Adsorption and bacterial adhesion characteristics of proteins, microbial growth media and milk on abiotic surfaces under static and laminar flow conditions

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ABSTRACT

Understanding the adhesion of bio-molecules and microorganisms (known as biofilms) to abiotic surfaces is an important consideration in food industry. The formation of these biofilms can have deleterious effects leading to contamination of food or materials coming into contact with them, causing spoilage &/or safety concerns. Among the biomolecules proteins are known to adhere to the surface very rapidly, followed by other biomolecules and microorganisms.

In this study the two abiotic model surfaces, mica and polystyrene, were conditioned with proteins [bovine serum albumin (BSA), sodium caseinate (CAS)], microbial growth media [tryptic soya broth (TSB)] and milk [whole milk (WML), skim milk (SML)] under static and dynamic flow processing conditions. The extent of protein adsorbed to surfaces was quantified using the micro BCA protein assay. The food contact surfaces conditioned or without conditioning, were characterized using contact angle measurements to obtain various surfaces thermodynamic properties (surface energy and its components, and absolute hydrophobicity). The conditioning solutions were also characterized by dynamic light scattering and zeta potential measurements. The total free energy of interaction was calculated using the thermodynamic and the extended XDLVO models, and the adhesive potential of the biofilms was evaluated. The effect of preconditioning (under static and flow) on the adhesion of bacteria (*Listeria innocua* and *Listeria monocytogenes*) to mica and polystyrene were studied. In addition the distribution of conditioning solutions and bacteria on the surface was evaluated using atomic force microscopy.

The results under static conditions showed that the amount of proteins adsorbed on mica was much higher than polystyrene surfaces which may be because mica is more hydrophilic and polystyrene more hydrophobic. Sodium caseinate had the highest adsorption on both mica and polystyrene when compared to other conditioning solutions. The thermodynamic properties of conditioned surfaces showed that they favored electron-donating or Lewis base properties which are typical for proteins in hydrated states. DLS and Zeta potential results indicated that proteins were in aggregated states in solutions and had a negative surface charge. Preconditioning of mica and polystyrene surfaces altered their surface thermodynamic properties, especially, the electron donor and acceptor components of the surface free energy. This had a significant impact on the resulting hydrophobicity of the preconditioned surfaces as compared to the surfaces which were

not conditioned. The computed free energy of interaction using the two models indicated the adhesion potential of conditioning solutions (in most cases) on both surfaces, but failed to accurately predict the extent of protein adsorption on surfaces. Under laminar flow conditions the amount of protein adsorbed onto mica and polystyrene surface was approximately two times higher than those obtained under static conditions. The DLS measurements of conditioning solutions showed that particle sizes of the conditioning solutions were more polydisperse than under static conditions.

Dynamic flow condition also altered the surface thermodynamic properties of mica and polystyrene as observed with static conditions. The AFM analysis of both static and flow conditioned surfaces revealed that the conditioning film formed was not a continuous film but a heterogeneous deposit and attached in the form of aggregates.

The adhesion of *Listeria innocua* to mica and polystyrene surfaces that were preconditioned with BSA, TSB and CAS under laminar flow showed *lower* adhesion values in comparison with those under static settings. However, with whole milk and skim milk, *higher* adhesion values of *L. innocua* were observed under flow conditions as compared with static conditions indicating bio-film source dependent variations. It was also observed that *L. innocua* cell adhesion on mica and polystyrene surfaces preconditioned with CAS WML and SML under static conditions were lower than *Listeria monocytogenes* under similar conditions, indicating strain dependent variations.

RESUME

Comprendre l'adhérence des substances biologiques comme des macromolécules et des micro-organismes (biofilms) sur des surfaces abiotiques a des effets délétères pour l'environnement de l'industrie alimentaire à cause des problèmes de sécurité ou détérioration alimentaire. Parmi les biomolécules, les protéines sont connues d'être capable de conditionner les surfaces très rapidement, un procès qui est suivi par l'adsorption ou l'adhésion d'autres biomolécules et les micro-organismes.

Dans cette étude, deux surfaces abiotiques (mica et polystyrène) ont été conditionnés avec des protéines [sérum-albumine bovine (BSA), caséinate de sodium (CAS)], des milieux de croissance microbienne [bouillon trypticase soja (TSB)] et le lait [lait entier (WML), lait écrémé (SML)] dans des conditions statiques et sous flux. L'étendue de la protéine adsorbée sur des surfaces a été quantifiée en utilisant le teste micro-BCA. Les surfaces de contact alimentaire (mica et polystyrène), conditionnés et non conditionnés, ont été caractérisés par des mesures d'angle de contact pour obtenir des propriétés thermodynamiques (énergie de surface et de ses composantes et l'hydrophobie absolue) pour les deux surfaces. Les solutions de conditionnement ont également été caractérisées par diffraction dynamique de la lumière et des mesures de potentiel zêta. L'énergie libre totale d'interaction a été calculé en utilisant les modèles thermodynamique et XDLVO, et les prévisions d'adhésion de protéines et de lait à des surfaces de mica et de polystyrène a été faite sur la base des modèles. L'effet du pré-conditionnement (statique et sous flux) sur l'adhérence des bactéries (*Listeria innocua* et *Listeria monocytogenes*) aux deux surfaces ont été étudiés. En plus, la distribution sur la surface des solutions de conditionnement et de bactéries a été étudiée en utilisant la microscopie à force atomique.

Sous condition statique, les résultats ont montré que la quantité de protéine adsorbée sur le mica était beaucoup plus élevée que les surfaces de polystyrène, attribué a son propriétés hydrophiles. La quantité de protéines à partir de solutions à base de caséinate de sodium adsorbé sur les deux surfaces (mica et polystyrène) était le plus élevé par rapport à d'autres solutions de conditionnement. Les propriétés thermodynamiques des surfaces conditionné ont montré qu'ils étaient les donneurs d'électrons ou tenir le rôle d'un base Lewis, qui sont les propriétés typiques pour des protéines hydratés. Les mesures de potentiel DLS et Zeta ont indiqué que les protéines

étaient dans des états agrégés dans les solutions et avait une charge de surface négative. La pré-conditionnement des surfaces (mica et polystyrène) modifié leurs propriétés thermodynamiques, en particulier, les constituent donneur et accepteur d'électrons de l'énergie libre de surface, qu'avaient un impact significatif sur l'hydrophobie des surfaces pré-conditionnés par rapport à les surfaces non-conditionnée. L'énergie libre d'interaction, calculé avec les deux modèles, ont démontré une adhérence favorable de solutions de conditionnement (dans la plupart des cas) sur les deux surfaces, mais n'a pas réussi à prédire avec précision l'étendue de l'adsorption des protéines sur les surfaces. Dans des conditions de flux laminaire la quantité de protéines adsorbées sur le mica et le polystyrène surface était environ deux fois plus élevés que ceux obtenus dans des conditions statiques. Les mesures DLS des solutions de conditionnement ont montré que les tailles de particules des solutions de conditionnement ont été plus diffusées que dans des conditions statiques.

État de flux a également modifié les propriétés thermodynamiques des surfaces (mica et polystyrène) par rapport à des conditions statiques. L'analyse par AFM des deux surfaces statiques et conditionnés flux a démontré que le film de conditionnement formé n'était pas un film continu, mais un dépôt hétérogène et fixé sous la forme d'agrégats.

L'adhésion de Listeria innocua aux surfaces pré-conditionnée avec BSA, TSB ou CAS sous flux a montré des valeurs d'adhérence plus faibles par rapport aux surfaces qui ont été pré-conditionnés par méthode statique. Par contre, les surfaces pré-conditionnées par lait (entier et écrémé) sous flux ont démontré des valeurs d'adhérence plus élevées de L. innocua par rapport aux conditions statiques, indiquant des variations dépendantes de la source. En plus, il a été observé que le nombre de cellules de L. innocua adhéré sur des surfaces de mica et de polystyrène pré-conditionnés dans des conditions statiques avec CAS, WML et SML était inférieur à Listeria monocytogenes sur les surfaces qui ont été pré-conditionnés aux mêmes conditions, indiquant des variations dépendant de la souche microbienne.

CONTRIBUTION OF AUTHORS

Several presentations have been made based on the thesis research and some manuscripts have been prepared for publication. Some authors have been involved in the manuscript and their contributions are detailed as follows:

Nikhil Hiremath is the Ph.D candidate who planned and conducted all the experiments, in consultation with his supervisor, gathered and analyzed the results, and drafted all the manuscripts for scientific publications.

Dr. H. S. Ramaswamy is the thesis supervisor, under whose guidance the research was carried out, and who assisted the candidate in planning and conducting the research as well as in correcting, editing, reviewing and processing the manuscripts for publications.

Dr. Louise Deschênes supervised the candidate's research work at Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600, Casavant Blvd W, Saint-Hyacinthe, Québec J2S 8E3, Canada. She provided the laboratory facilities and funding, offered scientific advice, reviewed experimental procedures and supported the candidate to carry out the experimental study as well as corrected, edited, reviewed and processed the manuscripts for publications.

Dr. Mohammad Reza Zareifard helped the candidate in the fabrication of the glass vials and assembling the modified Robbins device. In addition he also supported and offered scientific advice, reviewed manuscript and provided technical inputs for data analysis and calculations.

Dr. Gilles Robitaille offered suggestions to the review paper and Dr. Akier assanta Mafu provided partial funding for the project and helped in microbiological procedures.

Mr. Francois St. Germain helped the candidate with protein quantification experiments and performed atomic force microscopy measurements. Mrs. Marie Josee Lemay helped the candidate to prepare *L. innocua* cells and performed all experiments related to *L. monocytogenes* at Microbiology division, Canadian Food Inspection agency, Saint-Hyacinthe, Québec, Canada.

LIST OF PUBLICATIONS AND PRESENTATIONS

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Nikhil Hiremath, Louise Deschênes, Hosahalli Ramaswamy, and Mohammed. Reza Zareifard. Adhesion of *Listeria innocua* and *Listeria monocytogenes* on mica and polystyrene pre-conditioned under static and laminar flow settings (in preparation)

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Nikhil Hiremath, Louise Deschênes , Hosahalli Ramaswamy and M. Reza Zareifard. 2011. Protein adsorption kinetics: Quantification of surface bound proteins on selected food contact surfaces; Northeast Agricultural and Biological conference, July 24 to July 27, 2011, South Burlington, Vermont, USA (Presentation).

Nikhil Hiremath, Louise Deschênes , Hosahalli Ramaswamy, M. Reza Zareifard and Tatiana Koutchma. 2010. Review on adhesion and biofilm formation under continuous flow conditions in food processing. Canadian Institute of Food Science and Technologists conference, May 31 - June 1, 2010, Winnipeg, Manitoba, Canada. (Poster)

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NOMENCLATURE

%	Percent
~	Approximately
°C	degree Celsius
Å	Angstrom(s)
AB	Acid base
AFM	Atomic force microscopy / micrograph
ATCC	American type culture collection
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAM	Contact angle measurements
CAS	Sodium caseinate
CCD	Charge-coupled device
Cfu	Colony forming unit
Cm	Centimeter(s)
DLS	Dynamic light scattering
DLVO	Derjaguin, Landau, Verwey, Overbeek
EPS	Extracellular polymeric substances / Exopolymeric substances
FTÅ	First Ten Angstorms
H	Hour(s)
HOPG	Highly oriented pyrolytic graphite
kDa	Kilo Dalton
LW	Lifshitz -van der Waals
Mg	Milligram
mg/m^2	Milligram per square meter
Min	Minute(s)
Ml	Milliliter(s)
Mm	Millimeter(s)
MΩ•cm	Mega Ohm centimetre
Nm	Nanometer(s)
PBS	Phosphate buffered saline
PS	Polystyrene
Re	Reynolds number
SD	Standard deviation
SML	Skim milk
TSA	Trypticase soy agar
TSB	Trypticase soy broth
VCG	van Oss, Chaudhary and Good
WML	Whole milk
XDLVO	Extended Derjaguin, Landau, Verwey, Overbeek
Mg	Microgram(s)
$\mu g/cm^2$	Microgram per square centimeter
Mm	Micrometer(s)

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Chapter 1. INTRODUCTION

In a natural environment, majority of microorganisms live together in large numbers, generally attached to a contact surface. It is now well recognized that most bacteria spend much of their lives in the microbial equivalent of a "gated community"—a “biofilm” rather than living as isolated cells (so-called planktonic form) (Harrison et al., 2005). Biofilm can be defined as the assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002). The polymeric matrix is primarily composed of polysaccharides, proteins, and nucleic acids which are collectively termed “extracellular polymeric substances” (EPS or gycocalyx). In addition, non-cellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, may be found in the biofilm matrix depending on the environment in which the biofilm has developed (Vu et al., 2009).

Bacterial adhesion and biofilm formation on surfaces is considered a major threat to safety in food industry applications (Fratamico et al., 2009; Srey et al., 2013). Bacteria can adhere to food processing equipment surfaces which can lead to serious hygiene problems and economic losses due to food spoilage and equipment impairment. Bacteria can adhere to form biofilms on food processing equipment, on conveyor belts, in pipes, on floors, and in drains. They can cause metal corrosion in pipelines and tanks, and they can reduce the heat transfer efficacy if they can become sufficiently thick in equipment’s such as plate heat exchangers and pipelines (Brooks & Flint, 2008; Kumar & Anand, 1998; Storgards et al., 1999). Besides, the inefficiency of cleaning agents (surfactants) and disinfectants or antimicrobials to efficiently remove biofilms is one of the major problems that is faced by the food industry (Simões et al., 2010; Vlková et al., 2008). Because of this, it is becoming increasingly necessary to gain a better understanding of microbial adhesion (especially pathogenic bacteria) and biofilm formation in food and dairy industry environments (Marchand et al., 2012).

During the early stages of microbial adhesion and biofilm formation on abiotic surfaces, macromolecules present in a bulk solution, adsorb to the contact surface forming a conditioning film (Carpentier & Cerf, 1993; Donlan, 2002; Percival et al., 2011). In fact, it is presently well recognized that the microorganisms do not adhere directly to a surface per se, but actually adhere

to a conditioning film. The formation of conditioning film on a surface can significantly impact bacterial initial adhesion by altering the physicochemical properties of solid surfaces such as surface tension, charge density, and roughness (Jullien et al., 2008; Parkar et al., 2001; Schneider, 1996). It may also serve as a source of nutrition for adherent bacteria especially in food industry environment and may increase the likelihood of contamination of the food products. Hence studying the formation of conditioning film in food industry conditions will provide a wider understanding towards microbial adhesion and formation of biofilms on surfaces.

The nature of the conditioning film may be quite different depending on the kind of environment to which the surface is exposed. In general the conditioning film formed from the adsorption of proteins onto surfaces has received considerable attention not only in food industry but also marine, dental and medical settings as it is a fundamental phenomenon and also of great technical significance. For instance, the fouling of stainless surfaces by whey proteins especially β-lactoglobulin (β-lac), in dairy industry particularly at pasteurization and sterilization temperatures (Bansal & Chen, 2006; Detry et al., 2010), the fouling of clinical materials by proteins upon contact with living tissues and biological fluids such as blood, tears, saliva in dental and medical settings (Kishen & Haapasalo, 2010) and fouling by ship hulls by proteins in aquatic environments have been well studied (Garg et al., 2009). The investigations have focussed on different aspects including total amount adsorbed, the adsorption kinetics, the reversibility of adsorption, morphology, thickness of the protein layer, organization and orientation of molecules on the surface, as well as the extent of structural alterations. Despite considerable research in this field understanding the adsorption of proteins onto surfaces still remains a challenge (Rabe et al., 2011).

Studying conditioning film formation, bacterial adhesion and biofilm formation in natural environments is a challenge due to the inherent degree of complexity associated within the system. To facilitate the study in the laboratory, simplified in-vitro model systems and experimental techniques have been developed (Kishen & Haapasalo, 2010; McBain, 2009). Unfortunately, no single optimal model can be universally applicable and the choice of the experimental technique or model to study biofilm is dependent on the type of nature of the problem that is in question (McLandsborough et al., 2006). Some common ways to understand

the adhesion phenomenon of conditioning film, bacteria and biofilm formation is to use advanced techniques such as atomic force microscopy (AFM) which can provide information at nanoscales. Usually, topography, structure and composition have been characterized (Morris et al., 2011). Other ways to understand adhesion phenomena is to use physicochemical theories to predict if adhesion is likely to occur. Three models, namely, a) a thermodynamic model, b) DLVO (Derjaguin, Landau, Verwey, Overbeek) model, and c) extended DLVO model (XDLVO), all of which are based on the classical colloidal theories, have been widely utilized (Bos et al., 1999; Missirlis & Katsikogianni, 2007). These theories provide various surface energetic concepts in relation to bacteria-material interactions and have been somewhat successful in predicting the adhesion behaviour of bacteria to surfaces. An extensive body of knowledge exists in the scientific literature with respect to these theories (Araújo et al., 2010; Busscher et al., 2010; Katsikogianni & Missirlis, 2004).

It is known that a number of factors affect the formation of conditioning film which in turn affects bacterial adhesion and biofilm formation on surfaces. In relation to the physicochemical properties of the bulk solution, the concentration of protein present in the bulk solution is one of the major factors that influence protein adsorption to surfaces. Most of the protein adsorption experiments on surfaces were done by exposing the surface to low bulk solution protein concentrations (usually up to 1 mg/ml) which is related to biomedical settings such as biosensors, biochips, and biomaterials for medical implants. In food processing situations, due to their continued accumulation, the conditioning film may provide a higher concentration of nutrients at the surface than in the liquid phase. However, protein adsorption studies related to food industry environments have also been usually performed using relatively low bulk protein solution concentrations. In addition these studies have been performed mostly under static conditions. Thus, complete characterisation of the composition and properties of the conditioning film formed under high bulk solution protein concentration under both static and flow conditions is desirable for wider understanding of bacterial adhesion to surfaces in food industry applications.

1.1 Objectives

1.1.1 Overall objective

The overall objective of this study is to evaluate and characterize the formation of conditioning film (under solution protein concentration imitating bovine milk) on model surfaces and study its effect on bacterial adhesion in food industry settings. To do so, the following specific objectives were defined:

1.1.2 Specific objectives

a. Quantify the surface bound proteins onto mica and polystyrene surfaces after conditioning with proteins, microbial culture media and milk
    i. under static
    ii. flow conditions

b) Evaluate and characterize the surface energy parameters and apply thermodynamic concepts to predict the protein absorption behaviour

c) Investigate the adhesion of *Listeria innocua* and *Listeria monocytogenes* on mica and polystyrene surfaces preconditioned under static and flow settings

d) Utilize atomic force microscopy to image and characterize conditioning film and bacterial adhesion on mica and polystyrene surfaces

PREFACE TO CHAPTER 2

Bacterial adhesion to surfaces is the first step in the formation of a biofilm and has been studied extensively over the past decades. Biofilm formation has been a subject of study in various areas such as oral microbiology, biomaterial associated infection and food-processing equipment. Adhesion of bacteria and subsequent formation of biofilms on food processing surfaces is a major problem for food industries. The deleterious effects of biofilm growth on the food contact surfaces are numerous. They include energy losses due to increased fluid frictional resistance or increased heat transfer resistance corrosion induced by microorganisms, loss of optical properties, quality control and safety problems and huge economic losses. Several reviews and textbooks discuss biofilm formation, but very few of them have addressed specific concerns related to food processing conditions, particularly under flow conditions. Therefore, the experimental knowledge about bacterial adhesion and biofilm formation on abiotic surfaces in the food industry with special reference to flow conditions was reviewed.

Part of this review was presented at the Canadian Institute of Food Science and Technologists (CIFST) conference held from May 31 - June 1, 2010, in Winnipeg, Manitoba. One manuscript has been prepared from this Chapter.

Nikhil Hiremath, Louise Deschênes, Mohammad Reza Zareifard, Akier Assanta Mafu, Gilles Robitaille and Hosahalli Ramaswamy (2014). Bacterial adhesion and biofilm formation: A review with special reference to flow conditions and food processing (prepared for submission).

The review was carried out by the candidate under the supervision of Dr. Louise Deschênes and Dr. H. S. Ramaswamy.

Chapter 2. LITERATURE REVIEW

2.1 Abstract

Bacterial adhesion and biofilm formation is a serious hygienic problem in food industry causing processing contaminations. It is well established that the presence of bacteria on food contact surfaces affects the quality and safety of the food products. Several reports pertaining to adhesion of bacteria and subsequent biofilm formation on food contact surfaces under static conditions exist in the literature. Flow conditions are considered one of the dominant factors that strongly influence the number of attached microbes as well as the biofilm structure, performance and formation. Although, the influence of flow on adhesion and microbial biofilm formation on contact surfaces has been studied in medical and industrial settings, limited studies are available with respect to food industry conditions. This article reviews the experimental knowledge about bacterial adhesion and biofilm formation on abiotic surfaces in the food industry with special reference to flow conditions and conditioning films.

2.2 Introduction

Bacterial adhesion and biofilm formation on abiotic surfaces has been a hot topic of research over the past several decades and this phenomenon has been investigated across various scientific and engineering disciplines mainly because of its industrial, environmental and medical relevance. In fact it has been recommended, that researchers, in the coming decades should recognize the study of biofilms, as "biofilmology", as it has evolved into a discipline in its own right with significant research potential in the coming years (Karunakaran et al., 2011).

In relation to the food industry, the adhesion of bacteria and biofilm formation continue to pose concerns to food manufactures as they can lead to serious problems related to hygiene, food quality and food safety (Srey et al., 2013). Besides it can also create enormous increases in fluid frictional resistances, unacceptable reductions in heat transfer efficiency, enhance material deterioration and accelerate corrosion resulting in huge economic repercussions for the industry (Brooks & Flint, 2008; Kumar & Anand, 1998). Several attempts have been made to understand the adhesion and biofilm forming capabilities of several microorganisms (especially food borne pathogens) and spores including *Listeria monocytogenes* (Bonsaglia et al., 2014; Chavant et al.,

2002; Dubravka et al., 2009; Møretrø & Langsrud, 2004), *Escherichia coli O157:H7* (Mafu et al., 2011; Ryu et al., 2004), *Salmonella spp*.(Oliveira et al., 2007; Steenackers et al., 2012), *Yersinia enterocolitica* (Coquet et al., 2002; Herald & Zottola, 1988), *Campylobacter jejuni* (Moe et al., 2010; Sanders et al., 2008) , *Bacillus cereus* (Flint et al., 2001; Husmark & Ronner, 1990) and *Staphylococcus aureus* (Herrera et al., 2007; Marques et al., 2007) on food contact surfaces such as stainless steel, polypropylene, polycarbonate, high-density polyethylene, polyvinyl chloride, Teflon, ethylene propylene diene monomer rubber, nitrile butyl rubber, and silicone rubber. However, understanding bacterial adhesion and biofilm formation to surfaces is difficult, as the process of bacterial adhesion and biofilm formation is known to be complex and mediated by a number of factors, including both bacterial and material physicochemical surface properties, nutrient conditions, pH, temperature and hydrodynamic conditions (Boulangé-Petermann, 1996; Donlan, 2002; Katsikogianni & Missirlis, 2004; Palmer et al., 2007; Parkar et al., 2001; Verran & Whitehead, 2005).

In general bacterial adhesion and biofilm formation investigations have been performed using several experimental approaches and methods wherein model surfaces are exposed to microbial suspensions either under static or continuous flow conditions for a desired period of time, followed by enumeration, visualization and/or characterization of bacteria adhered to the surface. Static conditions are obtained by exposing the test surface to a stationary or slightly agitated bacterial suspension whereas bacterial adhesion and biofilm formation under flow is studied using different experimental growth model systems or flow systems. After exposure of the test surface to either static or flowing microbial suspensions, the test surfaces are rinsed to remove bacterial cells that are “unattached” or “loosely bound” either by successive dipping in the relevant fluid, mild shaking of the container with progressive replacement of the liquid, or washing under flow. Finally the bacteria on the surface are quantified (either in situ or after dislodging the cells from the surface) or detected using several imaging or molecular techniques. Several experimental growth models on flow systems, methods and techniques used to study bacterial adhesion and biofilm formation have been reviewed (An & Friedman, 1998; Kishen & Haapasalo, 2010; McBain, 2009; McLandsborough et al., 2006). In this review some technical details of few commonly used experimental flow models (including novel models) have been presented.

In the food industry, it is logical that many processes are flow dependent and flow is often considered an essential operation. Flow or hydrodynamic conditions, such as flow velocity and shear stress, will determine the rate of mass transport, i.e. the rate of transport of cells, oxygen and nutrients to the surface, and thus influence bacterial attachment and biofilm formation on abiotic surfaces. Furthermore, changes in fluid velocity frequently occur in food processing environments. In the food industry, therefore, it is important to consider how the behavior of the liquids flowing within the system influences the process of bacterial adhesion and biofilm formation on abiotic surfaces. Although several investigations have been performed under static conditions, attempts to characterize bacterial adhesion and biofilm formation under flow in food industry settings are much fewer (Brugnoni et al., 2007; Fratamico et al., 2009). Moreover, it is difficult to draw general conclusions, as ambiguous opinions exist concerning different variables that influence bacterial adhesion and biofilm formation on food contact surfaces (Brooks & Flint, 2008; Palmer et al., 2007; Verran et al., 2008). The objective of this article is to review the experimental knowledge about bacterial adhesion and biofilm formation in food and dairy industry with special reference to flow conditions. The information gained over the past several years in static conditions with emphasis on data concerning food pathogens is also highlighted.

In this chapter, in order to avoid the confusion, the terms attachment and adhesion are considered as synonymous. The term adsorption has been primarily used for surface conditioning by organic molecules. In addition, with respect to biofilm experimental methodologies, the mechanisms of agitation, shaking and stirring have been grouped under static conditions, although they create turbulent milieus.

2.3 Bacterial adhesion and biofilm formation

2.3.1 Definition of biofilm

Donlan and Costerton (Donlan & Costerton, 2002) gave a summary of the different definitions of biofilms that has been evolved over the past 25 years and presented a new definition by taking into account all readily observable and physiological characteristics (including non-cellular or abiotic components, altered growth rate etc.). According to them, a biofilm is defined as "a microbial derived sessile community characterized by cells that attach to

a surface or interface or to each other, with the help of gelatinous extracellular polymeric substances mainly composed of polysaccharides, proteins, and nucleic acids and exhibit an altered phenotype with respect to growth rate and gene transcription when compared to their free floating (so called planktonic form) counterparts. This definition gave a broader perspective of biofilms as previous definition did not take into account the biofilm phenotype and genotype characteristics (Donlan & Costerton, 2002).

2.3.2 Characteristics of a biofilm

Biofilms are complex, heterogeneous, dynamic community structures and not simply microbial cells that stick to surfaces. It is well acknowledged that as compared to the planktonic mode, a biofilm way of life is associated with a slower growth rate (Donlan, 2002), altered gene expressions (Sauer et al., 2007) and greater resistance to antimicrobials (Davies, 2003). In addition biofilm cells are known to communicate with each other using biochemical signaling mechanisms (Annous et al., 2009).

2.3.3 Basic steps of biofilm formation

Many physical, chemical and biological processes are involved throughout biofilm development depending on the environment and hydrodynamic conditions. From the initial adhesion of cells, their growth and dynamic maturity to detachment the process is said to be poetry in motion (Jenkinson & Lappin-Scott, 2001). A summary of process of microbial biofilm formation, as it is currently understood, is presented below (Bryers, 2008) and are illustrated in Figure 2.1.

