>43.5</td>
<td>51.2</td>
<td>133.5</td>
</tr>
<tr>
<td>Standard error of mean (AJ)</td>
<td>6.4</td>
<td>6.3</td>
<td>3.2</td>
<td>5.5</td>
<td>9.5</td>
</tr>
<tr>
<td># of adhesion energy states</td>
<td>200</td>
<td>179</td>
<td>195</td>
<td>173</td>
<td>170</td>
</tr>
<tr>
<td>$S = k_B \ln \alpha \ (10^6 \times \text{AJ}/\text{K})$</td>
<td>135.5</td>
<td>129.2</td>
<td>115.2</td>
<td>116.0</td>
<td>130.0</td>
</tr>
<tr>
<td>$L_0 \ (\text{nm})$</td>
<td>176 ± 13</td>
<td>68 ± 4</td>
<td>28 ± 1</td>
<td>52 ± 2</td>
<td>128 ± 6</td>
</tr>
</tbody>
</table>
Figure 5.1 Boltzmann distribution fitting to the probability histograms of bacterial adhesion adhesion for the cells grown at different ionic strengths 5.1A-E. F) A linear correlation between the number of molecules quantified from Boltzmann fitting and the mean adhesion energies measured by AFM.
5.4.2 Statistical thermodynamic interpretation of the entropy of bacterial adhesion. By applying the Boltzmann distribution to the probabilities of bacterial adhesion energies measured by AFM, the thermodynamic behavior of bacterial adhesion at the molecular level can be quantified in terms of the entropy or the disorder of the molecules at the most probable energy of the system. The Boltzmann’s definition of entropy was quantified by using the fitting parameter $\alpha$ as the number of different possible configurations of the system’s molecules $w$ given in equation 5.1.

Entropy determines that thermal energy ($k_B T$) always flows from the higher energy of state to the lower energy of state, and quantifies the favorability of a process\textsuperscript{10,27}. For the bacterial adhesion process, the thermal energy of the molecules which is related to specific configurations the molecules have on the bacterial surface indicates the strength or the favorability of the adhesion process. The entropy of the molecules involved in the bacterial adhesion process was found to be on average 12\% lower for the cells grown at IS of 0.1M and 0.3M compared to when grown at IS of 0.05M, 0.5M, and 0.003M, indicating that the bacterial adhesion was \textit{entropically less favored} when the cells were grown at IS of 0.1M and 0.3M.

The Boltzmann’s definition of entropy can also provide an explanation for the configuration of the molecules on the bacterial surface. The length to which bacterial surface brushes are extended is largely controlled by the free energy of the chains which is the summation of the entropic interactions and the energy caused by excluded volume interactions and incorporates both electrostatic and non-electrostatic effects\textsuperscript{28}. Figure 5.2 shows the dependence of the length of bacterial surface brushes to the entropic interactions at the molecular level which indicates that higher bacterial adhesion energies were associated to longer bacterial surface brushes, as we have also observed for the interaction between \textit{L. monocytogenes} EGDe
grown at different temperatures and silicon nitride in water\textsuperscript{21}. This study provides an evidence to our previous findings presented in Chapter 2 and 3, in which we have always observed that the higher adhesion was in part explained by longer bacterial surface brushes in which biopolymers furnish longer arms for the adhesion.

![Figure 5.2](image-url)

**Figure 5.2** The dependence of the length of bacterial surface brushes to the entropic interactions at the molecular level, indicating that higher bacterial adhesion energies were associated to longer bacterial surface brushes.

### 5.5 Conclusions.

Our results suggest that bacterial adhesion phenomenon can be explained in part by determining the total number molecules involved in the bacterial adhesion process, as by applying the Boltzmann distribution of energies to the probability histograms of bacterial adhesion energies measured by AFM. The total number of molecules involved in adhesion was used to estimate the entropy of the bacterial adhesion process which was used as an indicative of the favorability of bacterial adhesion process. Finally, we have shown that the length of bacterial surface biopolymer brushes linearly correlates with the entropic interactions of the system estimated at the molecular level.
5.6 Acknowledgement. We would like to thank Prof. Mark Lawrence; an associate professor at the Department of Basic Sciences, the College of Veterinary medicine at Mississippi State University for providing us with the *L. monocytogenes* strain EGDe used in the current study. We would like to thank as well the National Science Foundation grant EEC-0823901 for financial support of this study.
5.7 References