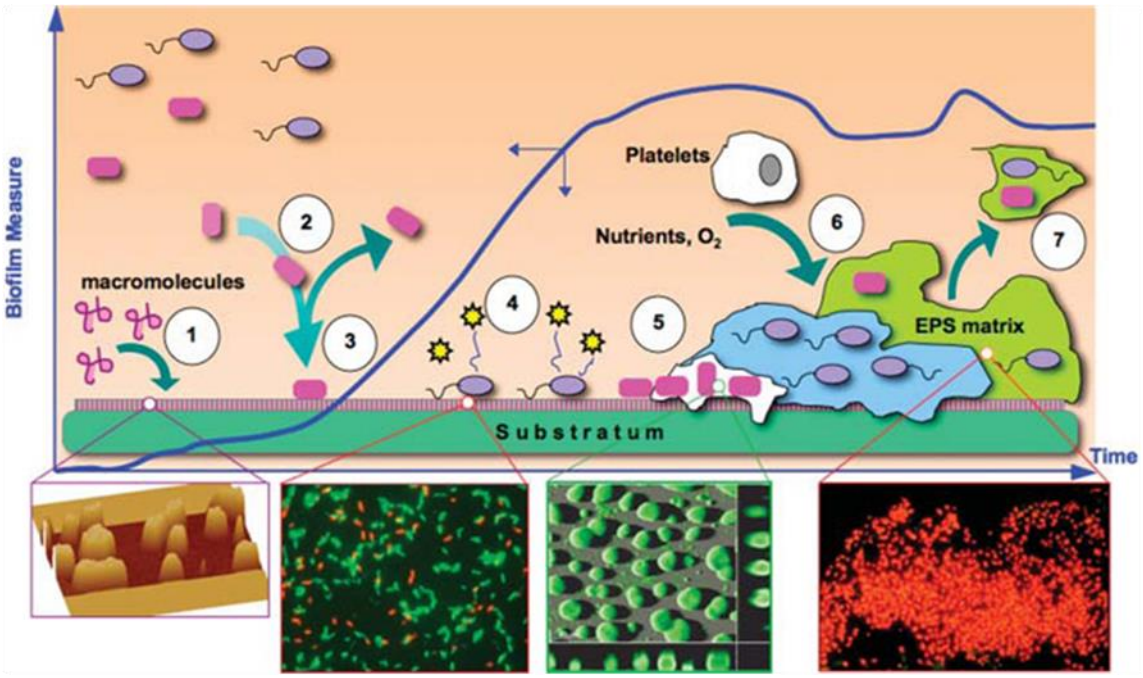

Figure 2.1: Basic steps in formation of a biofilm

Adapted from (Bryers, 2008)

The following steps are involved in the process:

(i) Conditioning of the surface: Surfaces are conditioned due to the adsorption of macromolecules thus changing the adhesion conditions for bacteria to this surface. The nature of the conditioning films may be quite different depending of the kind of environment the surface is exposed.

(ii) Transport of planktonic cells from the bulk liquid to the surface by a combination of many mechanisms, depending upon the hydrodynamic condition of the bulk liquid (such as Brownian motion, gravitation, diffusion, convection, or the inherent motility).

(iii) Cells attach reversibly on the surface bacterial cells and weakly held to a surface by physical attractive forces such as Van der Waals forces of mass attraction and electrostatic forces caused by ionic groups interacting on or around the approaching bacterial cells.

(iv) Bacterial cells become firmly attached to the surface as a result of the synthesis of extracellular polymeric substances (EPS) resulting in irreversible attachment of bacterial cells at a surface.

(v) Abundant amount of EPS are secreted forming a micro-colonies and three dimensional structures.

(vi) Biofilm continues to accumulate and begin to mature consuming nutrients, attracting other bacterial species or mammalian cells.

(vii) As a result of increases in shear stress or the onset of other cell-cell signaling events, portions or entire sections of biofilm can detach or slough off, to move downstream.

2.4 Conditioning of the surface for bacterial adhesion and biofilm formation

In a typical food industry, contact surfaces are either continuously or periodically in contact with organic biomolecules (proteins, lipids, carbohydrates etc.). Pristine surfaces are rapidly conditioned by organic material transported either by convection or diffusion forming "conditioning films" - the fundamental step for the formation of a microbial biofilm on a surface (Dunne, 2002; Goode et al., 2013a; Kumar & Anand, 1998). It is currently well recognized that the presence of conditioning film on food contact surface will deeply alter the physicochemical properties of the surface such as free energy, hydrophobicity and electrostatic charges and hence affect the hygienic status (presence of bacteria) of food contact surfaces (Jullien et al., 2003; Lorite et al., 2011; Verran & Whitehead, 2006).

2.4.1 Surface conditioning due to adsorption of proteins

Among the organic biomolecules, conditioning film formed due to protein adsorption to contact surfaces has received much attention for several decades in various fields such as biotechnology, environmental science, dentistry, biomedical, and industrial engineering. Proteins are well known for their interfacial properties and are adsorbed instantly to solid surfaces. The process of protein adsorption is multidimensional and depends on several factors such as size, charge, structural stability, unfolding rate of the protein molecule, energy, electrical

characteristics, topography, composition of the interacting surface and physicochemical characteristics of the surrounding fluid such as flow, pH, temperature and ionic strength under which the investigations are performed (Gray, 2004; Hlady & Buijs, 1996; Wahlgren & Arnebrant, 1991). Although considerable amount of information about the protein adsorption process, including the amount of adsorbed protein, the rate of adsorption, the conformation and/or orientation of the adsorbed protein layer and competitive adsorption from complex biological media have been acquired, understanding the complex phenomenon of protein adsorption to solid surfaces still remains a challenge (Nakanishi et al., 2001; Rabe et al., 2011). Generally, the primary step to understand the protein adsorption behavior is to quantify the amount of protein bound to the surface. The amount of protein adsorbed is obtained by measuring the adsorption isotherm which is a function that relates the measured adsorbed amount of a protein per unit area, to the solution concentration of protein. Typically, the amount of protein adsorbed increases abruptly at low solution concentrations and levels off at higher protein concentrations approaching a "plateau" or saturation value (Hlady et al., 1999). The amount of adsorbed protein at the "plateau" monolayer may vary from 1 to 5 mg/m^2 for single proteins like BSA and β casein depending on the diameter and orientation assumed for the protein (Fitzpatrick et al., 1992; Horbett et al., 1996; Lee et al., 2004; Terashima & Tsuji, 2003). In case of multilayer adsorption the amount of protein adsorbed on polymer surface was reported to be higher than a typical monolayer cover (Holmberg & Hou, 2009). A multilayer adsorption of lysozyme onto mica surfaces was also observed by direct imaging by atomic force microscopy (Kim et al., 2002). Competitive protein exchange on surfaces (“Vroman effect”) has also been extensively investigated from past 40 years but still not fully understood (Hirsh et al., 2013).

2.4.1.1 Protein adsorption on surfaces: food industry

In food industry, protein adsorption on equipment surfaces results in fouling leading to functional decline of the equipment, contamination, decreased product quality and effects the cleaning efficiency of detergents (Detry et al., 2010). Protein adsorption on food contact surfaces has been studied in great detail, especially in the dairy industry, given that milk protein, β-lactoglobulin (β-lac), causes significant fouling of contact surfaces (Bansal & Chen, 2006; Fryer et al., 2006; Lalande et al., 1985; Visser & Jeurnink, 1997). In addition to β-lac several other types of proteins on food contact surfaces have been investigated. Broadly speaking, protein

adsorption investigations have been performed under either under static or dynamic flow conditions.

2.4.1.1.1 Static Conditions

Table 2.1 shows typical experimental data on the adsorbed amounts of different proteins on variety of contact surfaces under static conditions. The amount of proteins adsorbed is difficult to compare as it varies with the kind of protein, type of surface, and adsorption conditions. In general, with respect to the solution properties the amount of protein adsorbed is higher at isoelectric point (the pH at which the molecule exhibits zero net charge), at elevated temperatures than at room temperature, and with increase in ionic strength of the medium. With respect to protein properties, proteins with low internal stability (known as "soft proteins" example bovine serum albumin (BSA), human serum albumin (HSA), immunoglobulin (IgG), α-lactalbumin, β-casein, haemoglobin, catalase and phytase) showed higher amounts when compared to "hard proteins" - example α-chymotrypsin, ribonuclease, lysozyme, and β-lactoglobulin) as "hard' proteins have strong internal coherence and structural rearrangements that make small contributions to the adsorption process when compared to 'soft' proteins, which have lower structural stability, and can be adsorbed even under more rigorous conditions (Kingshott & Höcker, 2006; Rabe et al., 2011).

Table 2.1: Reported values of proteins adsorbed onto variety of contact surfaces under static conditions

Protein	Contact surface	Buffer	pH	Concentration (g/100ml)	Temp (°C)	Incubation time (hours)	Adsorption conditions	Measurement method	Amt adsorbed (mg/m^2)	Reference
β-lac	SS particles	CB	6.8	0.0006	27	1.5	agitation	Depletion	1.5	(Kim & Lund, 1997)
β-lac	SS particles 316L	PB	6.9	1	25 75	3	agitation	Depletion	2.8 9	(Itoh et al., 1995)
β-lac α-lac	SS 304	PBS	6	0.2	25	1	immersion	Radio-labeling	0.5 1.5	(Addesso & Lund, 1997)
β-lac α-lac	Teflon								3 0.5	
α-lac β-lac BSA β-cas	Hydrophilic Silica	PB	7	0.1	Room	8	static	Ellip-sometry	1.4 1.5 1.4 2.6	(Krisdhasima et al., 1993)
α-lac β-lac BSA β-cas	Hydrophobic Silica								1.3 1.7 1.4 2.7	
β-lac	SS 304 & 316	NaCl (0.05M)	6	0.01	24 85	0.5	stirring	Ellip-sometry	3.2 5.8	(Karlsson et al., 1998)
BSA	SS particles 316L	KNO_3 (0.1M)	3 7 11	0.07	30	2	vigorous shaking	Depletion	1.5 0.3 Negligible	(Sakiyama et al., 2004)
BSA	SS 304	CB	6.8	0.1	4	0.6	immersion	Radio-libeling	2.1	(Vanencke vort et al., 1984)

Protein	Contact surface	Buffer	pH	Concentration (g/100ml)	Temp (°C)	Incubation time (hours)	Adsorption conditions	Measurement method	Amt adsorbed (mg/m^2)	Reference
BSA	316L SS	HBSS	-	1	37	12	End over end	Depletion	3	(Gispert et al., 2008)
BSA	SAM [&]	PBS	7.4	0.2	25	1	static	Nano-orange	2.4	(Roach et al., 2006)
	SAM[#]								2.18	
BFN	SAM [&]								3.77	
	SAM[#]								2.8	
BSA	Silica	KNO_3(0.001M)	5	0.2	40	2	reciprocal shaking	Depletion	2.2	(Fukuzaki et al., 1996)
	Titania								3.4	
	Zirconia								4.2	
	Alumina								4.7	
BSA	SS particles 316L	KNO_3 (0.001M)	3.5	0.2	40	2	reciprocal shaking	Depletion	1.15	(Fukuzaki et al., 1995)
			5						2.38	
			7.5						0.84	
			5		80				15	
Gelatin			3.5	0.5	40	14			1.97	
			5						3.87	
			7.5						2.08	
			3.5		80				2	
BSA		KNO_3 (0.1M)	4	0.2	40	2			15.5	
		KNO_3 (0.01M)							10.8	
		KNO_3(0.001M)							2.4	
Gelatin		KNO_3 (0.1M)	5	0.5	40	14			1.5	
		KNO_3 (0.01M)							2	
		KNO_3(0.001M)							3	
HPA	SAM[#]	[+]Aqueous solution	7.4	0.0039	22	2	static	Radiolabeling	1.9	(Van Dulm & Norde, 1983)
	SAM[&]	0.05M							0.5	
	Polystyrene	0.001M	7.4	0.0038					0.5	

Protein	Contact surface	Buffer	pH	Concentration (g/100ml)	Temp (°C)	Incubation time (hours)	Adsorption conditions	Measurement method	Amt adsorbed (mg/m^2)	Reference
	Latices	0.001M	4						0.8	
		0.1M	7.4						1	
		0.1M	4						2	
		0.01M	7.4						1	
		0.01M	4						2	
α-lac (Ca containing)	Hematite	PB	7	0.1	22	20	static	Depletion	0.9	(Norde & Anusiem, 1992)
	Silica								0.15	
α-lac (Ca delpleted)	Hematite								0.8	
	Silica								0.5	
BSA	Hematite								1	
	Silica								1.5	
LYS	Hematite								1.2	
	Silica								1.8	
LYS	Silicon dioxide	PB	7	1	25	1	static	Ellipsometry	5	(Wahlgren et al., 1995)
Protein from pink shrimp	SS	-	-	0.2	25	1-2	vigorous shaking (120 rpm)	Depletion	2.5	(Thammathongchat et al., 2010)

Protein: BSA(Bovine Serum Albumin), β-lac (beta lactoglobulin), α-lac (alpha lactalbumin), BFN (bovine fibrin), HPA (Human Plasma Albuni), LYS (Lysosyme); **Surface:** SS (stainless steel), SAM (self-assembled monolayers), [&] Hydrophilic, [#] Hydrophobic; **Buffer**: CB (Cacodylate buffer), PBS (phosphate buffer saline), HBSS (Hank's balanced salt solution), PB (phosphate buffer), KNO3 (potassium nitrate), AA (acetic acid buffer), TB (Tris buffer), NaCl (sodium chloride), [+]Aqueous solution (pH adjusted using AA and TB).

2.4.1.1.2 Flow conditions

In many food processing applications one is interested in protein adsorption taking place from a flowing rather than from a quiescent solution. Santos et al. (2006a) investigated the effect of turbulent flow conditions on the adsorption of whey protein to stainless steel surfaces at two temperatures (72 and 85°C). At both temperatures the amount of protein adsorbed increased with increase in Reynolds number and the adsorbed amount was almost an order of magnitude larger at 85°C when compared to 72°C. At both temperatures, longer protein solution residence times decreased the amount of protein adsorbed. The effect of the time of residence of proteins in the system (and so at elevated temperature) had a strong impact on the amount of adsorbed proteins. It was demonstrated that proteins aggregated on the surfaces, creating multilayers. In another study from the same group, the amount of protein adsorbed increased with the increase in protein concentration at a Re = 11300. In addition, the effect of flow (Reynolds numbers of 3,800, 11, 300 and 17,100) was investigated at different concentrations (Santos et al., 2006b) at 85°C. For the protein concentration of 3 mg/ml the amount of protein adsorbed was observed to increase with increase in Reynolds number, whereas for a protein concentration of 3 mg/ml the adsorbed amount was not influenced in turbulent regimes. However, at this concentration when the flow changed from the transition to turbulent regime, an increase in adsorbed amount was observed. In another study the effect of Reynolds number on fouling of a 1.5 m long stainless steel tube by whey proteins (10 mg/ml) at 83°C was investigated (Belmar-Beiny et al., 1993). They observed that total amount of deposition decreased with the increase in Reynolds number.

Under laminar flow conditions, Shirahama et al. (1990) studied the adsorption of lysozyme, ribonuclease, and α-lactalbumin on hydrophilic silica and on hydrophobic polystyrene-coated silica. They concluded that the adsorbed amounts never exceeded values corresponding to monolayer coverage (i.e. 1–2 mg/m^2) under flowing conditions. In addition it has been reported that type of flow (i.e. impinging jet or laminar flow) does not have a strong influence on the size of the protein adsorbing fraction and (hence the amount adsorbed), but instead it was determined by the physico-chemical properties of the proteins and surfaces that was used in the study (Norde et al., 1991).

From both the static (Table 2.1) and dynamic flow protein adsorption quantification studies, it can be seen that investigations have been performed from bulk solutions over a wide range of protein concentrations (ranging between 0.006 – 10 mg/ml). The results indicate that even for low protein concentrations of bulk solutions and relatively short contact time the surface coverage of the order of monolayer saturation are usually obtained. Experimental isotherms of proteins at the air-water interface indicate that the surface concentration corresponding to saturation for BSA (Sanchez-Gonzalez et al., 2003), β-casein, (Maldonado-Valderrama et al., 2004) β-lactoglobulin and α-lactalbumin (Cornec et al., 1999) at room temperature vary between 1.3 and 2 mg/m^2. Foods such as milk contain high concentrations of nutrients. The protein content commonly present in bovine milk is around 33 mg/ml. Hence, the degree to which the protein is adsorbed onto the surface from relatively high concentration protein solutions could provide additional information regarding the protein adsorption and interaction phenomena to surfaces in food and dairy industries. Also there are very few studies that have investigated the effect of flow on protein adsorption at room temperature conditions, especially in food industry settings.

2.4.2 Conditioning from adsorption of organic food soils

In addition to protein adsorption, several studies have investigated the adsorption of organic food soils. For example, Whitehead et al. (2009) examined the effect of concentration (10%, 1%, 0.1%, 0.01% and 0.001% w/v solutions) of food soils on stainless steel surface physiochemical properties (surface hydrophobicity, surface free energy, Lifshitz van der Waals, Lewis acid base electron acceptor and electron donor measurements). Food soils such as raw meat (beef extract), cooked fish (cod extract), cottage cheese (cottage cheese extract), proteins (BSA, fish peptones, casein), oils (cholesterol, fish oil), mixture of three fatty acids, (myristic, palmitic, stearic), carbohydrates (glycogen, starch and lactose) were spread across the surface and dried under flow hood. Although changes (increases or decreases) in physicochemical values were detected for all types of fouled surfaces when compared to the control surface, no general trend was observed. Scanning electron microscopy (SEM) analysis of the surfaces with dried food soils showed heterogeneous coverage of organic material. In another study the surface properties of glass and poly (methyl methacrylate) (PMMA) soiled by spraying a mixture of gelatinized starch, whole fat milk powder, and vegetable oil were investigated. The soils were

suspended in distilled water and the resultant mixture was diluted down, in 1:2, 1:10, and 1:50 concentrations, to provide soiling layers of differing thickness. The soil was even coated and dried at 50-60°C for 30 min. Results of the XPS (X-ray photoelectron spectroscopy) spectra showed lower concentration of proteinaceous material on glass than PMMA and the concentration increased with thickness of the soiling material for both the substrates. Analysis of soiled surfaces using TOF-SIMS (time-of-flight secondary ion mass spectrometry) showed that the fatty acid esters of glycerol, from the vegetable oil and milk powder, dominated the surface chemistry of PMMA. Soiling produces a surface of different topography and chemical nature from the original smooth substrate surface, with a complicated surface structure (Boyd et al., 2000). The amount of oil in water emulsions simulating salad dressings on surfaces of different surface tensions namely PTFE (polytetrafluoroethylene), low-density polyethylene (LDPE), polyethylene terephtalate (PET), stainless steel (SS), and glass was tested (Michalski et al., 1999). The study revealed that the amount of emulsion adhered on solid surface was found to increase with solid surface tension. In certain studies the work of adhesion of various milk products on glass was calculated using Young and Dupré Equation. The results revealed that chocolate milk had the strongest whereas 2% reduced fat milk had the weakest adhesion force (Handojo et al., 2009).

Hence, the understanding of adsorption phenomena of food materials and components that occur at interfaces still remains an actual subject of interest for the food and dairy industry and should be taken into account when characterizing the hygienic status of various materials employed in food processing environments.

2.5 Bacterial adhesion and biofilm formation: static condition

Various research groups have performed bacterial adhesion and biofilm formation studies under static conditions. The effect of variables such as conditioning film, bacterial properties (strain variability, cell surface hydrophobicity, surface structures), abiotic surface properties (surface roughness, surface finish, surface topography), external parameters (pH, temperature, ionic strength, initial bacterial concentration, suspending medium) on microbial adhesion and biofilm formation has been extensively investigated. Typical microbial strains, contact surfaces, techniques used, experimental conditions along with some important findings are illustrated in

Table 2.2. This collection of data has been selected with special focus on pathogen adhesion and biofilm formation on abiotic surfaces in the food and dairy industry.

Table 2.2: Studies on microbial adhesion and biofilm formation under static conditions

Micro-organism	Surface	Medium	Factors studied	Adhesion and biofilm formation conditions	Technique	Important findings reported	References
E. coli (NBRC 3301)	SS	PS, PBS, PHS	ICC. SM, SR	➢ 25° C for up to 6 h.	PC	• Adhesion was seen after 0.5h attaining plateau after 3 - 4 h. • Cells in PS adhered in higher numbers (ICC of 10^3 - 10^4 CFU/mL). • No significant difference in cell adhesion to SR.	(Ortega et al., 2010)
E. coli O157:H7	SS	PBS	CD, CP	➢ Adhesion: 4°C for 24 h ➢ Biofilm – 22 °C for up to 6 days (dTSB 1:10).	PC	• CD and CP strains showed no difference in adhesion • Only CP strains formed biofilms.	(Ryu et al., 2004)
E. coli (CUETM 98/10), *B. cereus* and *B. subtilis* spores	SS TF PEHD PVC GL	CD-NaCl (*E.coli*) & Saline (spores)	CSH, SFE	➢ Adhesion – RT for 2 h ➢ Biofilm (only for *E.coli*) –RT for 22 h	PC	• Higher number of *B.cereus* adhered to all materials when compared to *B. subtilis*. • Higher number of *E. coli* cells found on the PEHD and TF • Higher number of *E.coli* adhered after 24 h when compared to 2 h.	(Faille et al., 2002)
Entero-bactersakazakii (5 strains)	SS	TSB, IFB, LJB (for adhesion) PBS (for biofilm)	Temp, NA	➢ Adhesion –12 and 25°C. for 4 h ➢ Biofilm – 4°C for 24 h, followed by transfer into TSB, IFB, or LJB, 12 or 25°C for up to 10 days.	PC	• Higher populations of bacteria adhered at 25°C than at 12°C after 4hours. • Biofilms were not produced at 12°C but were formed on SS immersed in IFB at 25°C	(Kim et al., 2006)

Micro-organism	Surface	Medium	Factors studied	Adhesion and biofilm formation conditions	Technique	Important findings reported	References
L. monocytgenes (44 strains)	GL, PS, SS	Fresh TSB	Temp , CM, CSH	➢ 4, 12, 22 and 37 °C for 24h	CVS	• Biofilm levels were higher on GL at 4, 12 and 22 °C, as compared with PS and SS. • At 37° C, biofilm production was higher on GL and SS, as compared with PS. • CSH was significantly higher at 37°C than at other temperatures. • No correlation between cell motility and biofilm production.	(Di Bonaventura et al., 2008)
L. monocytgenes (4 strain cocktail)	SS	TSB	SR , SF	➢ Adhesion - 5 min. ➢ Biofilm - 32°C for 48 h. (1:20 d TSB)	PC	• The level of *Listeria* present in biofilms after 48 h was significantly higher than the attached cells after 5 min • *Listerial* adhesion and biofilm formation were independent of SR and SF.	(Rodriguez et al., 2008)
L. monocytogeenes Scott A	SS	Milk, Vanilla, Custard Yogurt	Temp, Food matrices	➢ 5 and 20°C for 7 days.	PC	• Bacterial attachment was more favorable at 20°C than at 5°C. • At 5°C, the lowest level of attached cells was observed in yogurt, whereas milk and custard resulted in similar levels of attachment.	(Poimenidou et al., 2009)
L. monocytogenes (ATCC 19111)	SS	BHI, MM	GM, Temp	➢ 4 , 23 , 30 , 35 , or 42 °C for 3 h (saturated humidity)	SEM	• The number of attached cells increased with increasing temperature. • The attachment of cells to SS was higher when cultivated in BHI than in MM	(Mai & Conner, 2007)
Salmonella spp.(122 strains) & L. *monocytogenes* (48 strains)	PS	BHI, TSB, MB, dTSB (1:20)	SM	➢ 35°Cfor 24 h	MMP	• *Salmonella spp*. produces more biofilm in nutrient-poor medium, while *L. monocytogenes* produce more biofilm in nutrient-rich medium. • Diluted TSB was the most effective in promoting biofilm production by *Salmonella* spp., followed by TSB, BHI and MB. • *L. monocytogenes* produced the highest quantities of biofilm in BHI, followed by TSB, MB, 1/20-TSB	(Stepanovic et al., 2004)

Micro-organism	Surface	Medium	Factors studied	Adhesion and biofilm formation conditions	Technique	Important findings reported	References
L. monocytogenes -(4 strains)	PP, SS	MCJ	SM, CF	25°C for 60 to 80 h with ➢ MCJ carrying *Listeria* cells or with ➢ *L. monocytogenes* after formation of MCJ conditioning film (CF)	PC	• Adhesion to PP was higher than SS except one strain • Adhesion was initially higher in the presence of CF, but numbers of adherent cells decreased sharply after ~ 24h in three of eight cases	(Saa et al., 2009)
S. Enteritidis (EMB, MUSC, AL, PC)	SS	PBS	SFE, CSHSEC	➢ 25ºC for 1 h with constant shaking at 100 rpm	EM	• *Salmonella* strains showed similar elemental composition and cell surface physico-chemical properties. • *S. Enteritidis* MUSC presented higher adhesion ability to SS. • Physico-chemical properties of the strain did not explain the ability of adhesion to stainless steel.	(Oliveira et al., 2007)
Salmonella serovars (Twenty five strains)	TF, SS, NBR, PU, GL	PBS	SFE, SR, CSH	➢ RT for 20min swirling 10s at 5 min interval	EM	• *S. Sofia* isolates adhered in higher numbers to all materials compared to other serovars. • *S. Soifa* isolates attaching to TF was significantly higher compared to all materials except SS. • Significant correlation between CSH and attachment numbers of bacteria to materials.	(Chia et al., 2009)
*S. aureus (*5 strains)	PP	PBS, TSBG, MCJ dried (CF)	NA, CF	➢ 25°C for upto 26 h	PC	• Adhesion was higher in the presence of TSBG than in PBS, and decreased sharply in both cases after 10–15 h. • Adhesion increased exponentially during 25 h in the presence of dried MCJ.	(Herrera et al., 2007)
S. aureus (ATCC 25923)	SS, GL	BHI	Temp	➢ 37°C for 15 days	MDT	• Number of viable cells adhered onto the GL surfaces was one log higher than on SS • Incubation temperature was one of the factors influencing the intense adhesion on surfaces	(Marques et al., 2007)

Micro-organism	Surface	Medium	Factors studied	Adhesion and biofilm formation conditions	Technique	Important findings reported	References
A. hydrophila, E. coli O157:H7,S.Enteritidis, and *S. aureus*	PS, GL	Buffers (pH 6,7&8)	pH, SFE	➢ 24 h at ambient temperature (20 ± 2◦C) under low agitation (90 rpm).	PC	• pH influenced the adhesion of *A. hydrophila*, *E. coli*, and *S. aureus* to surfaces whereas *S. Enteritidis* was not affected by the type of surface or pH. • The rate of bacterial adhesion could not be correlated to the physicochemical properties of the materials (except for *S. aureus*).	(Mafu et al., 2011)
Thermophillic *bacilli* spores & vegetative cells	SS	SDW, SMP	PDA, PRA, CF	➢ RT for 30min. ➢ CF -1% and 10% SMP – 2 h at RT with swirling at 40 rev per min on a rotary shake	EM	• Spores attached more readily to SS than vegetative cells. • Attachment was less affected by various treatments of the spore surface than the vegetative cells. • Coating the stainless steel with skim milk proteins decreased the attachment of vegetative cells and spores.	(Parkar et al., 2001)

Food contact surfaces: SS (stainless steel),SSC (stainless steel coupons), PS (polystyrene), GL (glass), PP (polypropylene), TF (teflon), NBR (nitrybutly rubber), PU(polyurethane), PEHD (polyethylene high density),PVC (polyamide-6, polyvinyl chloride). **Suspending medium**: PS (peptone saline), PHS (physiological saline), PBS (phosphate buffered saline) CD-Nacl (sterile pancreatic digest of casein supplemented with NaCl, pH adjusted to 7.2), TSB (trypticase soya broth), dTSB (diluted TSB), BHI (brain heart infusion), MM (minimal media - 10% filter sterilized pond water and 90% sterilized distilled water), MB (meat broth), MCJ (Mussel cooking juice), TSBG (TSB plus 1% glucose), SMP (skim milk powder), SDW (sterile distilled water), infant formula broth (IFB), LJB (lettuce juice broth). **Factors**: Temp (temperature) CF (conditioning film), ICC (initial cell concentration). SM (suspending medium), SR (surface roughness) SF (surface finish), CSH (cell suface hydrophobicity), CM (cell motility) SFE (surface free energy), GM (growth medium), NA (nutrient availability), PDA (protein denaturing agents: sodium dodecyl sulphate (SDS) and trypsin), SEC (surface elemental composition), PRA (polysaccharide removing agents: sodium metaperiodate, trichloroacetic acid (TCA), and lysozyme). **Measuring method**: PC (plate count), SEM (scanning electron microscopy), EF (Epifluoroscence microscopy), MMP (modified microtitre plate), MDT (micro drop technique), CVS (crystal violet staining).

2.5.1 Effect of conditioning film on bacterial adhesion and biofilm formation under static conditions

The effects of different conditioning films on bacterial adhesion and biofilm formation on food contact surfaces under static conditions have been reported by several authors. Fletcher (1976) reported that the presence of proteins such as albumin, gelatin and fibrinogen inhibited attachment of a marine *Pseudomonas* to polystyrene. Individual milk proteins, α-casein, β-casein, k-casein, and α-lactalbumin (protein concentration 0.05mM) and skim milk reduced the adhesion of *S. aureus*, and *L. monocytogenes* to stainless steel coupons. The coupons were incubated for 2 h at 20°C under static conditions to allow bacterial adhesion (Barnes et al., 1999). Similar results were reported by Helke et al. (1993) who found that individual milk components such as casein and β-lactoglobulin decreased the attachment of *L. monocytogenes* and *S. typhimurium* to stainless steel. Conditioning of stainless steel coupons with 10% and 1% skim milk powder suspensions for 2 hours at ambient temperature with swirling at 40 revolutions per min on a rotary shaker reduced the attachment of spores and vegetative cells of thermophilic strains of *Bacillus* species (Parkar et al., 2001). Stainless steel coupons conditioned with acidic skim milk (pH 3.8 to 5.5) for 30 minutes showed lower adherence of *Lactobacillus paracasei subsp. paracasei* in comparison with the untreated surface and with basic skim milk (pH 5.5-9.5) samples (Nguyen et al., 2010). They attributed the lower bacterial adherence to the thicker conditioning layers formed from the precipitation of milk proteins at acidic pH on the stainless steel coupons. In another recent study, the effect of bacterial adhesion on stainless steel was determined by coating with different extracts of different food commodities of animal and plant origin. The study considered the adhesion of six bacteria *Pseudomonas fluorescens, S. enterica, L. monocytogenes, E. coli* MG1655, *S. aureus* and *Vibrio parahaemolyticus* at room temperature under stirring (250 revolutions per min) conditions. The authors concluded that the presence of conditioning film reduced the attachment of bacteria to stainless steel (Bernbom et al., 2009). The same authors also demonstrated that coating stainless steel with aqueous cod extract significantly decreased the bacterial attachment by a factor of 10–100 when compared to surfaces coated with tryptic soya broth (Bernbom et al., 2006). In addition coating albumin on titanium (An et al., 1996; Kinnari et al., 2005) and with human plasma on poly (vinyl chloride) has shown to reduce bacterial adhesion.

On the contrary, other researchers have found that coating stainless steel, rubber and glass surfaces with whey protein enhanced the attachment of milk-associated bacteria (Speers & Gilmour, 1985). Al-Makhlafi et al. (1994) investigated the effects of 'preadsorbed' milk proteins (α-lactalbumin, β-lactoglobulin, β-casein and BSA) on adhesion of *L. monocytogenes* to hydrophobic and hydrophilic silica surfaces. All proteins inhibited attachment of *L. monocytogenes* on hydrophobic surface whereas all proteins except BSA enhanced attachment on hydrophilic surface when compared to bare hydrophobic and hydrophilic surfaces respectively. *S. typhimuriurn* inoculated onto stainless steel, formica, polypropylene, or wooden surfaces (25 cm^2) in the presence of protein (tryptic soy broth supplemented with 5% horse serum) at room temperature significantly increased the number of bacteria recovered from all surface types after surface sampling with sterile cotton tipped swabs (Moore et al., 2007). Stainless-steel surfaces preconditioned by immersion in growth media such as tryptic soy broth (TSB), 1% reconstituted skim milk (RSM) and RSM with 1% sucrose (RSM + S) showed that *Pseudomonas fragi, L. monocytogenes* and *S. typhimurium* cells adhered in the higher numbers when compared to surfaces that were not preconditioned (Hood & Zottola, 1997).

2.5.2 Effect of surface properties

Modern food processing equipment is constructed of many different materials to which bacteria can attach – 304 and 316 SS, glass, plastics, rubber and even polytetrafluoroethylene (PTFE) where the plant may have large surface areas suitable for rapid colonization. Various groups have observed greater cell attachment on surfaces with high surface roughness (Leclercqperlat & Lalande, 1994; Pedersen, 1990). On the other hand authors have reported no correlation between surface roughness and bacterial attachment to inert surfaces (Flint et al., 2000; Hilbert et al., 2003; Mafu et al., 1991). Similar trends have been reported by several authors with respect to surface topography. Surface irregularities such as cracks, crevices, pits (close to the diameter of the bacterial cells) may cause the cell entrapment and provide them with some degree of protection from cleaning agents (Verran et al., 2008). Surface chemistry also influences bacterial adhesion and proliferation. Materials with different functional groups change bacterial adhesion in a manner depending on material hydrophobicity and charge. Generally, metal surfaces have a high surface energy and are negatively charged and hydrophilic as shown by water contact angles, while and polymers such as ultrahigh molecular weight polyethylene or

Teflon® have low surface energy and are less electrostatically charged and hydrophobic. Different studies show opposing results for the effect of surface hydrophobicity, but there is a tendency for hydrophobic surfaces to attract more bacteria (Bendinger et al., 1993; Sinde & Carballo, 2000).

2.5.3 Bacterial surface properties

Bacterial cells generally have a net negative charge on their cell wall at neutral pH (Rijnaarts et al., 1995). However, the magnitude of the charge varies from species to species and is depends upon cultural conditions, age of the culture, ionic strength and pH. Generally, bacteria with hydrophobic properties prefer hydrophobic material surfaces; the ones with hydrophilic characteristics prefer hydrophilic surfaces. In addition the presence of fimbriae and flagella, and production of EPS all influence bacterial surface properties and hydrophobicity (An & Friedman, 1998). A study by (Parkar et al., 2001) showed that the hydrophobicity of thermophilic strains of *Bacillus* species did not have an effect on the degree of bacterial attachment to stainless steel. Similarly no correlation was found between *L. monocytogenes* hydrophobicity and attachment to glass (Chae et al., 2006). However a strong correlation between hydrophobicity and cell attachment have been by reported many authors (Gilbert et al., 1991; Liu et al., 2004). Thus, the correlation between surface charge or hydrophobicity and adhesion is not simple. The difficulty in relating cell surface characteristics to adhesion performance for different bacterial strains is due to the heterogeneity of the cell surface, in which many components will differ between various strains. In addition the different growth conditions (nutrients, pH, salt etc) may also induce changes in the biochemical composition and physico–chemical cell surface characteristics which impact bacterial adhesion to surfaces. Moreover, microorganisms are not inert colloids and bacteria can produce secretions that facilitate irreversible adhesion and biofilm formation (Araújo et al., 2010).

2.6 Studies on bacterial adhesion and biofilm formation under flow condition

The past three decades have seen several published reports demonstrating the influence of flow on bacterial adhesion and biofilm formation on surfaces using different experimental flow systems mainly in industrial, marine, and medical settings. The essential flow parameters in

experimental flow systems including the mechanisms of mass transport, flow rate and velocity (defined by dimensionless numbers such as Reynolds and Peclet number), wall shear rate and other hydrodynamic forces have been extensively defined and explained for cylindrical and rectangular configuration flow type experimental systems (Busscher & van der Mei, 2006). In the literature, various studies are available reporting the effects of flow velocity, wall shear stress and mass-transfer rate on biofilm structure, thickness, nutrients, density, phenotype, stability, activity and EPS formation. These investigations have been performed using different experimental flow system. In the following section an attempt has been made to summarize some commonly used experimental flow systems in biofilm research and to report findings and observations with respect to the

- effect of conditioning film on bacterial adhesion and biofilm formation under flow conditions
- effect of flow on number of bacteria attached to the abiotic surface
- effect of flow on biofilm characteristics

2.6.1 Experimental flow systems

Studying bacterial adhesion and biofilms under different flow conditions can be difficult because of the heterogeneous nature of these phenomena (Costerton et al., 1995). Therefore, simplified model systems that offer control of flow conditions and growth environment while providing ease of analysis are required. (Sjollema et al., 1989). Depending on the nature of the problem that is in question, several types of flow reactor models for monitoring bacterial adhesion and biofilm properties in laboratory and industrial conditions have been developed. These have been classified and described based on their nutrient availability as “closed” system models (batch culture) and “open” system models (continuous culture) (McBain, 2009). The most common models which allow control of flow conditions are

- Suspended substratum reactor (developed by Centers for Disease Control and Prevention, marketed by BioSurface Technologies Corp. Bozeman, MT, USA),
- Rotating reactors (available at BioSurface Technologies Corp)
- The modified Robbins device (MRD),

- Flow cell reactors
- Microfluidic Devices

Among these, the modified Robbins device (MRD) and flow cell reactors are commonly used to study bacterial adhesion and biofilm formation on different surfaces.

The modified Robbins device. The modified Robbins device (MRD) which was developed by Jim Robbins at the University of Calgary is a well-known system that has been used extensively to study bacterial colonization and biofilm formation. This laboratory flow model simulates flow regimes in industrial pipelines and tubular devices such as catheters. The MRD is a rectangular Perspex block measuring 44 cm long, 2 cm high and 2.5 cm wide, with a central lumen measuring 2 mm high by 1 cm. All along the length of the central lumen are multiple sampling ports to which different surfaces can be fitted. The surfaces can be removed and replaced aseptically. The MRD is relatively inexpensive and can be used in conjunction with continuous and batch culture systems. Microscopic analysis is possible using conventional staining techniques with slide-mounted samples or electron microscopy of the colonized surfaces. Quantification of several aspects of the biofilm, such as viable and total cell counts and total protein and carbohydrate content, is possible. However, destructive sampling techniques are required for quantitative analysis of the biofilm, and these devices do not allow *in situ* visualization of the biofilm (Hall-Stoodley et al., 1999; Jass et al., 1995; Kharazmi et al., 1999).

Flow Cells. In order to directly visualize the development of biofilms microscopically, glass flow cells that are capable of being mounted on microscopes were created. Flow cell reactors allow *in situ* real-time visualization of the fundamental processes of biofilm formation and development when reactors are used in association with high-resolution imaging techniques such as confocal laser scanning microscopy. Several different types of flow cell reactors have been designed and may be used in adhesion studies depending on the nature of the problem (Palmer, 1999). Among them the parallel plate flow chamber (PPFC) reactor has been commonly used (Bos et al., 1999). Different PPFC designs such as those that provide the automated image analysis for real-time enumeration of the microorganisms or invasive sampling for further chemical and biological analyses of test surfaces have been established (Huang et al., 1992; Sjollema et al., 1989). Special designs that suit laboratory and industrial conditions and allow

periodic sampling of colonized slides without stopping the flow have also been used for biofilm studies (Pereira et al., 2002b). More recently, the use of PPFC systems combined to cell tracking by microcopy with fast imaging capabilities have revealed to be extremely useful in bringing new insights on the initial steps of bacterial attachment to surfaces under flow conditions (Busscher & van der Mei, 2006; Nejadnik et al., 2008). Microfluidics development for biofilm investigation applications area appears to be a very promising approach enabling a better control of parameters (Kim et al., 2012). It allows special designs for the study of the effects of complex configuration creating vortex and other fluid dynamics and presence of confinements (Kumar et al., 2013; Yazdi & Ardekani, 2012). The PPFC devices are very flexible systems which could be adapted to a large variety of experimental designs. They could even be used to assess the efficacy of particles in bacterial removal (van der Mei et al., 2008).

It should be kept in mind that the configuration of the experimental flow device would have an effect on the biofilm formation. For instance, Simoes et al. (2012) reported evidences that the number of adhering cells is reactor-type dependent in the case of drinking water bacteria attachment on SS and polyvinyl chloride (PVC). In their study, a flow cell system was compared to Propella™ type bioreactor, both operated under laminar (Re: 2000) and turbulent (Re: 11000) flow conditions. The authors report that in the flow cell, more bacteria were found to adhere on PVC compared to SS. The bacterial attachment was similar for both types of surfaces in the Propella™ system regardless the flow regime. More adhering cells were observed under turbulent flow in the flow cell compared to laminar flow conditions. In fact, the colonization of the surface by the bacterial cells took longer time to establish in the flow cell under laminar flow.

Another factor that can vary from one system to another is the formation of nano and micro-bubbles in the system. The presence of such bubbles can have a significant impact on the morphology of adhering bacterial colonies and its spatial distribution on surfaces under high hydrodynamic conditions. This concern should be addressed in experimental flow cell methodologies used to investigate biofilms. A recent paper supplies useful guidelines for flow cell operation in order to avoid random bubble formation (Crusz et al., 2012).

It can be concluded that the choice of the experimental flow device for studying bacterial adhesion and biofilm formation depends on nature of the problem that is in question. Therefore

to study bacterial adhesion and biofilm formation in food industry settings the selection of the experimental flow model should be based on reproducing hydrodynamic food processing conditions at the laboratory scale.

To complete this section, it is worth mentioning that special models have been developed to study bacterial mobility and biofilm formation in porous media. These approaches are of great interest for the study of biofilms in systems such as membranes, filters and other like-type of equipment which are common in many food processing operations. For more details on this specific topic, the readers are referred to recent reviews addressing this specific configuration (Bottero et al., 2013; Bozorg et al., 2012; Tufenkji, 2007).

2.6.2 Effect of conditioning film on bacterial adhesion and biofilm formation under flow conditions

The effects of conditioning of a surface on bacterial adhesion and biofilm formation under flow conditions have also seen varied results depending on the type, method and conditions under which the investigations have been performed with Bagge et al. (2001) reporting no difference in numbers of adherent bacteria with respect to preconditioning of stainless steel with diluted tryptic soya broth when compared to SS that was not conditioned. Szlavik et al. (2012) demonstrated a lower initial adhesion rate (IAR) of *L. monocytogenes* (eight strains) at high shear stress except for strain 4446 on glass coated with unhomogenised milk which showed significantly higher IAR at high shear stress when compared to pretreated glass with beef extract, casein, and homogenised milk. In general, both strain and surface was found to significantly influence the initial adhesion rate (IAR) at both low and high shear stress but no clear correlation between shear stress and initial adhesion was seen.

The impact of alginate conditioning films on motile and non-motile (strains devoid of flagella) *Pseudomonas aeruginosa* strains on quartz cover slips demonstrated an increase in adhesion of both motile and non-motile strains when compared to bare quartz surfaces. However, deposition efficiency of motile strains was less sensitive to the presence of conditioning film than those of non-motile strains (de Kerchove & Elimelech, 2007). Similarly, in another study, in relation to the marine industry the presence of alginate and SRNOM (Suwannee River natural

organic matter) conditioned glass was shown to significantly improve *Burkholderia cepacia* initial adhesion at low ionic strength. They also studied the presence of BSA conditioning films and reported that it significantly hindered bacterial adhesion at high ionic strength, compared to bare glass slides (Hwang et al., 2012).

2.6.3 Effect of flow on the number of bacteria attached to a surface

The effect of flow rate, flow velocity, shear stress on bacterial adhesion and biofilm formation has been investigated by several authors. Both laminar and turbulent flow regimes have been used to study bacterial adhesion and biofilm formation. However, whether flow rate, flow velocity and shear stress enhances or hinders biofilm development remains under debate as seen from the Table 2.3.

Table 2.3: Effect of flow on the number of bacteria attached to a surface

Microorganism	Surface	Medium	Flow device	Flow rate/shear rate	Contact time	Technique	Effect on flow on the number of bacterial adhesion and biofilm formation	Reference
S. putrefaciens	SS	PBS	MRD	10 mL/min	72 h	FM IC	Reduced	(Bagge et al., 2001)
P. fluorescens	SS 316	MSM	RFGC	5 L/min	4 to 90 h	FM	Drastic decrease up to a "critical" range of shear stress (6-8N/m^2) above which the rate of reduction of attachment levelled off	(Duddridge et al., 1982)
S. epidermidis	GL, OS	PBS	PPFC	50, to 2000 s^{-1}	2h	PC SEM	Decreased , with increase in shear (especially at 2000 s^{-1})	(Katsikogiannni & Missirlis, 2010)
Yeast Species	SS 304	Apple juice	FC	3.6 L/60 min Re=66	2 h.	EM SEM	*K. marxianus*, *C. krusei* and *R. mucilaginosa* reduced one log cycle whereas *Zygosaccharomyces* spp. increased 0.5 log as compared with static conditions.	(Brugnoni et al., 2009)
Heterotrophic bacteria	SS 304 (2B)	Water	AR	20, 40 and 60 cm s^{-1}	25 days	PC	Significantly reduced the HPC from 10^6 to 10^3/10^4 CFU/cm^2	(Tsai, 2005)
P. aeruginosa	GL	DC	FCR	Re 96 to Re 2220	3 days	OMV	Rate of accumulation of cells decreased as the surface shear increased.	(Boyle & Lappin-Scott, 2006)
L innocua	SS	TYM	BA	Re- 9500 to 16,500	7 days..	SEM	Increase in Re number from 9500 to 16,500 resulted in less attachment (day1). After 4 days, surface attachment increased and at day 7 days biofilms became more established at all Re numbers	(Perni et al., 2006)
P. fluorescens	RB	Sodium citrate	MRD	Re - 2, 17, 51.5	~ 30 h	PC SEM EM	Colonisation by *P. fluorescens* increased with increasing Re number	(Brading et al., 1995)
P. aeruginosa	GL	MG & BS	AR	1.44, 2.20, 2.97 N/m2	24 h	OM	Shear stress had no effect on biofilm thickness biofilm thickness increased significantly with increase in substrate loading rate	(Peyton, 1996)

Microorganism	Surface	Medium	Flow device	Flow rate/shear rate	Contact time	Technique	Effect on flow on the number of bacterial adhesion and biofilm formation	Reference
C. krusei	SS 304 2B	Apple juice	RDS	Re- 15,000 - 136,000	Up to 96 h	EM	Low shear stress had no effect after 24 and 48 hours. whereas high shear stress resulted in an increase after 48 hours when compared to 24 hours	(Brugnoni et al., 2011)
Heterotopic plate count and Total bacteria	Cu and PE	Water	PWDS	Re ~ 420 to 2750	3 week	PC AO	Increase in flow rate did not significantly affect the HPC in Cu pipes, but increased in the PE pipes; Total number of bacteria in biofilms increased with increasing flow rate for both pipes up to a flow rate of 0.8 L/min	(Lehtola et al., 2006)
Total bacteria	SS 314 & 316	Potable Water	ERS	0.32, 0.96 and 1.75 m/s	5 month	PC EM SEM	Viable and total cell counts on all grades and surface finishes were significantly higher at 0.96 and 1.75 m/s than 0.32 m/s	(Percival et al., 1999)
Pseudomonas (4 strains); *Coryneform* (8 strains)	TF, GL	PBS	Column method	16 - 21 cm^3/hr	1h	OM	Approx 4 times higher in dynamic systems than in static systems despite shorter contact times for 15 out of 22 strain surface combinations studied	(Rijnaarts et al., 1993)
L. monocytogenes (7 Strains)	Polished SS 304	NaCl	PRC	0.75 ml/min; 8.40 ml/min	30 sec to 15 min	FM	High flow rate resulted in significant increase in initial adhesion rate of all strains except for strain 412.	(Skovager et al., 2012)
B. cereus	Silicone	KNO_3	CFSS	1.3 mL/min	Up to 48 h	PC	Significantly greater biofilm volume was observed under flowing conditions than under static conditions.	(Ksontini et al., 2013)

Surface: SS (stainless steel), GL (glass), OS (organosilanes), TF (teflon), Cu (copper), PE (polyehtylene); RB (Rubber). **Suspending medium**: MSM (mineral salt medium), PBS (phosphate buffered saline) DC (diluted culture), TYM (tryptone yeast medium), MG (mixed glucose and buffer solutions) NaCl (sodium chloride), KNO_3 (Potassium Nitrate). **Flow device**: MRD (modified Robbins device), RFGC (radial flow growth chambers). BA (biofilm apparatus), PPFC (parallel plate flow chamber), AR (annular reactor) RDS (rotating disk system), PWDS (pilot water distribution systems), ERS (experimental rig system), PRC (perfusion chamber) FC (flow chamber), CFSS (continuous flow silicon system), FCR (flow cell reactor), AR (annular reactor). **Technique**: FM (fluoroscence microscopy), IC (indirect conductance) PC (plate counting), SEM (scanning electron microscopy), EM (Epifluoroscence microscopy), OMV (optical microscope linked to video camera), MDT (micro drop technique), CVS (crystal violet staining) AO (acridine orange staining).

2.6.4 Effect of flow on biofilm characteristics

The effects of flow on the morphology, structure, activity (such as metabolic, respiratory) and stability of biofilms has been the subject of numerous studies (Table 2.4). Generally, the structure of biofilms has been investigated under both laminar and turbulent flow conditions. Most studies of this type have focused on the *pseudomonads* because certain species such as *P. putida* and *P. fluorescens* are good biofilm formers. It has been observed that flow generated biofilms show greater architectural complexity, are metabolically more active when compared to statically grown biofilms. In addition, laminar flow generated biofilm are thicker whereas turbulent flow generated biofilms are denser and more stable and the shapes of cells clusters in laminar flow were circular, whereas in turbulent flow the biofilms became elongated and formed filamentous "streamers"(Hall-Stoodley et al., 1999).

Few studies have also focused on the development and characterization of yeast biofilm model on food grade stainless steel in order to better understand biofilm formation in the food industry conditions (Brugnoni et al., 2009, 2011; Brugnoni et al., 2007). In general, it was observed that yeast cells exposed to higher shear stress were aligned to the direction of flow and consisted of larger microcolonies when compared to cells exposed to lower shear stress which generally consisted of spherical or hemispherical mound-shaped microcolonies and had an increased tendency to aggregate. They also observed that turbulent flow gave significant differences in mass for biofilms formed at different Reynolds number and the influence of hydrodynamic drag.

In the case of *L. monocytogenes*, in comparison to static condition, the presence of a flow of nutrients induces changes in the metabolism and the expression of *agr*, particularly in the cells at the contour of the colonies. Evidences have also been reported that the biofilm formation is not only influenced by the presence of a flow of nutrients, but also their concentration. For instance Teodosio et al. (2011) observed that an increase in nutrient concentration result in thicker but less dense biofilms. In addition to the effect of concentration during flow, the type of nutrients available for the growth of *L. monocytogenes* prior to adhesion was demonstrated to significantly affect the ability of this pathogen to attach to solid surfaces. Glucose, mannose and L-leucine were identified as influencing the biofilm formation under flow. The cell adhesion is

increased for growth media supplemented in L-leucine and glucose, but comparatively reduced when glucose is substituted by mannose (Skovager et al., 2013). This type of findings is of great interest in identifying critical locations and conditions more prone to surface colonization by foodborne pathogens and biofilm persistence in food processing lines.

A recent study investigated the initial attachment of *S. epidermidis, P. aeruginosa, P. putida and E. coli* and polystyrene particles on hydrophilic and hydrophobic glass under flowing conditions. The results suggest that in most cases the attachment numbers decreased with increase in shear beyond a critical value. Further, the extended Derjaguin and Landau, Verwey and Overbeek theory could contribute in the understanding of the initial bacterial attachment in presence of shear (Wang et al., 2013). Finally at high shear rate, when turbulence takes place, the presence of air bubbles cannot be excluded. The capabilities of microbubbles to detach adhering bacterial cells from surfaces have been demonstrated in numerous studies (Gomez-Suarez et al., 2001; Sharma et al., 2005). The presence of nano and microbubbles on the solid surface, particularly hydrophobic ones but also on hydrophilic substrates has also been shown to affect protein adhesion (Kolivoska et al., 2011; Wu et al., 2006)

Table 2.4: Effect of flow on biofouling

Microorganism	Surface	Medium	Flow device	Flow rate/shear rate	Biofilm formation conditions	Technique	Observations on structure and properties of biofilm	Reference
P. fluorescens	SS	Sterile water	FCR	Re 2000 &Re 5500	10 days	CLSM	Laminar flow biofilms were thicker, less dense more fluffy when compared to biofilms turbulent flow biofilms which were denser and more stable	(Pereira et al., 2002a)
P. fluorescens	GL	NM	FA	0.5m/s (Re 9667) and 2.5 m/s (Re 32222)	20 days	IRD, SEM, TEM	Thicker and less stable film was formed at a velocity of 0.5 m/s compared to 2.5m/s Biofilm formed at 0.5 m/s velocity was more open and "fluffy" than the biofilm formed at 2.5 m/s.	(Santos et al., 1991)
P. fluorescens (ATCC 13525)	SS 316	PB	FCR	Re 2000 &Re 5500	Upto 10 days	BOM, EM, BCA, SEM	Cellular features: Laminar flow biofilm were more metabolically active, had a higher total protein and polysaccharide content when compared to turbulent flow bioflm cells Biofilm features: Turbulent flow biofilms were metabolically more active had a higher amount of mass , total proteins and smaller amount of polysaccharide when compared laminar flow biofilms	(Simões et al., 2007)
C. albicans	SE	RPMI-1640	LFM	0.75 ml/min	Up to 24 h	SEM, CLSM	Flow biofilms was at least 1.8-fold more metabolically active and greater in biomass than the corresponding static biofilms Flow biofilms showed increased architectural complexity and grew rapidly (8 h) showing characteristics similar to a 24 h statically grown biofilms	(Uppuluri et al., 2009)
P. aeruginosa(PAO1	GL	LB	BRS	Re = 100 and 3000	6 days	ISM	Laminar flow biofilms formed monolayers interspersed with small circular microcolonies whereas turbulent flow biofilms	(Purevdorj et al., 2002)

Microorganism	Surface	Medium	Flow device	Flow rate/shear rate	Biofilm formation conditions	Technique	Observations on structure and properties of biofilm	Reference
and JP1)							formed streamlined patches, and in some cases developed to ripple-like wave structures	
							At the end of day 6 thickness of all biofilms stabilized at ~20 µm and the surface area coverage was over 80% for both laminar and turbulent conditions	
E. coli JM109(DE3)	PVC	NM (high and low)	MRD	Re=6000	12 days. (every 24 h)	DM and WW	Thickness and wet weight showed an increasing trend with time for biofilms formed under both nutrient conditions	(Teodosio et al., 2011)
Mixed Species *(K pneumoniae, P. aeruginosa, P. fluorescens and S. Maltophilia)*	GL	NM (low and high)	BRS	Re = 100 and 3000	Run 1:Both types of flow 23 days on low NM	ISM	Cell clusters in laminar flow were circular in shape, whereas in turbulent flow the biofilms became elongated and formed filamentous "streamers"	(Stoodley et al., 1999)
					Run 2: Turbulent flow on low NM for 21 days, high NMfor the next 5 days and low NM for the last 3 days		Exposure of 21 day old biofilm growing in turbulent flow to high nutrient conditions increased its thickness from 30 µm to 130 µm within 17 h	
C. krusei	SS (AISI 304 2B food grade)	Apple juice	RDS	Re- 15,000 to 136,000 shear stress- 0 to 91 N /m2	Up to 96 h	EM	Cells exposed to higher shear stress: aligned to the direction of flow, consisted of larger microcolonies with fewer single cells Cells exposed to lower shear stress: showed no alignment relative to the direction, consisted of spherical or hemispherical mound-shaped microcolonies and increased tendency to aggregate	(Brugnoni et al., 2011)
C. krusei	SS (AISI 304 2B food grade)	Apple juce	RDS	Re – 294,000 to 1.2 X 106	Up to 96 h	EM	C. krusei biofilm mass increased with increase in Re number, and time.	(Brugnoni et al., 2012)
							Demonstrated distinct developemental phases with time : (i) at 24 hrs cells were present as adherent blastospores (ii) at 48 hrs they formed microcolonies and at (iii) at 72 hrs they formed cell agggregates and (iv) at maturation showed the presence of extracellular material.	