6.1 Conclusions

AFM has been successfully used to investigate the relationship between the physiochemical properties of *L. monocytogenes* EGDe and the DLVO and hydrogen bonding interactions with silicon nitride surface in water. Our studies in Chapters 2 and 3 were combined to study the effects of environmental stresses of growth (temperature and ionic strength) on the mechanical and physiochemical properties of *L. monocytogenes* EGDe surface biopolymers in relation to their overall adhesion strength to silicon nitride. In Chapter 2, we studied the role of the temperature of growth on the mechanics and adhesion of this microbe to silicon nitride surface. The biopolymers on the surface of *L. monocytogenes* EGDe were shown to undergo a temperature-induced structural change from extended and dense biopolymers at 30 °C to shorter and less crowded at lower and higher temperatures of growth. The enhanced adhesive capacity for the cells when grown at 30 °C was related to both their longer and denser surface biopolymers, higher proteins’ content, and their lower negative surface potential, compared to those observed for cells grown at 37, 40, 20 and 10 °C, respectively. In this study, we were able to display the strong influence of the bacterial temperature of growth on the mechanical and physiochemical properties of bacterial cells, and total proteins’ content on the bacterial initial attachment to silicon nitride. In addition our experimental results agreed well with the theoretical results predicted from the soft-particle DLVO theory in that lower values of the maximum energy barriers to adhesion were obtained for the cells grown at 30 °C.

In Chapter 3, we studied the role of ionic strength of growth on the mechanical and physiochemical properties of the surface biopolymers of *L. monocytogenes* EGDe by extending
our study presented in Chapter 2 through the evaluation of Donnan potentials and scaling relations of bacterial polyelectrolyte surface brushes. The structure and the electric properties of the surface polyelectrolyte brush layer of *L. monocytogenes* EGDe were shown to be dependent on the ionic strength of growth, suggesting variations in the array and/or amount of the surface molecules contributing both to charging of the bacterial surface and adherence of the microbe to inert surfaces. With this study, we were also able to show that the adhesion affinity of *L. monocytogenes* to inert surfaces can change when the microbe is transferred to a suspension with a different IS. This finding is important for the predictive physiochemical strategies for the control of the adhesion of this microbe to inert surfaces in water.

Our study in Chapter 4 is a statistical investigation for the effects of specific and nonspecific forces on the overall adhesion of *L. monocytogenes* EGDe to silicon nitride in water. We have performed our study for the cells grown at various temperatures. Our results revealed that specific hydrogen bonding forces were dominating the adhesion process over nonspecific DLVO forces at all investigated conditions, suggesting a means to decrease the adhesion of *L. monocytogenes* to inert surfaces should focus on preventing the formation of hydrogen bonds between the microbe and the surfaces. As a conclusion for our studies presented in Chapters 2, 3 and 4, the following startegiues can be used to effectively control the adhesion of *L. monocytogenes* to food processing surfaces:

a. A proper control of the cooling systems used in the food industry

b. A physiochemical strategy which takes into account the ionic strength of the solutions through which the bacteria transfer

c. Disrupting the formation of hydrogen bonds between the bacteria and the inert surfaces together with
d. Introducing an electrochemical approach that can manipulate the surface charge of food processing surfaces.

Finally in Chapter 5, we described a new thermodynamic approach to explaining the factors that affect the molecular-scale bacterial adhesion process at a fundamental level by utilizing statistical mechanics. Our results provided a thermodynamic evidence of the direct relationship between the strength of bacterial adhesion and the number of bacterial surface molecules available for interactions and the length of the bacterial biopolymer brush. Our results suggested that bacterial adhesion phenomenon can be explained in part by determining the total number molecules involved in the bacterial adhesion process. In addition, with the results we have presented in Chapter 5, we have provided evidences to our previous studies (presented in Chapters 2 and 3) in which we have always observed that the higher adhesion was in part explained by longer bacterial surface brushes.

The results presented in this dissertation provide new insights toward a better understanding of the fundamentals of adhesion processes between bacteria and surfaces which can potentially lead to the development of better strategies to avoid the problems associated with bacterial adhesion to inert surfaces.