Microorganism	Surface	Medium	Flow device	Flow rate/shear rate	Biofilm formation conditions	Technique	Observations on structure and properties of biofilm	Reference
							In all phases, the morphology of C. krusei biofilms in turbulent flow revealed the influence of hydrodynamic drag	
P. fluorescens (ATCC 13525, D3-348 and D3-350)	SS (AISI 316)	Diluted NM	FCR	Re=5200, u=0.532 m/s & Re=2000, u=0.204 m/s	Up to 7 days	Various methods	Turbulent biofilms were more active, had more mass per adhesion surface area, a higher number of total and culturable cells, a higher amount of total proteins per gram of biofilm, similar matrix proteins and identical or smaller total and matrix polysaccharides content than their laminar counterparts	(Simões et al., 2008)

Surface: SS (stainless steel), GL (glass), SE (silicon elastomer), PVC (polyvinly chloride). **Suspending medium**: NM (nutrient medium), PB (phosphate buffered) LB (diluted culture), RPMI (Roswell Park Memorial) LB (Luria-Bertani). **Flow device**: MRD (modified Robbins device), BRS (biofilm reactor system) LFM (laboratory flow model), AR (annular reactor) RDS (rotating disk system), FCR (flow cell reactor), FA (flow apparatus). **Technique**: CLSM (confocal laser scanning microscopy), BOM (biological oxygen monitor), ISM (in situ Microscopy) PC (plate counting), SEM (scanning electron microscopy), EM (Epifluoroscence microscopy), IRD (infra-red device), TEM (transmission electron microscopy), BCA (bicinchoninic acid assay), DM (digital micrometer), WW (wet weight).

2.7 Conclusions

Fluid flow is of great importance in many industrial processes and is believed to be one of the most important that influence cell attachment to contact surfaces. Cell adhesion to surfaces is a very complicated process affected by many factors, such as bacterial-material properties, environment, and, furthermore the experimental evaluation of the relative contributions of these factors is extremely difficult. Conflicting opinions exist in the literature with respect to many factors including flow conditions and comparison of results from different laboratories is difficult due lack of uniformity in experimental protocol. However, from the data collected and presented in this review, it is clear that in most of the cases, the fluid hydrodynamics impacts very significantly surface colonization by pathogens of interest for food industry.

Several studies have shown that the magnitude of the shear rate influences the morphology and the number of cells attached to food contact surfaces in monoculture. However investigations of this parameter in mixed culture situations are missing. This would be of great interest as it is the most likely scenario that exists in food processing plants. Further, the effect of flow on bacterial adhesion has not been well studied in the food and dairy industry. Thus, systematic compilation of more adhesion data, especially for different species, is necessary to discuss the effects of various factors comprehensively and would help in the design of future investigations

PREFACE TO CHAPTER 3

Bacterial adhesion and biofilm formation is a serious hygienic problem in food industry causing processing contaminations. It is well established that the presence of bacteria on food contact surfaces affects the quality and safety of the food products. In addition, bacterial adhesion is influenced by the physico-chemical properties of the food as well as the nature and chemistry of the food contact surface. Prior to understanding bacterial adhesion, understanding the adsorption of food components such as proteins is integral. Besides, protein fouling is also known to be severe problem in the food industry.

For studying food protein fouling of surfaces, gathering the extent of proteins adsorbed onto surfaces as is required. Further, to understand the nature of interactions between the food and the food contact surface and the subsequent biofilm formation or elimination surface thermodynamic analysis is generally considered useful.

Therefore, prior to embarking on bacterial adhesion studies on contact surfaces, the first task is to quantify the adsorption of proteins on model surfaces mainly in food industry environments. The interaction or adsorption behaviour of food proteins were further analysed using the thermodynamic analysis.

Part of this research was presented in 2011 in the Northeast Agricultural and Biological conference, South Burlington, Vermont, USA. Other parts have been presented in 2014 Institute of Food Technology conference, New Orleans, Louisiana, USA and in 2014 at Northeast Agricultural and Biological conference, Kemptville, Ontario, Canada. One manuscript has been prepared from this Chapter.

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The experimental work and data analysis were carried out by the candidate under the supervision of Dr. Louise Deschenes and Dr. H. S. Ramaswamy.

Chapter 3. ADSORPTION OF PROTEINS FROM CONCENTRATED PROTEIN SOLUTIONS, MICROBIAL GROWTH MEDIA AND MILK ONTO HYDROPHILLIC AND HYDROPHOBIC SURFACES

3.1 Abstract

Understanding protein adsorption to food contact surfaces under industrial environments is relevant as it is considered as the foremost step for bacterial adhesion and subsequent microbial biofilm formation. In this study, the extent of protein adsorbed from concentrated protein solutions (bovine serum albumin, sodium caseinate) microbial growth media (tryptic soya broth) milk (whole and skim) on mica (hydrophilic) and polystyrene (hydrophobic) under static conditions was investigated. Adsorption was performed at room and refrigerated temperatures with adsorption times ranging between 1 to 24 h. Quantification of surface bound protein was done using micro-bicinchoninic acid protein assay (mBCA). The surface thermodynamic properties were characterized and quantified by contact angle measurements and the surfaces were imaged and analyzed by atomic force microscopy. Thermodynamic and extended Derjaguin–Landau–Verwey–Overbeek (XDLVO) theory was applied to predict and evaluate protein adsorption mechanisms. The results of this study showed that, ~7-10 times higher amount of proteins was adsorbed on mica when compared to polystyrene surfaces. Adsorption of CAS was observed to be the highest on both polystyrene and mica surfaces. The conditioning of mica and polystyrene modified their surface thermodynamic properties upon adsorption and proteins in solution and on surfaces were found in aggregated states. The total free energy of interaction calculated using the thermodynamic and extended Derjaguin–Landau–Verwey–Overbeek (DLVO) model showed favorable adhesion (negative values) of all proteins on polystyrene (-18 to -6.1E+12mJ/m^2) and most proteins of mica surface (-11.66 to -8.1E+11mJ/m^2). However, both models could not accurately predict the amount of protein adsorbed onto mica and polystyrene surfaces.

3.2 Introduction

The formation of a conditioning film due to the adsorption of macromolecules is typically considered as the first and foremost step in the course of bacteria adhesion and biofilm formation on solid surfaces (Bryers, 2008; Donlan, 2002; Hwang et al., 2013; Kishen & Haapasalo, 2010; Lorite et al., 2011). Among the different macromolecules, the adsorption / conditioning of proteins on contact surfaces has received considerable attention in various scientific and engineering disciplines such as medicine & pharmaceutical sciences, analytical sciences, biotechnology, cell biology and food science as an very important and fundamental problem (Nakanishi et al., 2001; Norde & Lyklema, 1991; Rabe et al., 2011). For instance, in dairy industry the adsorption of whey proteins, especially β-lactoglobulin (β-lac), which causes significant fouling on stainless steel food contact surfaces (particularly at pasteurization and sterilization temperatures) has been extensively investigated (Bansal & Chen, 2006; Fryer et al., 2006; Hausmann et al., 2013; Kim et al., 2007; Lalande et al., 1985; Visser & Jeurnink, 1997). Despite considerable progress, understanding of the complex phenomenon of protein adsorption to solid surfaces still remains a challenge as various events like denaturation, structural rearrangement or conformation in the adsorbed state, reorientation, desorption &/or exchange can occur upon or during the adsorption process (Norde & Anusiem, 1992). Furthermore, these events are highly dependent on the properties of the food contact surface, (surface energy, surface charge, roughness, topography), nature & properties of the protein (type, size, structural stability, charge) and external solution properties (pH, temperature, ionic strength, concentration) and as such several investigations have been reported (Gray, 2004; Hlady & Buijs, 1996; Wahlgren & Arnebrant, 1991).

Generally, the first step in the understanding of the protein adsorption behaviour is to quantify the amount of protein adsorbed/bound to the surface. Table 2.1 given in the previous chapter shows typical amounts of proteins adsorbed on to contact surfaces under different experimental conditions. This amount adsorbed is commonly presented using adsorption isotherms, where surface concentration build up is plotted against the bulk protein concentration. As the protein concentration in the bulk (solution) increases, the amount of protein adsorbed per unit area increases sharply and reaches a plateau or

surface saturation (Hlady et al., 1999). Based on the saturation values of well-defined proteins and model surfaces, Norde and Lyklema (1991) outlined a general set of rules to predict the adsorption proteins to surfaces. Proteins will have higher affinity if (i) the protein have low internal stability [known as "soft proteins", for example, bovine serum albumin (BSA), human serum albumin, (HSA) immunoglobulin (IgG), α-lactoalbumin, β-casein, haemoglobin, catalase and phytase) or "hard proteins" - proteins with high internal stability, for example, α-chymotrypsin, ribonuclease, lysozyme, and β-lactoglobulin), (ii) the surface is more hydrophobic, (iii) the protein exterior is more hydrophobic and (iv) protein and surfaces are oppositely charged. , These outcomes could often fail in "real" systems in which composition of biological fluid is unknown and varied nature of the surface obscures explanation (Kingshott & Höcker, 2006).

The adsorption of biological colloids onto solid surfaces, particularly microbial cells, has been largely understood using surface thermodynamics concepts. Surface thermodynamic properties, such as surface energy and hydrophobicity, have often been characterized and used for predicting the adsorption of biological colloids via three main physicochemical theoretical models namely the thermodynamic, Derjaguin, Landau, Verwey, and Overbeek (DLVO) and Extended DLVO (XDLVO) models (Bos et al., 1999; Busscher et al., 2010; Missirlis & Katsikogianni, 2007) and extensive reviews and descriptions with regards to the models are available in the scientific literature. In the case of proteins, these models have been utilized to understand membrane fouling of micro- and ultra-filtration systems in the water treatment industry (Hong et al., 2013; Kuhnl et al., 2010), protein interaction during adsorption chromatography (Aasim et al., 2012, 2013) and protein-fouling related marine environments (Schneider, 1996). With respect to food industry environment, very few studies have recognized theoretical models for describing the adsorption or adhesion of proteins or food material, although surface thermodynamic properties of solid surfaces (such as stainless steel, Teflon etc) or surfaces adsorbed or conditioned with proteins or food material have been characterized (Keijbets et al., 2009; Whitehead et al., 2009). Additionally several imaging techniques such as AFM (atomic force microscopy) and SEM (scanning electro microscopy), X-ray and Fourier transform infrared spectroscopy have been extensively used to visualize and

characterize several types of protein and food soils on contact surfaces (Whitehead et al., 2006; Whitehead et al., 2010).

The objectives of the present study were to

i) Quantify the adsorption of proteins from bovine serum albumin (BSA), sodium caseinate (CAS) concentrated solutions, Tryptic soya broth (TSB) and milk on mica and polystyrene (PS) using micro bicinchoninic acid (BCA) protein assay technique, (ii) Characterize surface thermodynamic properties of proteins and milk by contact angle, particle size and zeta potential measurements (iii) Characterize surface thermodynamic properties of mica and PS surfaces conditioned with proteins and milk by contact angle measurements (iv) Utilize the thermodynamic and XDLVO models to predict protein adsorption to mica and polystyrene surfaces (v) Use AFM to image mica and polystyrene surfaces conditioned with proteins and milk.

3.3 Materials and methods

Analytical grade ultra-pure water (18.2 MΩ•cm at 25 °C) was used for all preparations (Barnstead Easy Pure 11 LF Ultrapure water system).

3.3.1 Protein solutions and milk

Protein concentration was fixed at 33 mg/ml to represent concentrations particularly relevant to dairy industry (Walstra, 1999). Most protein adsorption studies in the past have been performed using bulk solutions of much low concentrations (0.006 to 10 mg/ml) (shown in Table 2.1), and is not characteristics of food products which contain high concentrations of nutrients. The protein content in milk is commonly around 3.3%. CAS was selected as it is major protein in milk and milk products. Interestingly, BSA, also a milk protein, is often used as a model protein in numerous adsorption investigations. TSB was selected to represent a commonly used growth media for microbial cells and in microbial adhesion studies on abiotic surfaces. In addition whole milk (WML) and skim milk (SML) were also selected to perform adsorption experiments as they represent "actual" conditioning fluid system in the dairy and food industry.

BSA, CAS and TSB powders were weighed and dissolved in appropriate quantity of water to obtain the desired concentration (33 mg/ml). The measured crude protein content of TSB was 50.29%. The protein content of TSB was measured using Dumas method (LECO Corporation, USA). TSB solution was autoclaved and cooled to room temperature prior to use. All the solutions were prepared before a couple of hours prior (to allow for protein chains relaxation) before each adsorption experiment was performed and were never recycled. Whole milk (WML) and skim milk (SML) cartons were obtained from the local supermarket and irradiated one day prior to the experiment and stored in a refrigerator at 4 ± 1 °C. Source and approximate composition of BSA, CAS, TSB, WML and SML that were used in the study are listed in (Table 3.1).

3.3.1.1 Degassed BSA solution

Degassing of BSA solution was performed under vacuum for about 2 hours under atmospheric pressure. Degassing was done to check the effect of nanobubbles since nanobubbles are strongly related to the dissolved gas (Switkes & Ruberti, 2004; Zhang et al., 2004).

Table 3.1: Source and composition of BSA, CAS, TSB, WML and SML that were used in the study

Conditioning material	Composition	Supplier
Bovine Serum Albumin (BSA)	Fraction V, Cold-Ethanol Precipitated, purity > 98%	Fisher Scientific Company Canada
Sodium Caseinate (CAS)	92.2% protein	Nealanders International Inc. Dorval, Quebec
Tryptic Soya Broth (TSB)	Enzymatic Digest of Casein: 1.7% Enzymatic Digest of Soybean Meal: 0.3% Sodium Chloride: 0.5% Dipotassium Phosphate: 0.25% Dextrose: 0.25%	Bacto™ Canada
Whole Milk (WML)	Protein 3.6%, Fat – 3.25%	Local store
Skim Milk (SML)	Protein 3.6%, Fat -0%	Local store

3.3.2 Solid surfaces and surface preparation

Two types of solid surfaces with different wettability and surface energy were selected for this study. Mica was chosen as a model hydrophilic surface as they have an outstanding even surface, and are considered optically flat, clear, transparent, and scratch less. They are also widely used in electron microscopy, particle imaging, and cell growing and thin film coating research. Sheets of mica were obtained from S&J Trading Inc, Glen Oaks, N.Y., US. In simple terms, muscovite is a stack of 10-Å-thick sheets, with adjacent sheets held together primarily by K^+ ions. These sheets can be readily cleaved to produce atomically flat surfaces over a large area usually over tens of square micrometres (Czajkowsky & Shao, 2003). Mica was chosen because it has been extensively used as a substrate to study the adsorption of proteins and colloids. Polystyrene was selected as it is a widely used food contact material in addition to being a model surface for studying protein adsorption to biomaterials.

Mica and polystyrene were cut into rectangular coupons (3.8 cm x 1.9 cm) with a total surface area of 14.4 cm^2. The mica and polystyrene coupons were used only once. Mica was freshly cleaved to reveal clean surfaces. Polystyrene coupons were cleaned by sonication in methanol (Fisher Scientific, Canada) for 10 minutes, followed by sonication in water (Branson 2510) for 10 min. In addition, to remove any remaining traces of methanol, polystyrene surfaces were thoroughly rinsed with abundant quantities of water and dried under nitrogen. Polystyrene surfaces were stored in vacuum oven until use.

3.3.3 Adsorption (conditioning) experiments

Adsorption test were carried out under static conditions in 50 ml polypropylene centrifuge tubes (Fisher scientific, Canada) with treatment times ranging from 15 min to 24 h. Experiments were carried out at room (25°C) and refrigerated (4°C) conditions. Clean individual coupons of mica or polystyrene surfaces were immersed vertically in 25 ml (enough to cover the surface) of protein solution or milk and incubated for the specified time. Concurrently, surfaces were also immersed in water which served as a control/blank (surface blank). After the immersion treatment, the coupons were

withdrawn by forceps and rinsed with water (from a wash bottle) on both sides to remove any loosely adhered protein. Then the coupons were rinsed again twice, sequentially by dipping in 40 ml volumes of water (in centrifuge tubes) for about 10 s to further remove any loosely bound protein. The rinsed coupons were then placed in clean empty centrifuge tubes and surface dried by blowing nitrogen gas for 10 min. The dried surface was immediately subjected to the micro-bicinchoninic acid (BCA) protein quantification assay using rectangular glass vials.

3.3.4 Protein quantification assay

3.3.4.1 Glass vials

In order to perform protein quantification experiments rectangular glass vials were obtained from Soufflage de Verre estrie, Sherbrooke, Quebec. The vials had an inner dimension was 3x30 mm and height of 50 mm (Figure 3.1). The vials had a capacity of 3.6 ± 0.2 ml (in terms of volume of liquid that could be contained). The surface area to volume ratio provided an optimum surface area to volume ratio which helped to quantify the amount of proteins on the surface.

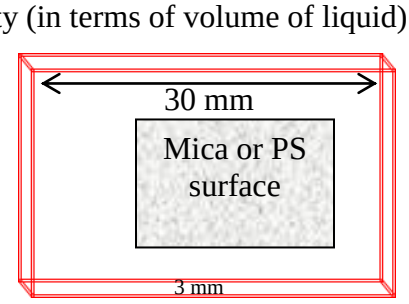

Figure 3.1: Glass vials for quantification of protein on surfaces

The vials were cleaned before and after each adsorption test run by sonication in methanol for 10 min followed by sonication in water for 10 min. To remove remaining traces of methanol, vials were thoroughly rinsed with abundant quantities of water and dried overnight by storing in an incubator (25°C). The cleaning procedure was validated by performing protein quantification experiment on empty glass vials.

The micro BCA protein assay quantification kit was used to quantify surface bound protein (Thermo Fisher Scientific, product number 23235). This kit has been optimized for use with dilute protein samples (0.5-20 µg/ml). The BCA assay measures the formation of Cu^{+} from Cu^{2+} by the Biuret complex in alkaline solutions of protein using bicinchoninic acid (BCA). A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+}). This water-soluble complex exhibits a strong absorbance at 562 nm (Smith et al., 1985).

The micro BCA working reagent was prepared according to manufacturer specifications. To minimize day to day variations (with respect to preparation of conditioning solutions) a standard curve of the applicable conditioning test solution namely BSA, TSB, CAS, WML and SML was prepared every time, for each adsorption condition, while performing the quantification experiment. To prepare the standard curve, conditioning test solutions were serially diluted in water to obtain concentrations ranging between 2.5 – 40µg/ml (dilution standard). Aliquot's of each diluted standard (500 µl) was pipetted in Eppendorf tubes and mixed with the micro BCA working reagent (500 µl) in the ratio 1:1 to obtain a protein concentration ranging between 1.25- 20µg/ml. A blank solution containing only water and reagent was also made (blank standard). Similarly, to quantify surface bound protein, micro BCA protein assay reagent (1800 µl) and water (1800 µl) was added to the special rectangular glass vials to completely cover the surface and covered using wax paper. Reaction was carried out by incubating the Eppendorf tubes and the covered glass vials in an air oven at 60°C for h. After cooling to room temperature, samples were thoroughly mixed (using a vortex for Eppendorf tubes and pipette tips for glass vials) and pipetted (200 µl) on to a microplate (96 well). The absorbance was read at 562 nm without any delay. The absorbance value of each dilution standard was subtracted from the absorbance of blank standard to construct the standard curve and the equation of the line was established. The amount of protein adsorbed on the surface was calculated by subtracting the absorbance value of each conditioned surface (surface in contact with conditioned test solution) from the average absorbance value of the surface blank (surface in contact with water). The value obtained, was converted to micrograms (µg) of protein adhered using the equation of line obtained from the standard

curve. The amount calculated was divided by the total surface area available for adsorption and finally expressed as microgram per square centimeter ($\mu g/cm^2$). All experiments were carried out in triplicate for each adsorption condition and repeated two times. Each measuring point presented in adsorption graphs from micro BCA experiments represent the average of measurements performed on six surfaces (3 measurements on two separate days) treated in the same way during the same adsorption experiments. The error bars (presented in the graphs) represent the standard deviation of six measurements.

3.3.4.2 SAS analysis

The data were analysed using the GLM (general linear model) procedure of SAS software Version 9.4 (SAS Institute Inc., Cary, NC). The influence of time, temperature , media and surface on the amount of protein adsorbed on both mica and polystyrene surface was investigated by analysis of variance. The Duncan's multiple ranges test was also used to separate means (only for influence of time and temperature). A confidence level of P = .05 was chosen during analyses.

3.3.5 Characterization of proteins, milk, mica and polystyrene surfaces

Protein solutions and milk (conditioning solutions), and mica and polystyrene surfaces, both blank (surfaces conditioned in water) and conditioned surfaces, were characterized by contact angle measurements (CAM) to obtain the various surface thermodynamic properties (surface energy and its components, and absolute hydrophobicity). Besides, the proteins and milk were subjected to dynamic light scattering (DLS) to obtain the size distribution (hydrodynamic radius) profiles and zeta potential measurements to know the charge on each of their surfaces. Atomic force microscopy (AFM) was also used to image both mica and polystyrene surfaces before and after conditioning.

Based on the surface thermodynamic properties the total free energy of interaction between conditioning solutions (proteins and milk), and solid surfaces (blank mica and polystyrene) was calculated utilizing the thermodynamic and XDLVO theories.

3.3.5.1 Contact angle experiments

Contact angle measurements (CAM) were made using the sessile drop technique (Busscher et al., 1984) using a FTÅ 200 system (First Ten Angstroms, Portsmouth, Virginia) equipped with a charge coupled device (CCD). A drop of a suitable diagnostic liquid (2-5 µl) was dispensed by a dosing device onto the sample surface. Probe liquids were water, formamide and α-bromonaphthalene (Aldrich Chemical Co., Inc., Milwaukee, WI). Surface properties of such liquids are available in the literature (van Oss, 1994). Images of drops were photographed, recorded and analyzed to obtain the contact angles using an image analysis program (FTA 32 ver 2.0 software package). Sixty images were captured for a total measuring time of 60 sec. Measurements of contact angles were taken at 30 sec after depositing the drop on the surface. At least three random locations were examined per sample surface and three independently prepared sample surfaces were analyzed.

Protein film layers" and "milk layers" were prepared for contact angle measurements (CAM) in 'hydrated states" using ultrafiltration membrane disc filters (Omega^{TM} Pall Corporation, USA) with a 43 mm diameter and 5 kDa molecular weight cut off rating according to the protocol described by (van Oss et al., 1985). Briefly, conditioning solutions were filtered (under 30 psi compressed nitrogen gas) through the ultrafiltration membrane discs to create "film layers of protein or milk". The membrane was then glued to a support using a double sided tape and air-dried (room temperature). The water contact angle on the "hydrated film layers" was measured as a function of time until a plateau value was obtained which was reached after 30 minutes in our case. This predetermination of drying time was done, to provide an accurate contact angles of each probe liquid on the "film layers' as the contact angle of the proteins in the hydrated state is dependent on the degree of hydration (Aasim, 2010).

CAM were also performed on both blank and conditioned mica and PS surfaces. Conditioning films on mica and PS surfaces were prepared by adsorption of proteins and milk according to the described protocol in Section 3.3.3. Briefly, mica and polystyrene were contacted with 25 mL of each protein solutions, milk or water (for blank), followed by

rinsing and drying under nitrogen. For these tests, the conditioning time was fixed at 1 h after which CAM were performed.

3.3.5.2 Calculation of surface thermodynamic properties

Surface thermodynamic properties (surface free energy and its components and absolute hydrophobicity) were calculated through the contact angles measured using the Lifshitz van der Waals acid–base approach which is also called the van Oss, Chaudhary and Good approach (VCG approach) in combination with the extended Young's equation (van Oss et al., 1988; van Oss et al., 1986). According to this approach the solid-liquid interfacial free energy was expressed as a sum of apolar Lifshitz -van der Waals (LW) and polar acid-base (AB) components (Equation 3.1). The acid-base (AB) polar components were further divided into electron accepting/ hydrogen (proton) donor parameter designated as γ_{SL}^{+} (Lewis acid) and the electron-donation/hydrogen (proton) acceptor parameter designated as γ_{SL}^{-} (Lewis base).

$$\gamma_{SL}^{TOTAL} = \gamma_{SL}^{LW} + \gamma_{SL}^{AB} \qquad \textbf{Equation 3.1}$$

γ_{SL}^{TOTAL}= Total free energy of solid-liquid interface

γ_{SL}^{LW}= LW component solid-liquid interfacial free energy

γ_{SL}^{AB}= AB component solid-liquid interfacial free energy

γ_{SL}^{+}= Electron acceptor – Lewis acid AB component solid-liquid interfacial free energy

γ_{SL}^{-}= Electron donor- Lewis base AB component solid-liquid interfacial free energy

The LW component of the solid-liquid interfacial free energy was combined using the geometric mean (Equation 3.2) combining rule whereas the AB component are non-additive and was combined as in Equation 3.3 (Missirlis & Katsikogianni, 2007).

$$\gamma_{SL}^{LW} = \gamma_{S}^{LW} + \gamma_{L}^{LW} - 2\sqrt{\gamma_{S}^{LW}\gamma_{L}^{LW}} \qquad \textbf{Equation 3.2}$$

$$\gamma_{SL}^{AB} = 2\sqrt{\gamma_S^+\gamma_S^-} + \sqrt{\gamma_L^+\gamma_L^-} - \sqrt{\gamma_S^-\gamma_L^+} - \sqrt{\gamma_S^+\gamma_L^-} \qquad \textbf{Equation 3.3}$$

The total solid-liquid interfacial free energy was combined with the extended Young's equation to obtain Equation 3.4 which was used to calculate the surface free energy components of a solid surface $\gamma_S^{LW}\ \gamma_S^+\ \gamma_S^-$ (or protein conditioned surface or "protein film layer" $\gamma_P^{LW}\ \gamma_P^+\ \gamma_P^-$) by measuring the contact angles (θ) using three different probe liquids (two polar and one apolar) of known parameters $\gamma_L^{LW}\ \gamma_L^+\ \gamma_L^-$ [acquired from van Oss (1994)] and solving the three equations of three probe liquids simultaneously (Sharma & Rao, 2002).

$$(1+\cos\theta)\gamma_L^{TOTAL} = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\ \gamma_L^-} + \sqrt{\gamma_S^-\ \gamma_L^+}\right) \qquad \textbf{Equation 3.4}$$

Based on the calculated surface free energy components the absolute hydrophobicity of any given surface was determined. van Oss expressed the absolute hydrophobicity (ΔG_{SWS}) between surface molecules (S) immersed in water (W) using Equation 3.5**.** A surface is classified hydrophilic when ΔG_{SWS} is greater than zero or hydrophobic when ΔG_{SWS} is less than zero (van Oss, 1995).

$$\begin{aligned}\Delta G_{SWS} &= -2\gamma_{SWS} \\ &= -2\left[\left(\sqrt{\gamma_S^{LW}} - \sqrt{\gamma_W^{LW}}\right)2\right. \\ &\left. + 2\left(\sqrt{\gamma_S^+} - \sqrt{\gamma_W^+}\right)\left(\sqrt{\gamma_S^-} - \sqrt{\gamma_W^-}\right)\right]\end{aligned} \qquad \textbf{Equation 3.5}$$

3.3.5.3 Particle size distribution

Particle size distribution (PSD) measurements of the protein solutions and milk were made by using Zetasizer Nano ZS Malvern instrument (Malvern Instruments, UK) operated with Mastersizer 2000 software with a measurement range 0.6 nm to 6 µm.. The Nano ZS instrument incorporates non-invasive backscatter (NIBS^{TM}) optics, which is a technique that measures time-dependent fluctuations in the intensity of scattered light that

results from the Brownian motion of particles and relates this to the size of the particles. The scattered light was detected by means of a detector that converts the signal to a size distribution by intensity. Note BSA, TSB, CAS, WML and SML samples were diluted from water to obtain 1mg/ml solution.

3.3.5.4 Zeta potential measurement

Zeta potentials of the protein solutions and milk (1 mg/ml) were measured using a Zetasizer Nano ZS electrophoretic light scattering spectrophotometer (Malvern Instruments, UK). The measurements were based on electrophoretic mobility using the laser Doppler velocimetry (LDV) technique within a range of 5 nm to 10 µm. The LDV technique applies a voltage across a pair of electrodes placed at both ends of a cell containing the conditioning solution. The instrument calculates the zeta potential by determining the electrophoretic mobility and then applying the Henry's equation (Zetasizer Nano Series User Manual, Malvern Instruments, UK)

3.3.5.5 Atomic force microscopy

The AFM images of surfaces (conditioned and not conditioned) were obtained using a Brucker -Veeco Multimode 8 Nanoscope 5 (Brucker, Santa Barbara, CA, USA) operated at room temperature in air. The scans were carried out in tapping mode using an Arrow™ Non-contact / Tapping™ Reflex coating (NCR) probe (NanoWorld®, Switzerland) . The probes contained a rectangular cantilever made from monolithic silicon with a thickness, length and width of 4.6 µm, 160 µm and 45 µm respectively. The average resonant frequency of the cantilever was 285 kHz with a spring constant of 42 N/m.

During the tapping mode, images were captured digitally using the computer software connected to the AFM instrument. The surfaces were imaged in different scan sizes of 1 µm X 1 µm and 5 µm X 5 µm at scan speeds of 1 Hz. Image analysis was carried out using NanoScope Analysis software Version 1.40. Duplicate samples were made and representative images selected for discussion. All images were flattened using a third-order least squares polynomial fit using the Nanoscope Analysis software. This

function is known to remove any tilt to the images while preserving essential features of the surface such as protein molecules and steps in the surface (Gettens et al., 2005).

3.3.5.6 Calculations of total free energy of interaction between conditioning solutions and solid surfaces based on thermodynamic theory

According to the thermodynamic model, the process of protein adsorption is considered as creating a new interface: protein (P) - solid surface (S), by disruption of the two pre-existing interfaces [protein (P)-liquid (L) and solid (S)-liquid (L)]. The total free energy of interaction (ΔG_{PLS}^{TOTAL}) of a protein (P) to a solid surface (S) in a suspending liquid (L) can be calculated as the sum of the LW component (ΔG_{PLS}^{LW}) LW and the AB component (ΔG_{PLS}^{AB}) as in Equation 3.6.

$$\Delta \mathbf{G_{PLS}^{TOTAL}} = \Delta \mathbf{G_{PLS}^{LW}} + \Delta \mathbf{G_{PLS}^{AB}} \qquad \textbf{Equation 3.6}$$

where,

$$\Delta \mathbf{G_{PLS}^{LW}} = \left[\left(\sqrt{\gamma_P^{LW}} - \sqrt{\gamma_S^{LW}}\right)^2 - \left(\sqrt{\gamma_P^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2 - \left(\sqrt{\gamma_S^{LW}} - \sqrt{\gamma_L^{LW}}\right)^2\right] \qquad \textbf{Equation 3.7}$$

and

$$\Delta \mathbf{G_{PLS}^{AB}} = 2\left[\left(\sqrt{\gamma_P^+} - \sqrt{\gamma_S^+}\right)\left(\sqrt{\gamma_P^-} - \sqrt{\gamma_S^-}\right) - \left(\sqrt{\gamma_P^+} - \sqrt{\gamma_L^+}\right)\left(\sqrt{\gamma_P^-} - \sqrt{\gamma_L^-}\right) - \left(\sqrt{\gamma_S^+} - \sqrt{\gamma_L^+}\right)\left(\sqrt{\gamma_S^-} - \sqrt{\gamma_L^-}\right)\right] \qquad \textbf{Equation 3.8}$$

Adsorption or adhesion of protein is favored if ΔG_{PLS}^{TOTAL} is negative and unfavored if ΔG_{PLS}^{TOTAL} is positive as predicted by the second law of thermodynamics (van Oss, 1995).

3.3.5.7 Calculations of total free energy of interaction between conditioning solutions and solid surfaces based on XDLVO theory

In the classical DLVO theory, the total free energy of interaction is described as a balance of attractive Lifshitz–van der Waals force and repulsive or attractive electrostatic forces. Classical DLVO theory was "extended" by including short-range acid-base (AB) energy component and is expressed according to Equation 3.9 (van Oss, 1994; van Oss, 1995).

$$G_{PLS}^{XDLVO}(d) = G_{PLS}^{LW}(d) + G_{PLS}^{EL}(d) + G_{PLS}^{AB}(d) \qquad \textbf{Equation 3.9}$$

where G_{PLS}^{XDLVO} is the total interaction energy, G_{PLS}^{LW} is LW interaction energy component, G_{PLS}^{EL} is the EL interaction energy component and G_{PLS}^{AB} is the AB component of the interaction energy. The subscript P refers to protein, L refers to aqueous environment, and S refers to solid surface. In this study, we considered the proteins as perfectly smooth spheres and the solid surfaces as flat planes as assumed by the XDLVO theory (Brant & Childress, 2002; van Oss, 2006).

The individual components were calculated using Equation 3.10, Equation 3.11 and Equation 3.12.

$$G_{PLS}^{LW}(d) = -{}^{Ar}/_{6d} \qquad \textbf{Equation 3.10}$$

$$G_{PLS}^{EL}(d) = \pi\varepsilon R\left[2\Psi_P\Psi_S \ln\left(\frac{1+e^{-kd}}{1-e^{-kd}}\right) + (\Psi_P^2\Psi_S^2)\ln(1-e^{-2kd})\right] \qquad \textbf{Equation 3.11}$$

$$G_{PLS}^{AB}(d) = 2\pi r\lambda\Delta G_{PLS}^{AB}\left[\frac{d_0-d}{\lambda}\right] \qquad \textbf{Equation 3.12}$$

where, A is the Hamaker constant, calculated using, $A = -12\pi d_0^2 \Delta G_{PLS}^{LW}$; d = the separation distance between the adhering particle and the solid surface; d_0 = 0.158nm the minimum equilibrium separation distance (considered as the distance between the outer electron shells of adjoining non-covalently interacting molecules) ; r = the radius of the protein (meter); ε = 80 *8.854 *10^{-12} dielectric permittivity of water (C^2 J^{-1} m^{-1}); Ψ_P , Ψ_S = protein and solid surface zeta-potential (millivolt); k = inverse Debye length calculated as k= 0.328*$10^{10}$$(I)^{1/2}$($meter^{-1}$), (I) is the ionic strength of the solution; λ = correlation length of molecules in a liquid medium (0.6 nm); ΔG_{PLS}^{LW}= LW component of the total free energy of adhesion (mJ/m^2); ΔG_{PLS}^{AB} = AB component of the total free energy of adhesion (mJ/m^2).

From a thermodynamic outlook, adhesion or attraction between two interacting surfaces occurs when the total energy G_{PLS}^{XDLVO} is negative, and repulsion occurs when G_{PLS}^{XDLVO} is positive.

3.4 Results and Discussions

3.4.1 Amount of protein adsorbed on mica and polystyrene surfaces

The amount of proteins adsorbed from conditioning solutions on mica and polystyrene at room (25°C) and refrigerated (4°C) temperatures with adsorption times ranging between 15 min to 24 h are shown in Figure 3.2 and Figure 3.3, respectively, for mica and polystyrene coupons. Figure 3.2 (a - e) shows the amount of protein adsorbed on mica and Figure 3.3 (a – e) displays the amount of protein adsorbed on polystyrene from aqueous solutions of BSA, TSB, CAS (of 33 mg/ml concentration), WML and SML. The SAS analysis showing the influence of the time on the amount of protein adsorbed on mica and polystyrene at room and refrigerated conditions are presented in Table 3.2 and Table 3.3, respectively.

The amounts of BSA adsorbed onto mica increased significantly ($p<0.001$), with increase in incubation time from 15 min to 24 h. The amount of BSA adsorbed at room temperature was 1.74 $\mu g/cm^2$ at 15 min which increased to 2.53 $\mu g/cm^2$ after 24 h. Similarly in case of TSB (1.13 to 1.87 $\mu g/cm^2$) and SML (1.45-1.91 $\mu g/cm^2$), the amount

adsorbed on mica increased ($P<0.05$) with respect to time (15 minutes and 24 hours). However, in case of CAS (1.97.to 2.86 $\mu g/cm^2$) and WML (1.32 to 1.82 $\mu g/cm^2$) the amount adsorbed on mica did not show statistical significance ($p>0.05$) with respect to time (15min and 24 h).

In case of polystyrene and the amount of BSA (0.16 to 0.32 $\mu g/cm^2$), and SML (0.32 to 0.35 $\mu g/cm^2$) adsorbed increased significantly ($p<0.001$). An increase was also seen ($p<0.05$) for TSB (0.15-0.20 $\mu g/cm^2$), CAS (0.32 to 0.44 $\mu g/cm^2$) and WML (0.21-0.22 $\mu g/cm^2$), with respect to time (1 and 24 h) at room temperature.

At refrigerated temperatures the amount of CAS, WML and SML adsorbed on mica showed no significant difference ($p>0.05$) with respect to time of adsorption. The same trend was also seen with polystyrene with BSA, TSB, CAS, WML except SML which showed significance ($p<0.05$).

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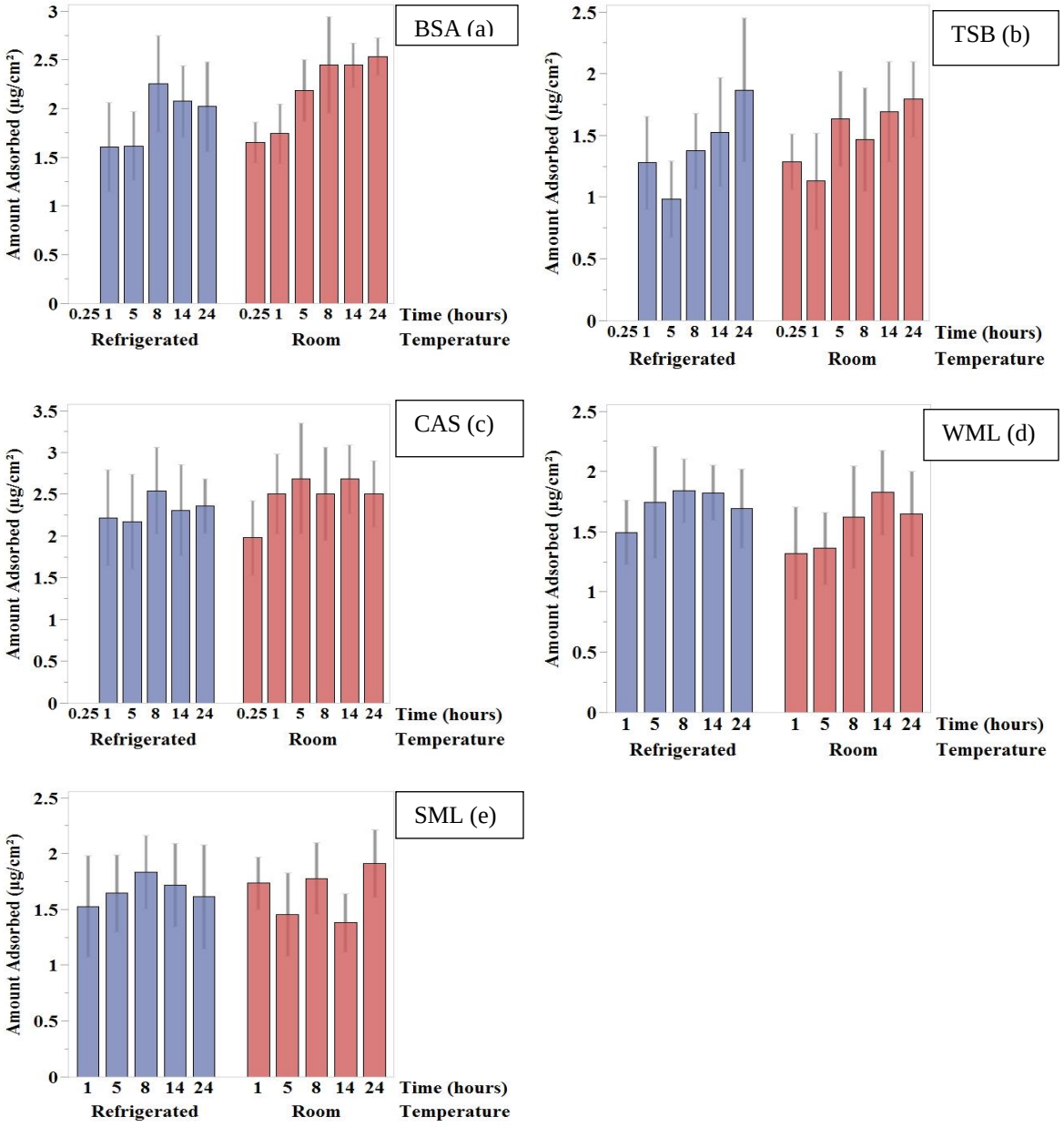


Figure 3.2: Amount of protein adsorbed to mica surfaces from BSA (a), TSB (b), CAS (c), WML (d) and SML (e)

*The error bars represents standard deviation

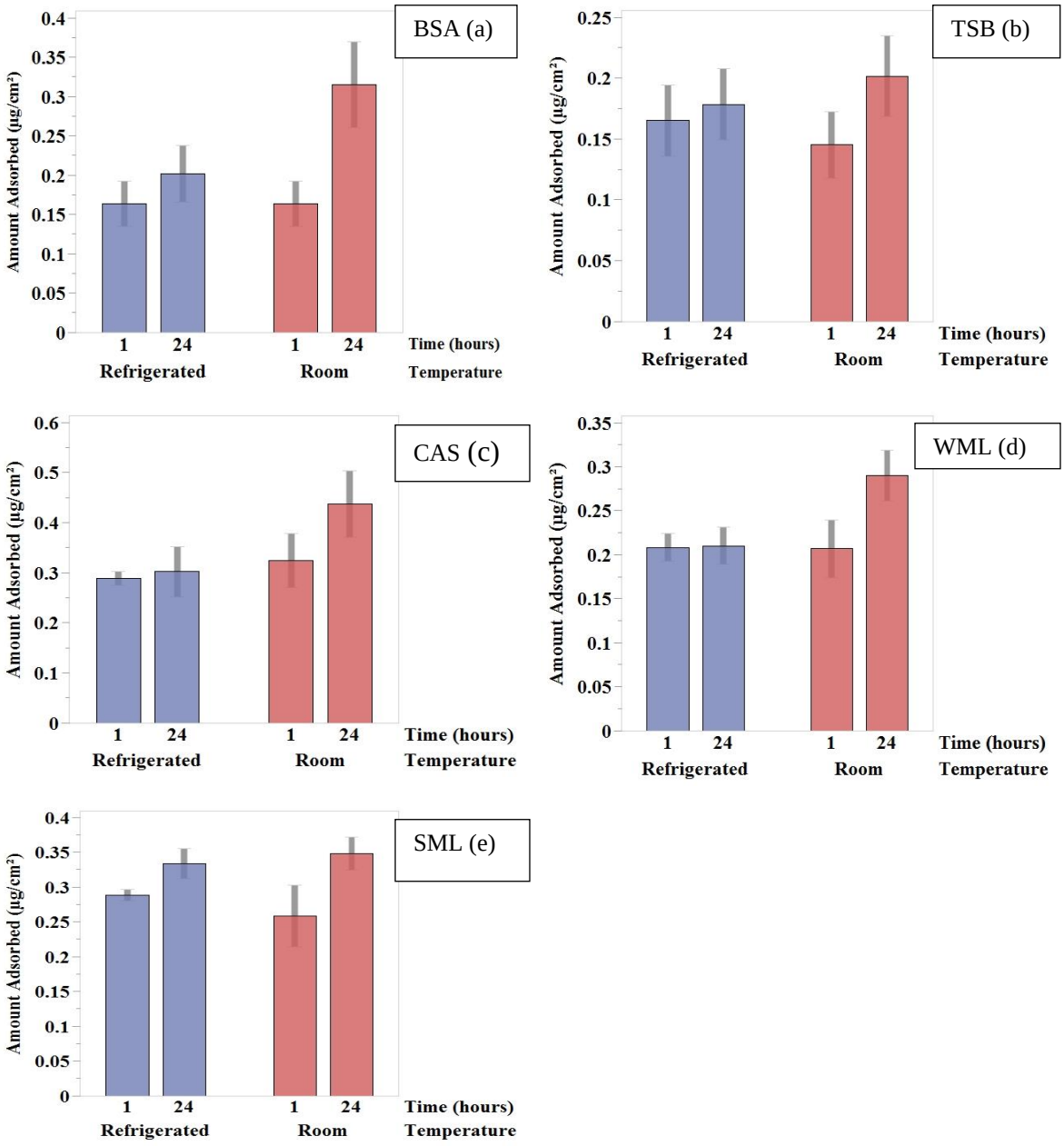


Figure 3.3: Amount of protein adsorbed to polystyrene surfaces from BSA (a), TSB (b), CAS (c), WML (d) and SML (e)

*The error bars represents standard deviation

Table 3.2: SAS analysis (ANOVA and Duncan's grouping of means) showing the influence of time on the amount of protein adsorbed on mica and polystyrene surfaces at room temperatures

	Time (hours)	
	Mica	**PS**
BSA	***	***
TSB	**	**
CAS	Ns	**
WML	Ns	**
SML	**	***

***** = p<0.001, ** = p<0.05, ns= not significant (p>0.05).**

Duncan's grouping of means at room temperature

	Mica						**PS**	
	0.25	**1**	**5**	**8**	**14**	**24**	**1**	**24**
BSA	1.65 B	1.74 B	2.18 A	2.44 A	2.44 A	2.53 A	0.16 B	0.31 A
TSB	1.28 BC	1.12 C	1.63 AB	1.46 ABC	1.69 AB	1.79 A	0.14 B	0.20 A
CAS	1.97 B	2.50 AB	2.68 A	2.50 AB	2.68 A	2.50 AB	0.32 B	0. 43 A
WML	-	1.32 B	1.36 B	1.62 AB	1.82 A	1.64 AB	0.29 B	0. 20 A
SML	-	1.73 ABC	1.45 BC	1.77 AB	1.38 C	1.91 A	0.25 B	0. 34 A

Means with the same letter are not significantly different

Table 3.3: SAS analysis (ANOVA and Duncan's grouping of means) influence of time on the amount of protein adsorbed on mica and polystyrene surfaces at refrigerated temperatures

	Time (hours)	
	Mica	**PS**
BSA	**	ns
TSB	**	ns
CAS	Ns	ns
WML	Ns	ns
SML	Ns	**

***** = p<0.001, ** = p<0.05, ns= not significant (p>0.05).**

Duncan's grouping of means at refrigerated temperatures

	Mica					**PS**	
	1	**5**	**8**	**14**	**24**	**1**	**24**
BSA	1.6 B	1.61 B	2.25 A	2.07 AB	2.01 AB	0.16 A	0.20 A
TSB	1.27 BC	0.98 C	1.37 BC	1.52 AB	1.86 A	0.16 A	0.17 A
CAS	2.21 A	2.16 A	2.54 A	2.3 A	2.35 A	0.28 A	0.30 A
WML	1.49 A	1.74 A	1.84 A	1.82 A	1.69 A	0.20 A	0.21 A
SML	1.52 A	1.64 A	1.83 A	1.71 A	1.61 A	0.28 B	0.33 A

Means with the same letter are not significantly different

The amount of BSA adsorbed onto mica and polystyrene surfaces from degassed solution of BSA is shown in Table 3.4. Adsorption was carried out for 1 h. It is clear that the amount BSA adsorbed was higher ($P<0.05$) on both surfaces when the solution was degassed (Table 3.4). Previous studies have shown that nano-bubbles inhibit protein the adsorption of BSA on mica and HOPG (highly oriented pyrolytic graphite) surfaces (Wu et al., 2006). This suggests that the existence of nano-bubbles in the conditioning solutions studied may reduce the amount of protein adsorbed on mica and polystyrene surfaces.

Table 3.4: Amount of protein adsorbed on to mica and polystyrene surfaces from BSA solutions before and after degassing

	Amount adsorbed (µg/cm^2)	
Surface	BSA	Degassed BSA
Mica	1.74±0.40	2.32±0.07
Polystyrene	0.16±0.03	0.44±0.08

BSA adsorption to hydrophilic and hydrophobic surfaces has been quantified widely by a number of experimental techniques. The broad range of literature values (from 0.03 µg/cm^2 to 0.29 µg /cm^2) for the amount of BSA adsorbed at room temperature on hydrophilic and hydrophobic surfaces found in the literature is almost certainly due to differences in experimental conditions (Table 2.1). Most of these studies are for pH~7 (buffer) and lower protein solution concentrations, whereas our results are for unbuffered BSA aqueous solutions and relatively higher protein solution concentrations which can explain the higher amounts of BSA on both hydrophilic and hydrophobic surfaces. Also in most cases, the equilibrium seems to be reached in about an hour on both mica and polystyrene surfaces. The amount of BSA adsorbed on to mica was higher than on polystyrene surfaces. Similar trends were also observed for other types of conditioning solutions that were studied. One reason is that a strong hydrophobic interaction may result in an unfolding and spreading of the protein on hydrophobic surface and thus result in less adsorbed amount of protein. Roach et al. (2006) reported that BSA and bovine fibrinogen had lower adsorption amounts but showed higher affinity on hydrophobic than on hydrophilic surfaces at room temperatures. They indicated that the lower amounts of

proteins on hydrophobic surface may be due to greater deformation of adsorbing protein molecules on these surfaces. The results of this study also show that higher amounts ($P<0.05$) of CAS were adsorbed on both mica and polystyrene when compared to BSA TSB, WML and SML. The higher adsorbed amount of CAS to both surfaces may be due to the presence of sodium ion which can modify the protein structure and properties and affect the adsorption dynamics (Lee et al., 2004). In addition, TSB, WML and SML contain more than one protein and hence, this implies competitive protein adsorption, where different proteins with different characteristics will compete with each other for the available space on the surface and as result may lead to lower amount of protein adsorbed on the surface (Holmberg & Hou, 2009).

Upon adsorption the structure of a protein layer can be a densely or loosely packed monolayer or even a multilayer (Rabe et al., 2011). In case of BSA because of the its ellipsoidal shape, two types of orientations on a solid surface namely side-on (if major axis is parallel) and end-on adsorption (if major axis is perpendicular) have been proposed. Fitzpatrick et al. (1992) determined the amount of BSA adsorbed on mica surfaces using XPS to be 0.57 ±0.1 µg/cm^2 under the condition where the mica surface was immersed in BSA at concentration of 0.005 mg/ml for 19 h at room temperature conditions. They concluded from their results that end-on adsorption of BSA molecules prevailed under such experimental conditions. On the contrary Gallinet and Gauthiermanuel (1992) measured the thickness of BSA layer adsorbed on mica surface and concluded that BSA molecules were adsorbed side on. In this case mica surfaces were exposed to a BSA solution at a concentration of 0.04 mg/ml for a period of 45 min. Terashima and Tsuji (2003) studied the adsorption of BSA (0.002 mg/ml) onto mica surfaces by a direct weighing technique. Initially, BSA molecules adsorbed side-on directly onto mica surface to form monolayer. The amount of BSA adsorbed was reported to be 0.201 µg/cm^2. However, multilayer adsorption of BSA molecules was observed as the adsorption continued for a longer period (more than 16 hours). The amount of BSA adsorbed was reported to be 0.753 µg/cm^2. In this study, the amount of BSA adsorbed onto mica surface varied from 1.6 to 2.5 µg/cm^2 at room and refrigerated temperatures

for adsorption times ranging between 1 to 24 hours and therefore a multilayer adsorption on mica is suspected.

On polystyrene the amount of BSA adsorbed ranged from 0.16 to 0.3 µg/cm^2 and was expected to be a closely packed monolayer. In a previous study it was observed that BSA (1mg/ml) formed a monolayer with a side-on orientation on PS (Reimhult et al., 2008). The amount of BSA adsorbed was found to be 0.329 µg/cm^2. A monolayer coverage was also reported by Jeyachandran et al. (2009) when BSA was adsorbed onto polystyrene surfaces from solutions of high concentrations (10 mg/ml, PBS pH 7.4) was adsorbed.

3.4.2 Contact angles and surface thermodynamic properties of proteins and milk

The surface thermodynamic properties for the proteins and milk were derived from the plateau contact angles measurements of the "hydrated film layers" (Table 3.5). The water contact angles of BSA and TSB were lower than CAS and milk. The LW component of the surface energy ranged between 35 to 42 mJ/m^2 and the total surface energy ranged between 47 – 52 mJ/ m^2. The electron-donor component of the surface energy was much greater than the electron-acceptor component of the surface energy which suggest that the conditioning solutions have a strongly monopolar surface or that they favor electron-donating or Lewis base properties. This is typical for hydrated proteins, where the high electron donicity results from the mostly negative surface charge and the prevalence of oxygen groups in exposed surface groups (van Oss et al., 1997). Similar results were obtained by van Oss for hydrated human serum albumin, human fibrinogen and lysozyme (van Oss, 2006). Furthermore, the values of surface energy and its component were similar to those reported by (Aasim, 2010) for hydrated BSA layers who also calculated the surface energy components using the VCG approach.

The absolute hydrophobicity (ΔG_{SWS}) of protein and milk was derived based on the surface energy components as shown in Table 3.5. The positive sign indicates that the surface is hydrophilic, whereas the negative sign shows that the surface is hydrophobic. In addition, positive hydrophobicity values also indicate the stability in water and not easy to

aggregate, while the negative value suggest thermodynamic instability in water. Based on the values given in Table 3.5, BSA and TSB had a hydrophilic surface whereas CAS, WML and SML had a hydrophobic surface. Moreover, the, ΔG_{SWS}, of WML was more negative than that of SML, suggesting that the WML had more hydrophobic surface. Likewise, the, ΔG_{SWS} of TSB was more positive than that of BSA, suggesting that the TSB had more hydrophilic surface. There is lack of information concerning the hydrophobicity values of TSB, CAS, WML and SML. However, several earlier studies have reported the ΔG_{SWS} values of BSA. Ding et al. (2013) reported positive absolute hydrophobicity values which became significantly lower with the increase of ionic strength. Kim and Hoek (2007) and Subhi et al. (2012) also reported positive values hydrophobicity of BSA indicating that BSA was thermodynamically stable in the solution.

Table 3.5: Contact angles and surface thermodynamic properties of proteins and milk

Proteins and Milk	Contact Angles (°)			Surface Energy Components (mJ/m^2)					Absolute hydrophobicity (mJ/m^2)
	θ_{Water}	$\theta_{Bromonaphthalene}$	$\theta_{Formamide}$	γ^{LW}	γ^{+}	γ^{-}	γ^{AB}	γ^{TOTAL}	ΔG_{SWS}
BSA	34.65 ± 4.40	37.29 ± 4.19	26.32 ± 2.40	35.79	1.65	39.96	16.25	52.04	15.7
TSB	24.96 ± 3.09	32.41 ± 1.71	23.31 ± 1.71	37.75	1.13	48.97	14.89	52.64	26.71
CAS	56.54 ± 3.06	32.49 ± 3.69	31.85 ± 2.15	37.72	2.12	16.37	11.79	49.51	-18.76
WML	71.21 ± 2.93	20.2 ± 1.38	39.49 ± 2.39	41.71	1.41	5.63	5.63	47.34	-47.78
SML	67.99 ± 1.49	17.94 ± 2.14	33.72 ± 1.07	42.27	1.92	6.3	6.96	49.23	-43.91

3.4.3 Contact angles and surface thermodynamic properties of mica and polystyrene surfaces

Table 3.6 shows the contact angle and surface thermodynamic properties (surface energy components and absolute hydrophobicity) of conditioned and blank mica and polystyrene surfaces. Water contact angles less than 90° correspond to high wettability, while contact angles greater than 90° correspond to low wettability (Rios et al., 2007). The water contact angle blank mica surface is 14°, indicating that the surface is very hydrophilic and almost completely wettable whereas the water contact angle of polystyrene is 91° indicating that the surface was moving toward more hydrophobic. Previously reported water contact angles for freshly cleaved mica surfaces ranged between 0 to 14° (Feng et al., 1996; Ong et al., 1999; Yang et al., 2007). For polystyrene surfaces contact angles ranging between 88 -90°, has been reported. (Detry et al., 2007; Husmark & Rönner, 1993). Conditioning of mica and polystyrene surfaces resulted in changes in the water contact angle values. The conditioning film decreased the wettability of mica and increased the wettability of polystyrene respectively for the different types of conditioning solutions used in the study. In case of mica, the highest increase in water contact angle values was observed in the case of mica conditioned with WML followed by CAS SML, BSA and TSB. The water contact angle values of polystyrene conditioned with BSA, TSB, CAS, WML and SML ranged between 78-83° which was lower than the blank polystyrene surfaces suggesting that the conditioned polystyrene became more wettable.

With regards to surface thermodynamic properties the calculated surface energy and its components, the electron donor component (γ^-) was observed to be greater than electron acceptor component ($\gamma+$) for blank mica and polystyrene surfaces which means that mica and polystyrene in water were negatively charged. Also, blank mica was more electron donating than blank polystyrene surfaces. The total surface energy of blank mica (53.29 mJ/m^2) was higher than blank polystyrene (42.46 mJ/m^2) surfaces. Previously reported total surface energy values calculated using the VCG approach ranged between 43.2 to 64.09 mJ/m^2 (Ong et al., 1999; van Oss & Giese, 1995) for mica and was approximately 42 - 46 mJ/m^2 for polystyrene (Mafu et al., 2011; van Oss, 1991).

Upon conditioning, the Lifshitz -van der Waals (LW) component (which is considered to be always attractive at shorter separation distances between two neutral stable molecules) and the total surface energy values showed very little change. The only parameters affected by the conditioning treatment were the electron donor and acceptor components of the surface free energy which had a tremendous impact on the resulting hydrophobicity of the coated surfaces compared to blank mica surface. Similar trends with respect to surface energy components have been observed for stainless steel surfaces conditioned with milk , meat, fish extract, cottage cheese cholesterol, fish oil, mixed fatty acids, BSA, casein and fish peptone when compared to clean stainless steel surfaces (Jullien et al., 2008; Whitehead et al., 2009).

Based on the surface free energy components, absolute hydrophobicity (ΔG_{SWS}) of blank and conditioned mica and polystyrene surfaces was calculated and the compiled data are also included in Table 3.6. As expected, blank mica surface was hydrophilic and blank polystyrene surface was hydrophobic, as the calculated ΔG_{SLS} was positive in case of mica and negative for polystyrene surfaces. After conditioning of mica with BSA CAS, WML and SML, the calculated ΔG_{SLS} was found to be negative indicating that the surfaces became hydrophobic except in the case of mica conditioned with TSB which retained a hydrophilic character. For polystyrene conditioned surfaces, the calculated ΔG_{SLS} were negative in all cases demonstrating a strong hydrophobic surface.

Pervious works have reported the absolute hydrophobicity of proteins and organic material adsorbed on solid surfaces in dehydrated states and the ΔG_{SWS} of stainless steel coupons immersed in water has been found to be positive showing a hydrophilic character. However, upon immersion in different milk samples (UHT, whole milk, chocolate milk and infant formula) negative values have been observed (Bernardes et al., 2012). Similarly, changes in the absolute hydrophobicity of stainless steel surfaces were observed by Whitehead et al. (2009), after conditioning with various other complex organic food soils such as (milk, carbohydrates etc) in comparison with clean stainless steel surfaces. In the case of stainless steel conditioned with BSA with a (concentration range of 0.1% to 10%), a surface hydrophobicity of approximately 80 mJ/m^2 was reported. In another study, hydrophilic glass became hydrophobic after covalent

modification of glass with casein (Han et al., 2011). Szlavik et al. (2012) observed that hydrophilic glass when coated with beef extract, casein, un-homogenized milk became more hydrophilic and when coated with homogenized milk showed similar value as glass. In general changes in the absolute hydrophobicity values were seen upon conditioning of mica and polystyrene surfaces.

Table 3.6: Contact angles and surface thermodynamic properties of blank and conditioned mica and polystyrene surfaces

Surface	Conditioning solution	Contact Angles (°)			Surface Energy Components (mJ/m^2)					Absolute hydrophobicity (mJ/m^2)
		θ_{Water}	$\theta_{Bromonaphthalene}$	$\theta_{Formamide}$	γ^{LW}	γ^{+}	γ^{-}	γ^{AB}	γ^{TOTAL}	ΔG_{SWS}
Mica	BSA	47.74 ± 1.52	16.87 ± 0.89	31.55 ± 3.66	42.51	0.66	27.18	8.49	51.0	-4.08
	TSB	39.56 ± 3.95	13.09 ± 4.29	23.19 ± 1.70	43.25	0.88	33.05	10.8	54.05	4.22
	CAS	61.97 ± 1.63	21.43 ± 2.69	20.16 ± 2.14	41.38	3.57	7.61	10.42	51.8	-35.2
	WML	66.86 ± 1.74	16.23 ± 2.89	28.28 ± 0.90	42.65	2.6	5.71	7.71	50.35	-43.51
	SML	59.68 ± 2.80	22.22 ± 1.73	17.57 ± 2.12	41.16	3.67	9.00	11.49	52.65	-31.81
	Water	14.68 ±1.99	14.33 ±2.59	18.36 ±1.45	43.03	0.48	55.13	10.26	53.29	34.27
PS	BSA	82.01 ± 1.45	10.95 ± 0.74	62.51 ± 0.97	43.59	0.18	6.39	-2.14	41.45	-62.67
	TSB	83.61 ± 0.61	11.95 ± 0.91	61.86 ± 2.25	43.44	0.08	4.86	-1.27	42.17	-68.15
	CAS	81.37 ± 1.81	14.19 ±0.99	58.83 ± 2.49	43.05	0.004	5.24	-0.31	42.74	-63.66
	WML	79.51 ± 2.66	15.4 ± 0.73	53.51 ± 1.77	42.82	0.17	3.48	1.52	44.34	-66.18
	SML	78.77 ± 3.20	14.97 ± 0.71	51.15 ± 0.73	42.91	0.26	4.19	2.08	44.98	-61.62
	Water	91.23 ± 0.67	8.01 ± 0.55	67.06 ± 0.92	43.97	0.27	2.09	-1.51	42.46	-88.04

3.4.4 Particle size distribution and zeta potential measurements

The particle size distribution profile of the proteins and milk solutions was measured using dynamic light scattering (DLS) technique whereas the zeta potential was derived from electrophoretic mobility measurements.

DLS measurements showed a multimodal distribution profile for BSA and TSB and monomodal distribution profiles for CAS, WML and SML solutions. In all cases, poly-disperse (the level of homogeneity of the sizes of the particles) profiles and heterogeneous distribution of distinct sizes were found. Table 3.7 summarizes the particle sizes (hydrodynamic radius) of the proteins and milk. The size distribution profile curves for BSA showed two peaks with a mean radius of 8.87 nm and 49.97nm respectively which was smaller than its average radius of 3.3 to 4.3 nm reported in the literature (Jachimska et al., 2008). The particle size distribution analysis of TSB revealed two mean peaks at around 30 nm and 115 nm. For CAS, a mean radius around 110 nm was observed which was higher than those found in the literature. Using static light scattering Lucey et al. (2000) showed that the radius of sodium caseinate varied between 20 and 70 nm. Other studies have also observed that sodium caseinate solutions were found to contain a small weight fraction of particles with a radius of about 75-80 nm (Chu et al., 1995; Nash et al., 2002). In milk, caseins are the main proteins and present in aggregated states called "casein micelles", with an average radius of 75-100 nm (Bouzid et al., 2008; Dalgleish & Corredig, 2012). In this study the particle size distribution of WML and SML samples showed a peak radius of 109 nm and 78 nm respectively. In conclusion, the DLS results of our solutions revealed that the solutions were highly polydisperse and a large fraction of particles were present under the form of aggregates.

The zeta potential indicates the charge on the surface and the potential stability (i.e., likelihood of aggregation) of a suspension. The zeta potential of BSA, TSB, CAS, WML and SML was negatively charged (Table 3.7). The zeta potential values of BSA obtained in our experiments are comparable to those obtained in the literature (Ding et al., 2013; Hong et al., 2013; Kim & Hoek, 2007; Xiao et al., 2011). The negative charge of BSA is attributed to the carboxylate and other acid moieties. The zeta potential of

CAS, WML and skim milk was close to each other. This is in accordance with the results obtained with zeta potential of caseins, casein micelles and skimmed milk at pH 7.0 (Bouzid et al., 2008; Malvern, 2009). In general, a potential between +30 mV and -30 mV is regarded as instable (aggregated suspensions), whereas values lower than -30 mV or higher than +30 mV represent "homogenous" suspensions (Gauggel et al., 2012). Therefore, the zeta potential values of the proteins and milk solutions showed aggregated states in solution.

Table 3.7: Mean hydrodynamic radius at the peaks and zeta potential of proteins and milk solutions.

Proteins and milk	Hydrodynamic Radius (nm)		Zeta Potential (mV)
	Peak 1	Peak 2	ζ_P
BSA	8.87±0.90	49.97±0.72	-11.84±0.37
TSB	30.56±3.56	115.0±1.56	-20.97±1.49
CAS	110.0±4.35	-	-26.1±0.20
WML	109.70±1.1	-	-19.03±0.67
SML	78.83±1.17	-	-19.46±0.61

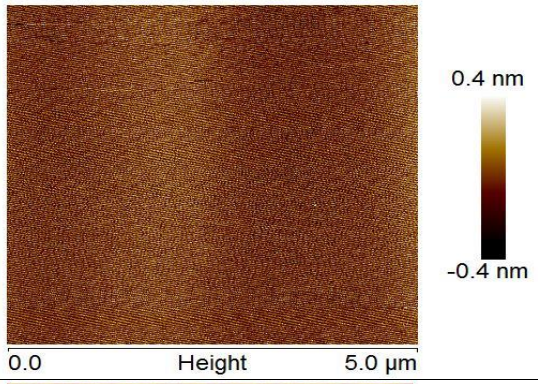
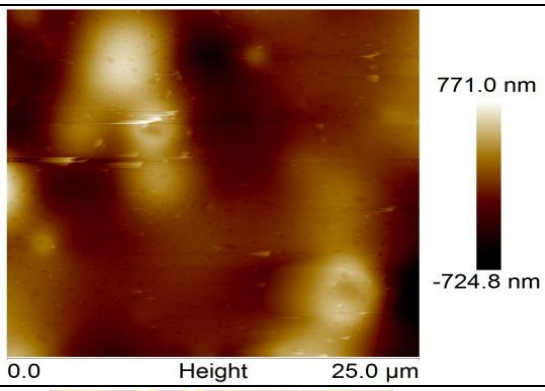
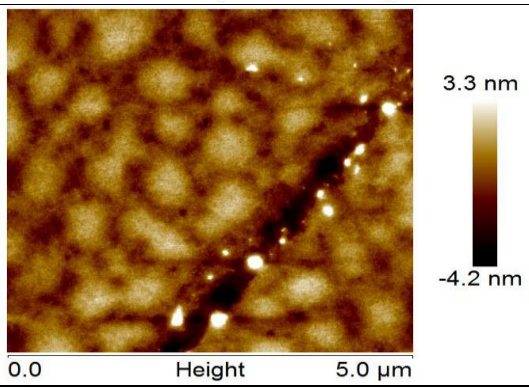
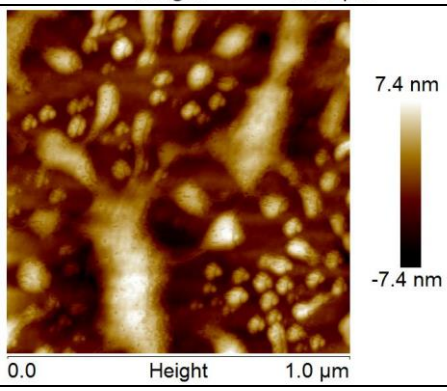
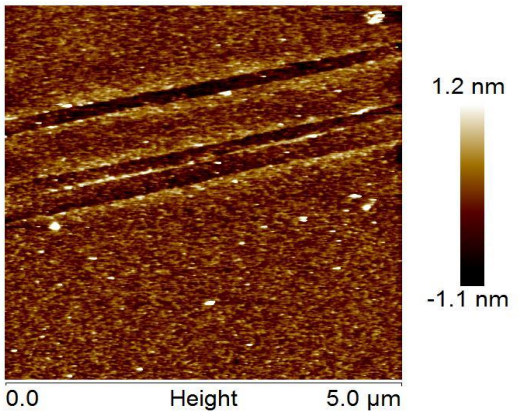
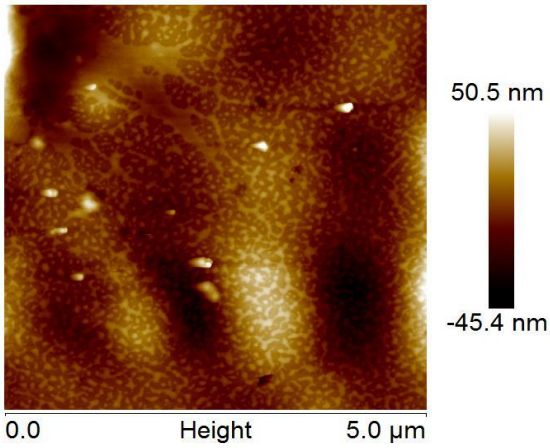
3.4.5 Atomic Force Microscopy

Atomic force microscopy (AFM) has been extensively used to visualize and characterize interfacial protein films, fouling deposits and solid surfaces parameters as it can provide information regarding molecular interaction, surface topography, and deposit characteristics. Handojo et al. (2009) used AFM to determine the film thickness and adhesive strength of chocolate milk, whole milk, 2% fat milk and skimmed milk residues on glass following washing. AFM results showed that whole and chocolate milk presented larger residue film thicknesses and had significantly higher adhesive strengths as compared to glass. Goode et al. (2013b) used AFM to study the effect of temperature on adhesion forces between microparticles surfaces (SS and Flouro-coated glass) and model foods (whey protein and sugar). At higher temperatures a significant increase in the adhesion force to both types of micro particles in the case of whey protein was observed. Elofsson et al. (1997) used AFM to study the coverage of β-lactoglobulin and

whey protein concentrate to mica sheets at different surface loadings in the range of 0.03 to 3 μg/cm^2. The use of AFM enabled the authors to characterize the different states of protein aggregation at the sub-micrometer level. In our study conditioning solutions adsorbed to mica and PS surfaces from bulk protein concentration of 33 mg/ml was imaged using AFM. Figure 3.4 shows the AFM images of before (bare) and after conditioning of mica and polystyrene surfaces with BSA, CAS, WML and SML solutions. Before conditioning adsorption, the mica surfaces were extremely smooth with almost no surface features whereas polystyrene surfaces showed undulations. Conditioning of BSA, milk and skim milk on both mica and polystyrene surfaces resulted in closely packed repeated structures with distinct domains at the surface. These domains were approximately circular in outline in case of mica, except with CAS which formed a thick layer on mica surface. On polystyrene, the conditioning solutions formed either dots or stripes connected to each other. Browne et al. (2004) used AFM to image albumin onto polystyrene surfaces from 0.1, 0.05 and 0.01 mg/ml solution concentrations and observed "peak and ridge' topography.

Quantitative analyses were performed using section analysis tool to obtain the diameter of the circular entities (mica) and inter distance between stripes (polystyrene). The results are shown in Table 3.8. The size of the deposit measured showed the conditioning solution, adsorbed in the form of aggregates onto both mica polystyrene surfaces. In case of mica, BSA aggregates were found to have a higher radius in comparison to skim and whole milk. CAS was found to be adsorbed in thick layer rather than circular or patchy entities and hence the radius could not be obtained. In case of polystyrene, BSA, WML and CAS showed strip like patterns whereas SML showed dot like patterns. The measured inter-distance for SML was higher than BSA and WML. The inter-distance of stripes observed in case of CAS was highly variable. Barnes et al. (2001) used AFM to image skim milk on stainless steel surfaces and reported approximately circular structures which occurred both individually and as clustered deposits which had highly variable dimensions. In another study, AFM analysis of stainless steel coupons coated with fish extract and 1:7 diluted TSB revealed that fish extract contained spherical structures of (approximately 30 nm in diameter), the 1:7 diluted TSB-coated surface had

a sponge-like structure with nanometer-sized holes (Bernbom et al., 2006). Protein aggregates on hydrophobic polymer surfaces using high concentrations of protein solution (0.1-10 mg/ml) have been observed using AFM (Holmberg & Hou, 2009). The roughness analysis tool was used to characterize the roughness R_q (root mean square average of height deviations taken from the mean image data plane). Verran et al. (2000) characterized the surface roughness and topographies of new and worn stainless steel surfaces by AFM and found similar roughness values for both surfaces, although the topography was clearly different. In our study, the roughness (R_q) values of bare and conditioned slides indicated that the surface roughness of both surfaces changed with the adsorption of conditioning films and showed no uniformity in the roughness values because of the heterogeneous nature of the conditioning films formed on the surface.. Similar results were observed in the case of R_{max}, which shows the difference in height between the highest and lowest points on the cross-sectional profile relative to the center line (not the roughness curve) over the length. We also observed the presence of nanobubbles on mica conditioned surfaces which have been reported to influence the adsorption of BSA on mica surfaces (Wu et al., 2006).

	Mica	Polystyrene
Bare		
BSA		
CAS		

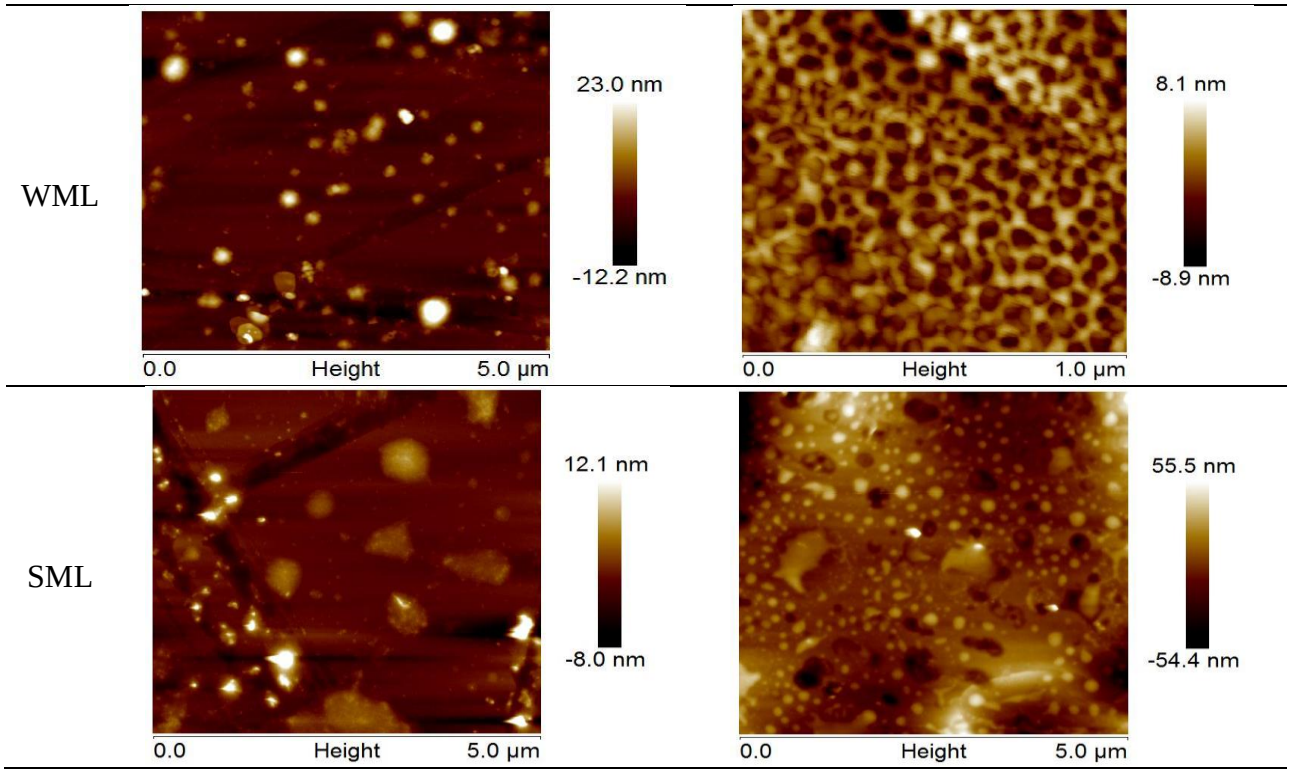


Figure 3.4: Tapping mode 2-dimensional AFM images of bare and conditioned mica and polystyrene surfaces

Table 3.8: AFM analysis

	Radius (µm)	R_q (nm)	R_{max} (nm)
Mica - Bare	-	0.11±0.03	0.32±0.04
Mica - BSA	14000 – 26000	1.04±0.01	1.28±0.12
Mica - CAS	-	0.30±0.01	1.33±0.36
Mica - WML	400 – 600	3.84±0.08	14.23±1.10
Mica - SML	7000 -12000	2.35±0.08	4.80±1.12
PS-Bare	-	14.75±0.78	28.67±2.68
PS - BSA	69 – 119	2.44±0.14	6.28±1.43
PS - CAS	-	15.50±4.95	13.33±2.35
PS- WML	57-361	2.39±0.22	6.18±0.69
PS - SML	282-357	12.15±2.47	18.28±2.75

3.4.6 Total free energy of interaction between conditioning solutions and solid surfaces

The total free energy of interaction represents the interaction energy per unit area of two planar surfaces brought into contact with each other. In this study, conditioning solutions which were either proteins (BSA and CAS) or solutions mainly containing proteins (TSB, WML and SML) were characterized with respect to surface thermodynamic properties. Although thermodynamic surface properties such as absolute hydrophobicity may provide some qualitative insight into potential interactions between solid surfaces and proteins in the conditioning solutions, it is unable to provide a quantitative assessment of the adhesive energy between the two surfaces. A quantitative assessment could be provided through the quantification of the total free energy of interaction per unit area between two surfaces (Brant & Childress, 2002). We used the thermodynamic and XDLVO theories to evaluate and quantity the interaction between proteins from conditioning solutions (BSA, TSB, CAS, WML and SML) and blank surfaces (mica and polystyrene). From thermodynamic prediction point of view the adhesion is favourable only when the total energy of interaction is negative. The values contained in Table 3.9 and Table 3.10 are valid at the shortest distance of approximation (0.157 nm) which may be considered as the distance between the outer electron shells (van der Waals boundaries) of adjoining non-covalently interacting molecules (van Oss et al., 1999).

3.4.6.1 Calculations based on the thermodynamic theory

Table 3.9 provides the values of the total free energy of interaction (ΔG_{PLS}^{TOTAL}), also called the Gibbs adhesion energy, between proteins in the conditioning solutions and solid surfaces calculated using the thermodynamic approach. The total free energy of interaction of adhesion between proteins with blank surfaces of mica and polystyrene were calculated. The values of apolar (ΔG_{PLS}^{LW}), acid/base (ΔG_{PLS}^{AB}) components are also shown. The ΔG_{PLS}^{TOTAL} were observed to be:

i. positive for BSA, TSB and CAS and negative for WML and SML - mica interaction combinations. This indicated that BSA, TSB CAS showed favorable whereas, WML and SML showed favorable adhesion to mica surfaces.

ii. negative for all proteins – polystyrene interaction combination, demonstrating that all proteins favored adhesion to polystyrene surfaces.

Further, in order to compare the relative strengths of the LW and AB interactions ΔG_{PLS}^{TOTAL} was broken into components apolar (LW) and polar (AB) components. LW is the sum of non-polar interactions, including orienting dipole–dipole interactions, orienting dipole-induced dipole (or induction) interactions, and fluctuating dipole-induced dipole (or dispersion) interactions (van Oss, 2006). Only the dispersion interactions have a considerable impact between macroscopic bodies, especially in aqueous media (van Oss, 2006) and for this reason LW interactions are mainly determined by London-dispersion forces. In this study, the values of the LW components did not differ appreciably for both mica and polystyrene conditioned surfaces while, ΔG_{PLS}^{AB} presented a high degree of variability and therefore controlled the overall adsorption. The ΔG_{PLS}^{AB} values for mica conditioned with CAS, WML and SML and polystyrene conditioned with all solutions were negative, leading to overall ΔG^{AB} values that were negative, thus favoring protein adsorption. For mica conditioned with BSA and TSB the increased polar interactions (i.e., positive values ofΔG_{PLS}^{AB}) caused the overall system to be unfavorable for protein adsorption. The ΔG_{PLS}^{AB} components (i.e. the electron-donor and electron-acceptor or polar interactions) of the total free energy of interaction involves hydrogen bonding and chemical interactions occurring in aqueous media and for protein molecules in hydrated state it is believed to increase inter-molecular repulsion and thus increase its solubility in aqueous media and is the most important type of interaction occurring in aqueous media (van Oss, 2006).

Table 3.9: Total free interaction energy between proteins in the conditioning solutions and solid surfaces calculated using the thermodynamic theory.

Solid surface	Conditioning Solution	Total free energy of interaction and its components (mJ/m^2)		
		ΔG_{PLS}^{LW}	ΔG_{PLS}^{AB}	ΔG_{PLS}^{TOTAL}
Mica	BSA	-4.96	28.97	24.00
	TSB	-5.58	35.92	30.34
	CAS	-5.57	8.32	2.75
	WML	-6.76	-4.99	-11.76
	SML	-6.93	-4.73	-11.66
PS	BSA	-5.15	-12.96	-18.11
	TSB	-5.79	-7.02	-12.81
	CAS	-5.78	-37.08	-42.86
	WML	-7.02	-57.69	-64.71
	SML	-7.19	-54.70	-61.89

3.4.6.2 Calculations based on XDLVO theory

The total free energy of interaction was calculated using the ΔG_{PLS}^{LW} and ΔG_{PLS}^{AB} values obtained in the preceding section along with radius of proteins and zeta potential values of both the proteins in the conditioning solutions and the solid surfaces. The values of the zeta potential of mica (-120 mV) and polystyrene (-35 mV) were obtained from the literature. (Chaves Simoes et al., 2010; Lackie, 1983; Ong et al., 1999; Zembala et al., 2001). Table 3.10 reveal the values of the total interaction energies and its components at minimal separation distance calculated using the XDLVO approach. The interaction energies between the proteins with blank surfaces of mica and polystyrene are shown. Calculations were performed assuming sphere-plate geometry. The total interaction energies were positive for BSA and TSB – mica interaction combinations indicating unfavourable adsorption while in case of CAS, WML and SML – mica interaction combinations a strong attraction was observed as the total interaction energy was positive. For protein - polystyrene interaction combinations the total interaction energies were negative indicating a strong attraction.

In comparison with the thermodynamic approach, the values of the energies calculated were much higher and showed similar trends, for all interaction combinations except mica – CAS interaction combinations. In this case, the total interaction energy was

negative and showed favourable adsorption to mica as the electrostatic interaction was stronger than the AB interactions.

Table 3.10: Total free interaction energy between proteins in the conditioning solutions and solid surfaces calculated using the XDLVO theory.

Solid surface	Conditioning Solution	Total free energy of interaction and its components (mJ/m^2)			
		G_{PLS}^{LW}	G_{PLS}^{AB}	G_{PLS}^{EL}	G_{PLS}^{XDLVO}
Mica	BSA	-10.04	242.28	-87.55	144.69
		-60.00	1364.91	-493.25	811.66
	TSB	-4.1E+10	1.0E+12	-1.1E+11	8.6E+11
		-1.6E+11	3.9E+12	-4.4E+11	3.3E+12
	CAS	-149.71	862.90	-2235.41	-1522.21
	WML	-1.8E+11	-5.2E+11	-4.7E+11	-1.2E+12
	SML	-1.3E+11	-3.5E+11	-3.3E+11	-8.1E+11
PS	*BSA	-10.427	-108.417	1.191	-117.653
		-62.30	-610.60	6.71	-666.20
	*TSB	-1.6E+11	-7.6E+11	9.6E+10	-8.3E+11
		-4.3E+10	-2.0E+11	2.5E+10	-2.2E+11
	CAS	-155.35	-3845.73	130.54	-3870.54
	WML	-1.9E+11	-6.0E+12	7.8E+10	-6.1E+12
	SML	-1.4E+11	-4.1E+12	5.8E+10	-4.1E+12

*Calculated using two values of hydrodynamic radius

3.4.6.2.1 XDLVO energy profiles

In the XDLVO approach, the total free energy of interaction can be calculated not only at the shortest distance of approximation, but also as a function of separation distance. Therefore, the total interaction energy is plotted as a function of the separating distance to obtain the interaction energy profiles. These profiles could explain the discrepancies between the XDLVO predictions at contact and the adsorption experimental findings as the adsorption process starts from a distance between the interacting surfaces and should not be considered only at contact. As a case study the interaction energy profiles of BSA and CAS interacting with mica and polystyrene are described. Comparisons between each component of the free interaction energies for BSA and CAS with mica are presented in Figure 3.5a-d and for polystyrene are shown in Figure 3.6a-d respectively. The interaction energy in kT – the product of the Boltzmann

constant, k, and the temperature T (y axis) versus separation distance (x axis) in nanometer (nm) is plotted. (1kT is equivalent to 4.11×10^{-21} J).

BSA and CAS interaction with mica and polystyrene surfaces

The profile of the van der Waals (LW) free interaction energy (Figure 3.5a) revealed that with the mica surface, both BSA and CAS showed a negative LW implying attractive interactions where the intensity of interactions decayed with separation distance and reached almost zero beyond 8 nm. Figure 3.5b shows the free interaction energy based on acid–base interactions (AB). The AB values were positive for both BSA and CAS indicating net repulsion. The influence of Lewis acid–base rapidly decreased with longer separation distance and reached almost zero at 7 nm. Figure 3.5c describes the free interaction energy based on electrostatic force (EL). BSA and CAS showed attractive interactions upon approach to the mica surface. In the case of BSA, the EL interaction energy decayed with separation distance and reached almost zero at 7 nm. However, in case of CAS a strong long ranged attractive force was observed. The profiles of the total interaction energies were similar to the AB interaction energies profiles in the case of mica – BSA and EL interaction energies profiles for mica – CAS interaction combinations. This showed that the total interaction energies were influenced mainly by AB interactions for BSA and EL interactions for CAS respectively. The values obtained showed favorable adhesion of CAS and unfavourable adhesion of BSA on mica surfaces respectively.

In the case of polystyrene (Figure 3.6 a-d), similar attractive LW interactions were seen for BSA and CAS as in seen with mica. However, the AB and EL interactions showed opposite trends. Highly attractive properties of the LW and AB interaction components in both the protein–polystyrene combinations were expected from the negative values in a short range (<5 nm). On the contrary, the EL interactions were repulsive as both polystyrene and the proteins were negatively charged. The total interactions were influenced by AB interactions for both BSA and CAS and showed negative values demonstrating the adhesion potentials of both proteins to polystyrene surfaces.

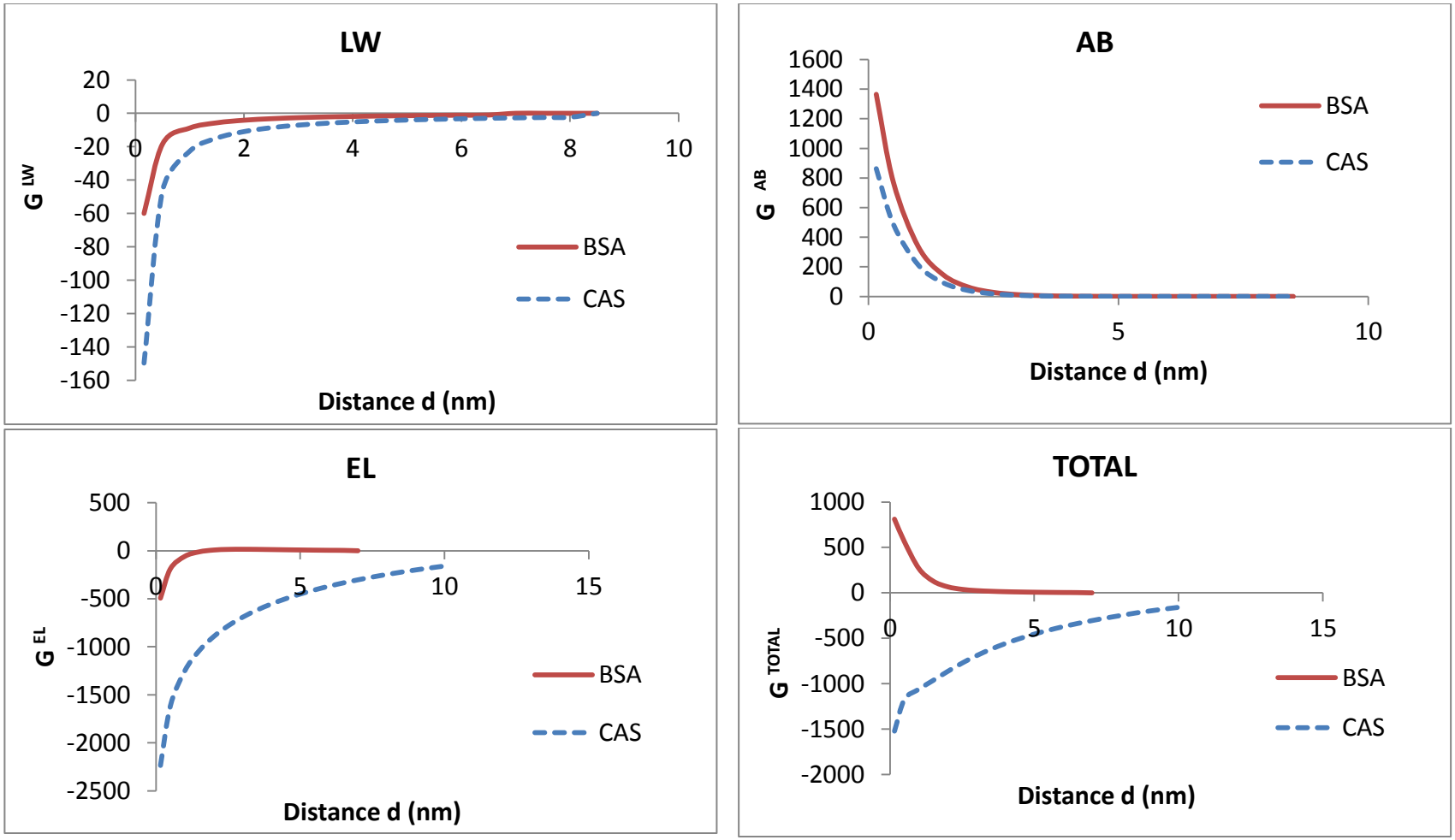


Figure 3.5: XDLVO energy profiles of (a) LW, (b) AB, (c) EL and (d) total interaction energies with separation distance between Mica -BSA and Mica -CAS

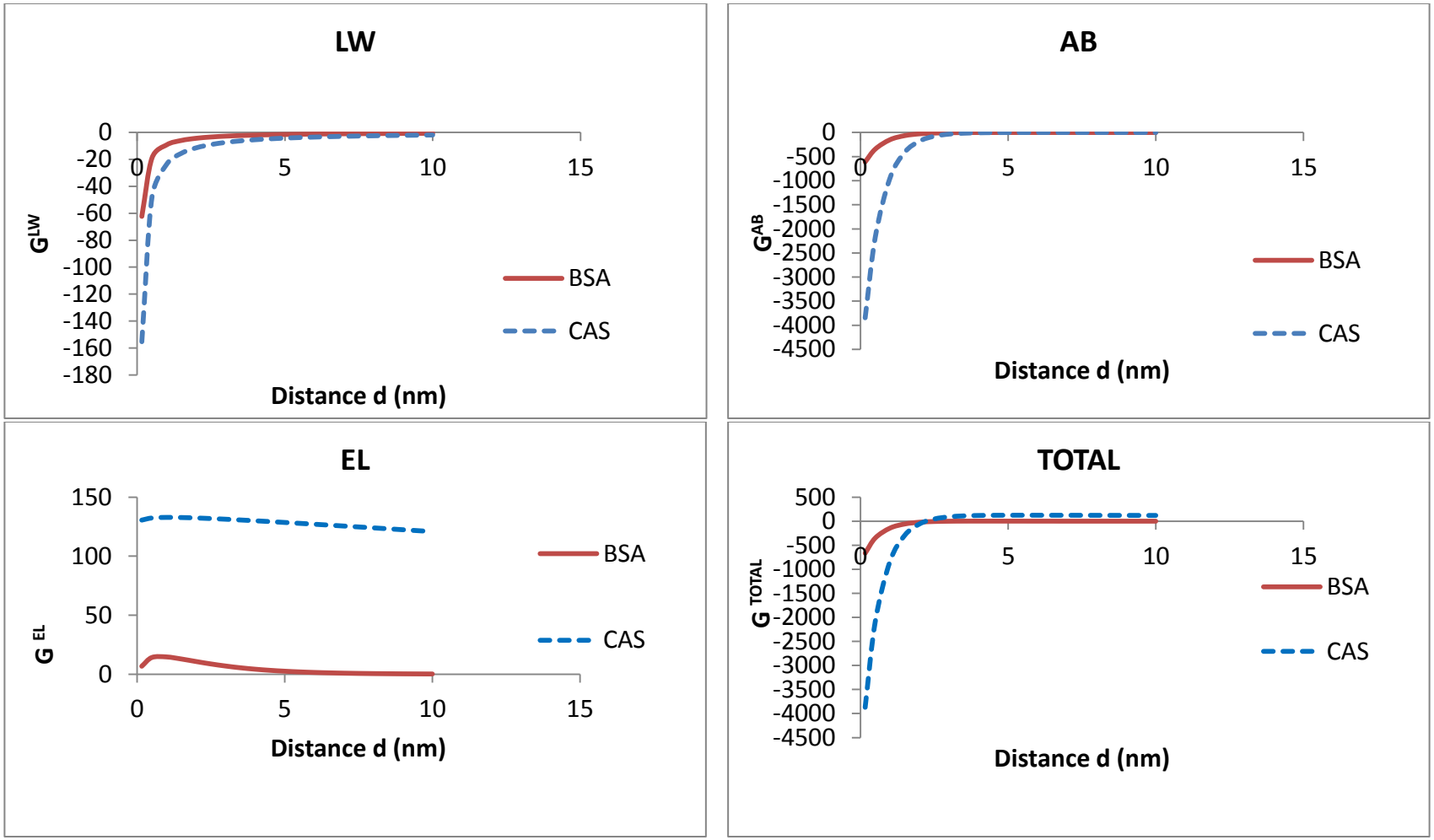


Figure 3.6: XDLVO energy profiles of (a) LW, (b) AB, (c) EL and (d) total interaction energies with separation distance between PS-BSA and PS –CAS

3.4.7 Adhesion predictions of proteins and milk to mica an polystyrene surfaces based on thermodynamic and XDLVO calculations

The amount of protein adsorbed to mica and polystyrene surfaces was predicted using the total free interaction energies of adhesion quantified using the thermodynamic and XDLVO theories. Our results with quantification experiments revealed that amounts of proteins adsorbed on mica were generally higher than polystyrene surfaces. Assuming a higher negative total free energy of interaction value should correspond to higher amount of adsorbed proteins, the calculated total interaction energies using thermodynamic and XDLVO theories predicted that the conditioning solutions adsorbed more on polystyrene surfaces in comparison with mica. Therefore, the theoretical predictions at contact between the surface and the conditioning films could not accurately verify the amount of protein adsorbed solid surfaces.

Although a few studies have demonstrated that the XDLVO theory could successfully predict fouling trends of biological colloids such as proteins and organic foulants especially to membrane surfaces used for filtration in the wastewater treatment industry (Subhi et al., 2012; Xiao et al., 2011) our study showed that theories cannot fully explain and predict the amounts adsorbed which is most likely due the high variation in chemical structures and conformations of the proteins on the surfaces and in solution. Another reason for the difference in the theoretical and the experimental results could be related to the surface roughness of the materials. Hoek et al. (2003) had found that surface roughness of a membrane could reduce the repulsive energy barrier height, thus rendering rough surfaces more favorable for particle deposition. Our AFM analysis results showed that the roughness of polystyrene was higher than mica and hence may result in more favorable adsorption of proteins.

3.4.8 Conclusions

Due to the adsorption of proteins, conditioning films are usually formed on solid surfaces in aqueous environments, which can significantly impact bacterial initial adhesion and biofilm formation through altering surface free energy, charge, roughness, and hydrophobicity of the surface. In the present study, surface conditioning of model hydrophilic and hydrophobic surfaces was investigated by immersion in relatively high concentrations of BSA, TSB, CAS,

WML and SML. The amount of protein adsorbed onto the surface was quantified and surface thermodynamic properties were characterized using contact angle measurements, zeta potential and dynamic light scattering measurements. Our results demonstrate that higher amount of protein was adsorbed on hydrophilic when compared to hydrophobic surfaces and adsorption of CAS was observed to be the highest on both surfaces. Proteins adsorbed with high favourability but with low amounts on polystyrene when compared to mica. Proteins were present in aggregated states in solution and also on the surface as seen by AFM images. The total free energy of interactions calculated using the thermodynamic and XDLVO model. Although the models showed favourable adhesion of conditioning solutions (in most cases) on both surfaces it could not accurately predict the amount of protein adsorbed on the surfaces.

PREFACE TO CHAPTER 4

The formation of unwanted layers of protein deposits on the surface of process equipment and their removal, as well as the attachment and inactivation of associated microbiological species, is of critical importance in the food industry. In food processing lines contact surfaces are exposed to varying hydrodynamic conditions caused by the flow of liquid food. Flow is also known to be one of the several factors that influence the protein adsorption on solid surfaces.

An effective tool for studying the adsorption of protein to surfaces under flow conditions in laboratory settings is the Robbins device. In this chapter, evaluation and quantification of the surface bound proteins under flow conditions using the modified Robbins device are highlighted. In addition the surfaces thermodynamic properties of mica and polystyrene surfaces conditioned with proteins and milk under flow conditions are characterized. Dynamic light scattering (DLS) and zeta potential measurements were done to know the size and charge of the proteins in solution. Experimental conditions were selected to closely mimic food industry environments.

Part of this research was presented in 2013 in the Institute of Food Technology conference in, Chicago, Illinois, USA (Poster).

Nikhil Hiremath, Louise Deschênes, Hosahalli Ramaswamy, and Mohammed. Reza Zareifard. Characterization of mica and polystyrene surfaces conditioned with proteins and milk under flow conditions (in preparation)

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. Louise Deschênes, and Dr. H. S. Ramaswamy

Chapter 4. CHARACTERIZATION OF MICA AND POLYSTYRENE SURFACES CONDITIONED WITH PROTEINS AND MILK UNDER FLOW CONDITIONS.

4.1 Abstract

Protein adsorption is the first stage in biological contamination of surfaces, with cells binding to a pre-adsorbed protein layer before proliferating and spreading. In the food industry adsorption of proteins can alter the surface physicochemical characteristics and can act as a nutrient source for adsorbed micro-organisms. In addition, several processes in the food industry are flow dependent and known to be important parameters that affect protein adsorption to surfaces. In this study hydrophobic and hydrophilic surfaces, were exposed to protein solutions and milk under laminar flow conditions using a modified Robbins device. The amount of protein adsorbed was quantified and surface thermodynamic properties of were characterized by contact angle measurements and compared with those obtained under static conditions. The size distribution in solution of the protein aggregates formed under the same conditions was determined by dynamic light scattering and zeta potential measurements. Atomic force microscopy (AFM) was used to image surface topography and surface roughness. In comparison with the adsorption of proteins from the same solutions, under static conditions, higher amount of protein was adsorbed onto both mica and polystyrene surfaces under flow conditions and changes in the surface thermodynamic properties were observed. Particle size distribution, zeta potential and AFM images showed that the proteins in solution and on surface attached in the form of aggregates. The findings from this study would help us further to understand bacterial adhesion behaviour to contact surfaces in the food industry settings.

4.2 Introduction

The adsorption of proteins onto solid surfaces is a fundamental phenomenon and is of vital importance to many disciplines including industrial, biomedical and environmental sciences. This complex phenomenon has been explored over several decades. Several factors governing protein adsorption, measurements and experimental methods, protein surface interactions, mechanistic aspects and protein resistant materials has been reviewed by several

authors (Banerjee et al., 2011; Barish & Goddard, 2013; Gray, 2004; Kingshott & Höcker, 2006; Nakanishi et al., 2001; Norde, 1996; Norde & Lyklema, 1979; Rabe et al., 2011; Wahlgren & Arnebrant, 1991). Despite considerable amount of progress in several fields, understanding the adsorption behaviour of protein still remains a challenge. Some aspects of protein adsorption on to surfaces were discussed in detail in the previous chapter.

Among the several factors, flow is considered to be one of the important factors that affect the adsorption of proteins to surfaces (Byrne et al., 2009; Klose et al., 2006; Li et al., 2009). The complexity of protein adsorption and the need to study them under laboratory conditions has led to the development of experimental flow systems such as parallel plate flow cell, rotating disk andradial flow chamber systems (Byrne et al., 2009; Detry et al., 2009). These systems enable well controlled and defined flow conditions. Typically, different aspects such the amount adsorbed proteins adsorbed, the morphology, the structure, rate, conformation or orientation of the adsorbed protein layers and their removal have been investigated and characterized under diverse experimental conditions (Kalasin & Santore, 2009; Karlsson et al., 2001; Norde et al., 1991; Salim et al., 2007; Shirahama et al., 1990).

In food processing and handling applications, the adsorption of proteins onto surfaces forms a conditioning film layer upon which microbial cells adhere before proliferating and spreading (Saa et al., 2009; Verran & Whitehead, 2005). The formation of protein film layers has been a chronic problem and is known to reduce the efficiency of equipment, compromises product quality, and reduces productivity of the industry due to shut down for cleaning and leads to higher investment, maintenance and operating costs (Fryer et al., 2006; Goode et al., 2013a). As a result, greater insight into the protein–surface interaction is necessary to understand protein adsorption especially under flow conditions as many processes in the food industry are flow dependent.

Several studies have been carried out on the adsorption of proteins under flow conditions especially in relation to the dairy industry. Santos et al. (2006b) investigated the effect of flow rate on the adsorption of whey protein adsorption on stainless steel surfaces under well-defined flow conditions. Below the unfolding temperature of β-lactoglobulin (72°C), the amount of protein adsorbed was reported to increase with an increase in Reynolds number. Above the

unfolding temperature (85° C), the amount of protein decreased with increase in Reynolds number although the adsorbed amount was significantly higher than at 72°C, and showed the formation of aggregates/multilayers. At both temperatures studied, longer protein solution residence times decreased the amount of protein adsorbed. The same authors also investigated the effect of bulk protein concentration and solution pH and under flow conditions. They monitored the adsorption at 85°C and a high Reynolds number of 11300. They observed an increase in the amount of protein adsorbed with an increase in bulk solution concentration from 0.3 to 3 mg/ml. A decrease in solution pH increased the amount of protein adsorbed for a bulk solution concentration of 0.3 mg/ml (Santos et al., 2006b). To study temperature and flow effects on whey protein fouling Belmar-Beiny et al. (1993) performed adsorption experiments in a tubular heat exchanger at an inlet temperatures of 83°C and Reynolds number in the turbulent region. The total amount of deposition increased with inlet temperature and decreased with the Reynolds number. Although there are numerous fouling studies using various whey proteins, there are limited reports about the adsorption behaviour of actual milk under flow conditions. For instance a recent study by Patel et al. (2013) studied the absorption behaviour of real milk and whey protein isolate on modified stainless steel surfaces under flow conditions with laboratory-scale and pilot scale equipment. It was concluded that the real milk system could mask the potential benefit of surface modification procedures that were used in the study. Other studies have used various fouling fluids such as simulated milk ultra-filtrate (SMUF), and SMUF with added whey protein to study their adsorption or fouling behaviour on modified stainless steel surfaces under flow conditions (Rosmaninho et al., 2008; Rosmaninho et al., 2007). In the dairy industry context most protein adsorption studies have used relatively low bulk solution concentrations ranging between 0.01 to 3.0 mg/ml and are usually conducted at higher temperatures due to the relevance of protein fouling in heat exchangers during milk processing. However, bulk of the handling of diary and beverage products (including handling of sterilized and pasteurized dairy and beverage products) takes place at room or refrigerated temperatures,) under dyamic flow situations.

The objective of this work was to quantify and characterize the surface bound proteins under well-defined laminar flow conditions using higher protein concentrations. Accordingly a modified Robbins device was used as an experimental flow cell.

4.3 Materials and methods

All experiments were carried out at room temperature (25^{o}C).

4.3.1 Protein solutions and milk

Protein solutions [BSA (Fraction V, Cold-Ethanol precipitated, purity > 98%, Fisher Scientific Canada), CAS (92.2% protein Nealanders International Inc. Dorval, Quebec, Canada), TSB (Difco, Fisher Scientific Canada)], and milk, [WML - 3.25% fat and SML - 0% fat (Quebon brand, purchased from local supermarket)] and were used in the study. Proteins (BSA, TSB, and CAS) were dissolved in analytical grade ultra-pure water (18.2 MΩ•cm at 25°C) to obtain the desired concentration (33 mg/ml). TSB solution was autoclaved. WML and SML were irradiated (25kGy) to obtain sterile milk. All the solutions were stored in refrigerator and thawed to room temperature before each adsorption experiment. Solutions were used within two days of preparation and new solutions were prepared for each batch of experiments.

4.3.2 Solid surfaces and surface preparation

Sheets of freshly cleaved mica (S&J Trading Inc, Glen Oaks, N.Y., US) and polystyrene weighing dishes (Fisher scientific, Canada product number S67090A) were cut into rectangular coupons of size 3.8 cm x 1.9 cm. Polystyrene was cleaned by methanol (Fisher Scientific, Canada) sonication for 10 minutes, followed by water sonication for an additional 10 min. To remove any remaining traces of methanol, polystyrene surfaces were thoroughly rinsed with abundant quantities of water. Polystyrene surfaces were then dried under nitrogen and stored in vacuum oven until use.

4.3.3 Modified Robbins device and pumping system

A modified Robbins device (MRD) was used as an experimental flow cell (Figure 4.1). This laboratory flow system is known to simulate flow regimes in industrial pipelines and has been extensively used to study microbial adhesion to surfaces under flow (McBain, 2009). The MRD (model number LAMR-2C5-PSF) made of polysulphone was obtained from Tyler research corporation Edmonton, Canada. The MRD was an autoclavable set-up which consisted of a

manifold which was 54 cm long x 10 cm wide with 2 removable cassettes each having a length of 17 cm and the width of 8 cm. Each cassette contained 5 slots into which the coupons of size 3.8 cm x 1.9 cm x 0.08 – 0.1.0 cm could be inserted. Inlet and outlet ports of the device were barbed fittings suitable for ¼ inch inner diameter tubing. The maximum pressure rating of this device was 30 psi at a flow rate of 2 liters per minute. The pumping head, tubing and console drive were purchased from Cole palmer, Canada. The Easy-Load® II Pump Heads rated to handle flow rates of 1.7 to 2900 mL/min with high-performance precision pump tubing (Norprene® Food grade A 60 F) with an inner diameter of 6.4 mm was used for circulation of the experimental liquids.

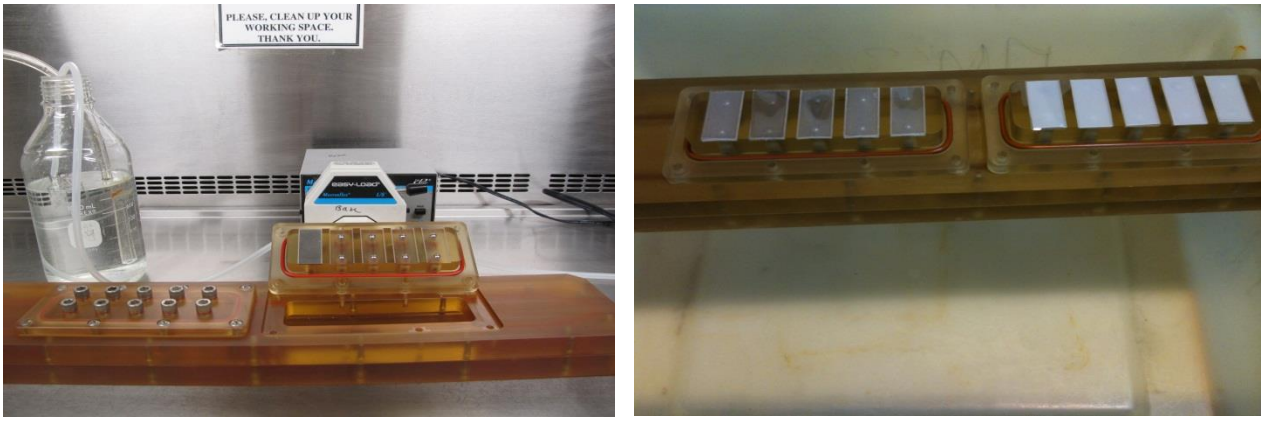


Figure 4.1: Pictures of the MRD system along pump and the tubing and inserted mica and polystyrene coupons in the cassettes

In this study, Reynolds number (Re) was used as the parameter (representing the fluid flow) to know the flow regime. This is given as $Re = V\rho D/\mu$ where ρ is the density of the fluid (kg /m^3), μ is the viscosity of the fluid (N s /m^2 or centipoise), v is the velocity (in m/s) and d is the diameter of the MRD (m). The Reynolds number was calculated using rectangular rather than circular in cross-section (Brading et al., 1995). The viscosity of each solution was measured using Ubbelhode viscometer (Cannon Instrument Company, PA, USA). The density of each solution was measured by weighing (kg) a precisely measured volume (m^3) of the solution.

4.3.3.1 Calculation of Reynolds number

The Reynolds number for each conditioning solution was calculated at the maximum possible flow rate that could be achieved by the pump head. Table 4.1 shows the Reynolds

number at the maximum flow rate for conditioning solutions. The Reynolds number was found to be less than 2000 showing laminar flow behaviour.

Table 4.1: Calculated Reynolds number of conditioning solutions at maximum flow rate

Conditioning Solution	Velocity (V) m/s	Density (ρ) kg/m^3	Diameter (D) m	Viscosity (μ) Pa.s	Re
					Amount of protein adsorbed (μg/cm^2)
BSA	0.06031	1048.2	0.0072	0.0013	1125
TSB	0.09649	1066.4	0.0072	0.0024	609
CAS	0.1864	1050.5	0.0072	0.0012	1157
WML	0.1864	1007.7	0.0072	0.0018	749
SML	0.1864	1003.3	0.0072	0.0017	812
Water	0.01672	1003.3	0.0072	0.0009	1523

4.3.4 Adsorption (conditioning) experiment

The conditioning of surfaces was tested simultaneously for each solid surface. Five coupons of each surface were mounted on previously cleaned supports (polystyrene supports for mica and glass supports for mica) and inserted into cassette of the MRD (Figure 4.1). The experimental fluids were circulated for 1 h at maximum available pump speed (still under laminar flow conditions). Before each experimental run, the MRD was cleaned with 1000 ppm sodium hypochlorite (pH adjusted to 12 using 0.1M sodium hydroxide solution). This was followed by rinsing with abundant quantities of water, autoclaving and overnight drying. The conditioning experiment was done twice on separate days with each conditioning fluid.

The conditioned coupons were removed by forceps and rinsed with water (with wash bottle) on both sides to remove any loosely bound protein. Then the coupons were rinsed twice, sequentially by dipping in 40ml volumes of water (in centrifuge tubes) for about 10 s in each centrifuge tube to remove any residual loosely bound solution. The rinsed coupons were then placed in clean empty centrifuge tube and dried by blowing nitrogen gas for 10 min to remove excess water remaining on the surface. Similar procedure was followed to obtain control/blank (surface blank) using water as the circulating fluid.

4.3.5 Protein quantification assay

The protein quantification assay was performed as described in Chapter 3. Briefly the flow treated surfaces were transferred to specially constructed glass vials. The surfaces in the vials were covered with 1:1 ratio of micro BCA reagent (Thermo Fisher Scientific, product number 23235) and water so as to completely cover the surface. The vials were covered with wax paper and incubated in an air oven at 60°C for 1 hour. After cooling to room temperature, samples were thoroughly mixed and pipetted (200 µl) on to a micro-plate (96 wells). The absorbance was read at 562 nm without any delay. A standard curve (using Eppendorf tubes) for each experimental conditioning solution was also performed at the same time with 1:1 ratio of diluted standard and micro BCA reagent. The standard curve was prepared each time for each experimental run to minimize day to day variations.

The amount of protein adsorbed on the surface was calculated by subtracting the absorbance value of conditioned surface from the absorbance value of the surface blank (surface in contact with water). The value obtained, was converted to micrograms (µg) of protein adhered using the equation of line obtained from the prepared standard curve. The amount calculated was divided by the total surface area available for adsorption and finally expressed as microgram per square centimeter ($\mu g/cm^2$).

4.3.6 SAS analysis

The amount of protein adsorbed onto mica and PS under flow and static (quantified in chapter 3) was compared using the GLM (general linear model) procedure for analysis of variance (SAS 9.4 Institute Inc., Cary, NC).

4.3.7 Characterization of proteins, milk, mica and polystyrene surfaces

Mica and polystyrene surfaces that were conditioned under flow were also characterized by CAM (to obtain various surface thermodynamic properties) and AFM which was used to image the flow conditioned surfaces. In addition proteins and milk (conditioning solutions) samples were circulated through the MRD for 1 h and subjected to dynamic light scattering (DLS) to characterize the size distribution (hydrodynamic radius) profiles.

4.3.7.1 Contact angle experiments

Contact angle measurements were performed using a goniometer (FTÅ 200 system, Portsmouth, Virginia) by the sessile drop method as described in chapter 3. Briefly, one drop of a probe liquid was deposited onto prepared "protein layers" as well as flow-conditioned mica or polystyrene surface and using three probe liquids, water, formamide and α-bromonaphthalene (Aldrich Chemical Co., Inc., Milwaukee, WI). Three to six contact angle measurements were made on surfaces for all probe liquids on three random locations on the surface. Three independently prepared sample surfaces were analyzed.

4.3.7.2 Calculation of surface thermodynamic properties

The surface thermodynamic properties (surface energy components and absolute hydrophobicity) were estimated from contact angle via the approach proposed by van Oss (van Oss et al., 1988). The surface thermodynamic properties were calculated as detailed in section 3.3.5.2. Briefly, the surface energy components were obtained by solving the extended Young's equation (Equation 3.4) obtained by three probe liquids simultaneously. The absolute hydrophobicity was calculated according to Equation 3.5.

4.3.7.3 Dynamic Light Scattering Measurements

Proteins and milk (conditioning solutions) samples were circulated through the MRD for 1 h and subjected to dynamic light scattering (DLS) using a Zetasizer Nano ZS Malvern instrument (Malvern Instruments, UK) operated with a Mastersizer 2000 software.

4.3.7.4 Atomic Force Microscopy

AFM was used to image and analyze the surface morphology and roughness of the flow conditioned surfaces. AFM experiments were conducted using a Veeco Multimode 8 Nanoscope (Brucker, Santa Barbara, CA, USA) which was operated tapping mode in air condition and room temperature. The surfaces were imaged in different scan sizes (from 1 µm X 1 µm and 5 µm X 5 µm). The monolithic silicon cantilever used was an Arrow™ Non-contact Reflex coating (NanoWorld®, Switzerland). The cantilever had an average resonant frequency, spring constant,

length and width of 285 kHz, 42 N/m, and 160 µm and 45 µm, respectively. The apex curvature radius was in the order of 10 nm.

Root mean square (or RMS) roughnesses as well as 2D surface pictures were obtained using this technique. The image processing was carried out using a NanoScope Analysis software Version 1.40. Duplicate samples were made and representative images have been shown.

4.4 Results and Discussions

4.4.1 Amount of protein adsorbed under flow conditions

Comparing the adsorption of proteins onto surfaces under dynamic flow conditions (with a protein concentration of 33 mg/ml) revealed that significantly higher amount of proteins were adsorbed on mica than on polystyrene surfaces. Among the conditioning solutions, higher amount of CAS ($P<0.05$) was adsorbed on both the mica and polystyrene (Figure 4.2). From previous experiments with BSA adsorption onto mica under static conditions (detailed in Chapter 3) it was seen that, the amount of BSA adsorbed increased at first and showed no significant difference after 1 h of incubation. Similar trends were observed with TSB and CAS adsorption on mica. Based on these observations the adsorption time was fixed at 1h for the adsorption under flow conditions.

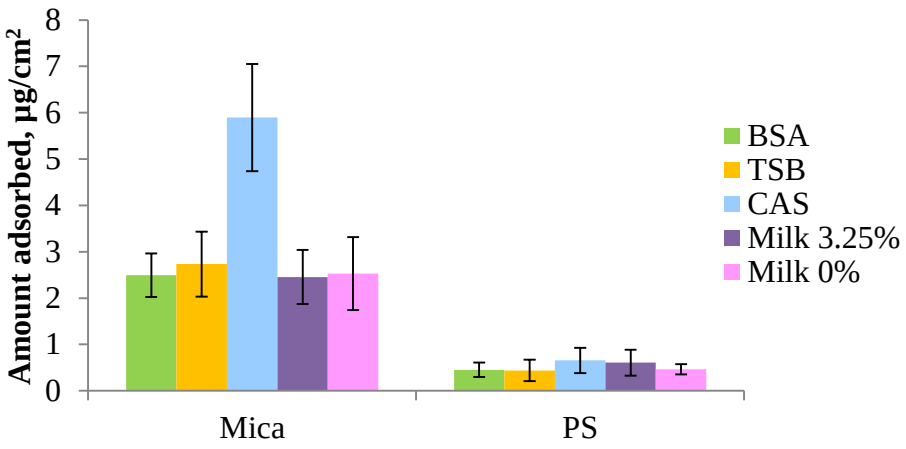


Figure 4.2: Amount of protein adsorbed from flowing conditioning solutions on mica and polystyrene surfaces

Varied protein adsorption amounts on surfaces have been reported in the literature. This is expected due to different measurement methods and different adsorption conditions that have used to quantify the surface bound proteins. Several authors have quantified surface bound BSA under laminar flow conditions using different measurement methods, several types of surfaces, experimental conditions and measurement methods. Gispert et al. (2008) reported an amount of 0.45 and 0.7 µg/cm^2 on alumina and stainless steel surfaces respectively from QCM (quartz crystal microbalance) measurements from a 15 mg/ml flowing solution of BSA. Using the same measurement method Roach et al. (2006) reported an amount of 0.8 and 0.7 µg/cm^2 on hydrophilic and hydrophobic surfaces respectively from a 2 mg/ml BSA solution. Hook et al. (2002) utilized in situ ellipsometry, optical waveguide lightmode spectroscopy, and quartz crystal microbalance/dissipation techniques to study the adsorption of 0.08 mg/ml BSA on titanium oxide surfaces and reported an adsorbed mass of 0.162, 0.214 and 0.333 µg/cm^2 respectively. Wertz and Santore (2001) studied BSA adsorption kinetics on hydrophilic surfaces under flow and varying solution concentrations of 0.01, 0.025, and 0.05 mg/ml using total internal reflectance fluorescence (TIRF), and reported a surface concentrations of 0.1, 0.2 and 0.3 µg/cm^2, respectively. The adsorption of BSA onto both mica and polystyrene surfaces has also been studied but mainly under static conditions and from low concentrations of proteins in

the bulk solution. The reported adsorbed amounts on mica range from 0.2 to 0.7 μg/cm^2(Fitzpatrick et al., 1992; Gallinet & Gauthiermanuel, 1992; Terashima & Tsuji, 2003) whereas in case of polystyrene it was found to be 0.329 μg/cm^2 (Reimhult et al., 2008) . In case of CAS, which contains a mixture of four caseins (αs1-, αs2-, β- and κ-), β-casein has been widely investigated. Also β-casein together with αs1-casein is the most abundant milk protein. Chandrasekaran et al. (2013) and Lee et al. (2004) used QCM to study the amount of β-casein on stainless steel and hydrophobic surface and reported a surface concentration of 0.6 and 1.05 μg/cm^2 from a bulk solution concentration of 0.05 and 2 mg/ml respectively.

Generally, based on the adsorbed amounts, more can be learned about the protein organization or orientation on solid surfaces. For BSA, because of the ellipsoidal shape, two types of orientation of adsorbed BSA molecules on surfaces have been proposed. "Side- on" and "end-on" orientations if major axis is parallel and perpendicular to solid surface, respectively. The final adsorbed amount of a protein monolayer in its saturation state is higher in the case of 'endon' oriented proteins than in the case of 'side-on' oriented proteins. The calculated monolayer capacity in the case of BSA on mica was reported to be 0.201 μg/cm^2 for side-on and 0.753 μg/cm^2 end-on orientations (Terashima & Tsuji, 2003). The maximum density of monolayer β-casein is approximately 0.23 μg/m^2 (Waninge et al., 2005). Comparing these values with the adsorbed amounts with our experiments the surface concentration corresponded to more than a monolayer in the case of mica, indicating a possible formation of protein agglomerates on the mica. In case of polystyrene a monolayer formation is expected.

Table 4.2 compares the adsorbed amount of proteins from different conditioning solutions previously performed under static conditions under the same experimental conditions. ANOVA (analysis of variance) performed using SAS comparing the amount of protein adsorbed from each conditioning solution onto mica and polystyrene surfaces is also shown. Static adsorption experiments yielded lower protein surface concentrations when compared to flow conditions on both mica and polystyrene surfaces. An explanation for that result might be that under flowing conditions the residence time of the protein on the surface is shorter than under stagnant conditions. Hence, structural rearrangements of proteins which is a time dependent process is expected to take place extensively under flowing than under stagnant conditions. In the case of proteins with low conformational stability the shorter residence time under flow

conditions leads to a more expanded and space-consuming spreading of the unfolded protein, resulting in a higher amount of adsorbed protein molecules. Salim et al. (2007) studied the adsorption comparison of fibrinogen under static and flow conditions on glass. Their studies also indicated that lower protein adsorption occurred during non-flow conditions which were in line with our current observations. Santos et al. (2006a) also reported that the amount of β-Lg adsorbed from 0.3 g/l and 3 g/l solution concentrations onto stainless steel at 85°C increased with increase in Reynolds number (from 3800 to 11300; in turbulent flow conditions) in both cases showing that an increase in both solution concentration and flow rate can result in higher amount of protein adsorbed onto a surface.

Table 4.2: Comparison of amount of protein adsorbed under flow and static conditions with ANOVA

		Amount of protein adsorbed ($\mu g/cm^2$) ±standard deviations		
Surface	**Conditioning Solution**	**Flow**	**#Static**	**ANOVA analysis**
Mica	BSA	2.49±0.47	1.77±0.44	**
	TSB	2.73±0.70	1.13±0.37	**
	CAS	5.90±1.16	2.27±0.65	***
	WML	2.45±0.58	1.32±0.36	***
	SML	2.53±0.79	1.66±0.32	**
PS	BSA	0.45±0.16	0.16±0.03	**
	TSB	0.44±0.23	0.15±0.03	***
	CAS	0.65±0.27	0.32±0.05	**
	WML	0.61±0.28	0.21±0.03	**
	SML	0.46±0.11	0.26±0.04	**

*** = $p<0.001$, ** = $p<0.05$, ns= not significant ($p>0.05$).

#Values taken from previous experiments

4.4.2 Contact angles and surface thermodynamic properties of blank and flow - conditioned mica and polystyrene surfaces

The contact angle values, along with the surface thermodynamic properties (surface energy parameters and absolute hydrophobicity) of mica and polystyrene conditioned under flow along with their respective blanks (surfaces obtained after circulation of water) are presented in Table 4.3. The water contact angle measurements show that the blank mica surface is hydrophilic while the blank polystyrene is hydrophobic. However, conditioning of mica and polystyrene surfaces resulted in changes in the water contact angle values. Contact angle measurements also show that conditioning film adsorption on the mica and polystyrene surfaces may occur in different conformations. Polystyrene conditioned surfaces are moderately less wettable than mica conditioned surfaces.

With regards to the surface energy and its components, γ^{LW}, showed marginal changes for both conditioned mica and polystyrene surfaces in comparison with their respective blank's. Jullien et al. (2008), described that the surface energy components of stainless steel conditioned with milk and meat showed no change in γ^{LW} values when compared to bare stainless steel surfaces and the only parameter affected by the conditioning treatment was the electron donor component of surface free energy. They observed that the electron donor component of surface free energy was higher than the electron acceptor component for both milk and meat conditioned stainless steel surfaces which were in-line with our observations. The electron donor component was greater than electron acceptor component for both mica and polystyrene surfaces conditioned with respective conditioning solutions, which means that both mica and polystyrene conditioned surfaces are negatively charged. The electron donor component of the surface energy has been reported as the most important factor and has been used as the characterizing factor of fouling deposits (Rosmaninho et al., 2008) The total surface energy values showed a slight increase after conditioning when compared to blank mica and polystyrene surfaces. In general the surface energy of conditioned mica was higher than polystyrene surfaces. In this work higher amounts of proteins were adsorbed on mica (higher surface energy). Although the surface free energy of the solid is known to be one factor that influences protein adsorption, no simple relation between surface free energy and protein adsorption is apparent. Some researchers found higher amounts of protein adsorbed on low energy surfaces (Krisdhasima et al., 1992;

Yoon & Lund, 1994) while others reported similar extent of protein adsorption at surfaces differing in surface energy (Addesso & Lund, 1997).

Blank mica and blank polystyrene surfaces were hydrophilic and hydrophobic respectively as the calculated ΔG_{SWS} was positive in case of mica and negative for polystyrene surfaces. After conditioning of mica with BSA CAS, WML and SML the ΔG_{SLS} was found to be negative indicating that the surface became hydrophobic except in the case of mica conditioned with TSB which retained a hydrophilic character. For polystyrene conditioned surfaces the calculated ΔG_{SLS} was negative in all cases demonstrating a strong hydrophobic surface. Previous works have reported the hydrophobicity of dehydrated conditioning film layers on solid surfaces. For instance, Hong et al. (2013) reported negative values of absolute hydrophobicity for dehydrated BSA on Dehypolyvinylidene fluoride (PVDF) membrane surfaces in context of membrane fouling. In food context, the free energy of cohesion of stainless steel coupons immersed in different milk samples (UHT,whole milk, chocolate milk and infant formula) and water was found to be positive and negative respectively (Bernardes et al., 2012) . Whitehead et al. (2009), reported changes in the absolute hydrophobicity for stainless steel surfaces conditioned with various organic food soils. In another study absolute hydrophobicity of hydrophilic glass became negative after covalent modification of glass with casein (Han et al., 2011). Therefore the quantification and characterization of surface hydrophobicity is necessary as these observations provide insights into the process of bacterial adhesion and biofilm formation.

Changes in the calculated values of surface energy components and absolute hydrophobicity for surfaces conditioned under flow when compared to surfaces that were conditioned under static condition were seen (Chapter 3). For instance the total surface energy for surfaces conditioned with respective solutions decreased for mica and increased for polystyrene. The ΔG_{SWS} values increased for both mica and polystyrene surfaces conditioned under flow except for mica conditioned with TSB which showed a slight decrease. This indicates that flow conditions may change the surface thermodynamic properties which can affect the process of bacterial adhesion and biofilm formation.

Table 4.3: Contact angles and surface energy components of blank and solid surfaces conditioned under flow

Surface	Conditioning solution	Contact Angles (°)			Surface Energy Components (mJ/m^2)					Absolute hydrophobicity (mJ/m^2)
		θ_{Water}	$\theta_{Bromonaphthalene}$	$\theta_{Formamide}$	γ^{LW}	γ^{+}	γ^{-}	γ^{AB}	γ^{TOTAL}	ΔG_{SWS}
Mica	BSA	47.11 ± 3.07	16.56 ± 1.85	33.95 ± 2.00	42.58	0.42	29.50	7.05	49.63	-0.16
	TSB	40.1 ± 1.20	10.84 ± 0.82	23.03 ± 1.03	43.61	0.87	32.30	10.59	54.20	2.96
	CAS	60 ± 3.92	30.84 ± 1.57	52 ± 1.80	38.34	4.62	8.62	12.63	50.97	-29.15
	WML	59.49 ± 1.22	13.66 ± 1.75	38.53 ± 1.04	43.15	0.51	16.63	5.84	48.99	-24.06
	SML	57.41 ± 0.74	20.22 ± 0.40	23.37 ± 3.93	41.71	2.50	12.52	11.19	52.89	-27.38
	Water	14.68 ±1.99	14.33 ±2.59	18.36 ±1.45	42.58	0.42	29.50	7.05	49.63	34.27
PS	BSA	76.4 ± 2.54	13.29 ± 2.20	62.19 ± 3.35	43.22	0.34	11.62	3.98	47.20	-36.57
	TSB	81.02 ± 2.89	14.72 ± 1.87	62.32 ± 0.75	42.95	0.16	7.12	2.11	45.06	-51.45
	CAS	78.25 ± 1.02	16.77 ± 1.58	60.02 ± 2.45	42.53	0.22	9.69	2.90	45.43	-42.38
	WML	80.75 ± 2.79	10.86 ± 1.02	63.01 ± 2.23	43.61	0.26	7.74	2.85	46.46	-48.64
	SML	74.37 ± 0.94	16.63 ± 0.43	58.79 ± 1.20	42.56	0.09	11.66	2.03	44.59	-37.96
	Water	91.23 ± 0.67	8.01 ± 0.55	67.06 ± 0.92	43.97	0.27	2.09	-1.51	42.46	-88.04

4.4.3 Dynamic Light Scattering

The particle size distribution was investigated for the conditioning solutions at 33 mg/ml protein concentrations. The conditioning solutions were collected from the MRD after circulation of the solution for 1 hour at room temperature conditions. The apparent hydrodynamic radius of the particles in solution is shown in Table 4.4**.** In comparison with particle size distribution of conditioning solutions under static conditions (Chapter 3) the particles sizes of conditioning solutions were reduced. Santos et al. (2006a) reported that increasing the protein solution residence time in a flow cell from 9 to 14 s at a Reynolds number of 11,300 resulted in smaller sized protein aggregates of β-lactoglobulin. In this study, the sizes of particles showed poly-disperse profiles and heterogeneous distribution and were present under the form of aggregates as observed under static conditions.

Table 4.4: Mean hydrodynamic diameter at the peaks of the conditioning solutions.

Conditioning solution	Hydrodynamic radius (nm)	
	Peak 1	Peak 2
BSA	6.08±0.04	86.29±5.55
TSB	37.71±6.93	166.30±11.17
CAS	87.10±3.71	
WML	115±2.55	
SML	76.74±0.90	

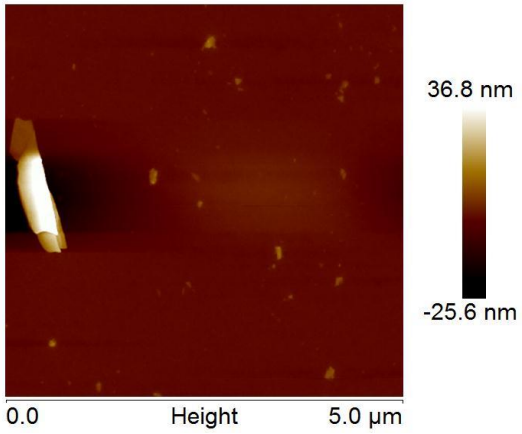
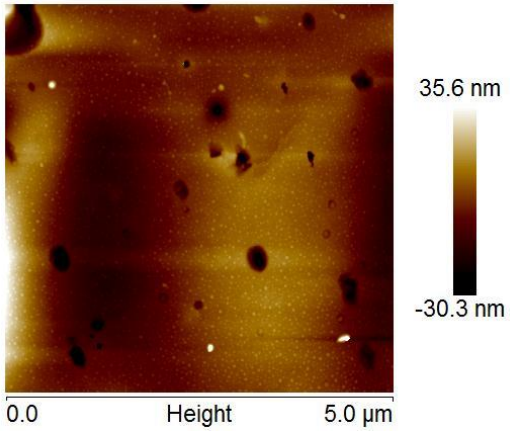
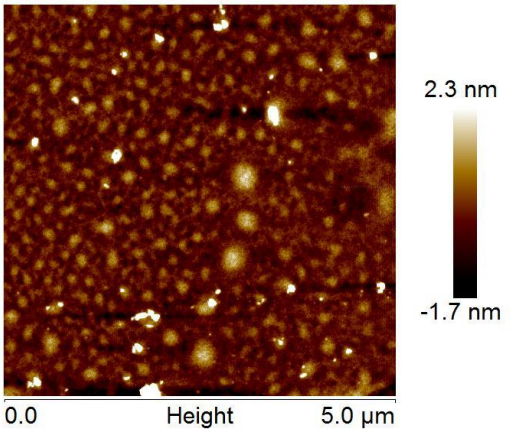
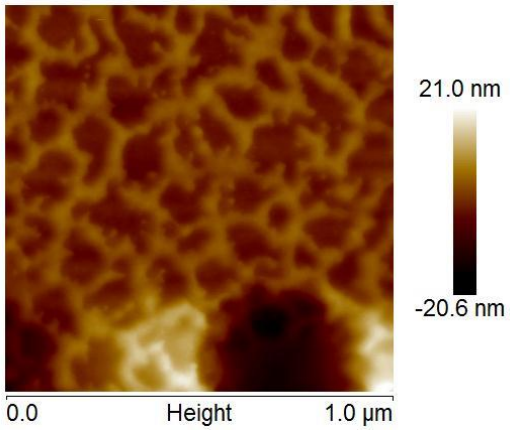
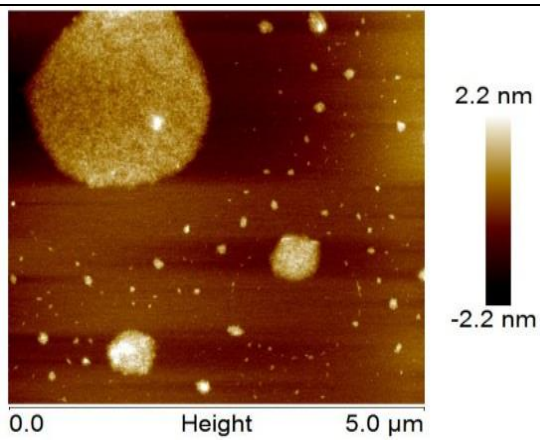
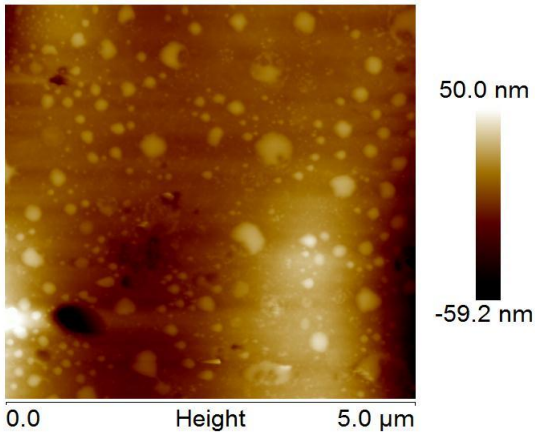
4.4.4 Atomic force microscopy of flow conditioned mica and polystyrene surfaces

Figure 4.3 shows 2D AFM images of the blank and conditioned surfaces mica and polystyrene surfaces obtained under flow. Conditioning of mica and polystyrene surfaces resulted in changes in morphology. In general the conditioning under flow shows deposits with regular patchy clusters with small particles, whereas on polystyrene the conditioning solutions appear to develop network type coverage. Jimenez et al. (2013) studied the microstructures of two dairy fouling deposits on stainless steel surface using AFM after passing the fouling liquid through experimental fouling equipment. The fouling layer obtained was quite homogeneous,

and the deposit covered the steel surface as well the fissures. The reported roughness of the fouled surface was 32 nm.

The Root mean square averages of height deviations taken from the mean image data plane (R_q), for mica and polystyrene surfaces are shown in Table 4.5. The difference in height between the highest and lowest points (R_{max}) on the cross-sectional profile relative to the center line (not the roughness curve) was also determined and shown in Table 4.5. Mica s showed very little surface imperfections. The surface roughness of bare mica surface was measured as 0.11 nm. The surface height is flat and uniform. The polystyrene had an uneven surface layer, with a significant amount of surface imperfections in a 5 μm by 5 μm scan. The roughness of polystyrene was measured as 14.75 nm. Upon conditioning the roughness (Rq) of both the surfaces was modified. The roughness of mica increased whereas the roughness of polystyrene seemed to decrease. AFM images of glass conditioned with Periwinkle wilt culture medium under flow showed Rq values decreased significantly when compared to bare glass (Lorite et al., 2011). Further cross-sectional analysis of these features shows the conditioning film formed from all the solutions on mica to be very heterogeneous. The largest height difference (R_{max}) was seen in the case of WML followed by SML and CAS. In comparsion with mica conditioned with respective solutions under static conditions (Chapter 3) the R_q and R_{max} values in general showed a decrease in most cases whereas the R_q and R_{max} values showed variability in case of polystyrene conditioned with respective solutions. In case of polystyrene because of the uneven nature of the surface it is difficult to determine whether the exact height difference between lowest and the highest points and hence comparison was between static and flow conditioned polystyrene was difficult.

In general the features on the surfaces reflect bulk incompatibilities of the protein solutions and demonstrate the heterogeneity of the surface which will be presented to micro-organisms for attachment.

	Mica	Polystyrene
Bare		
BSA		
TSB		

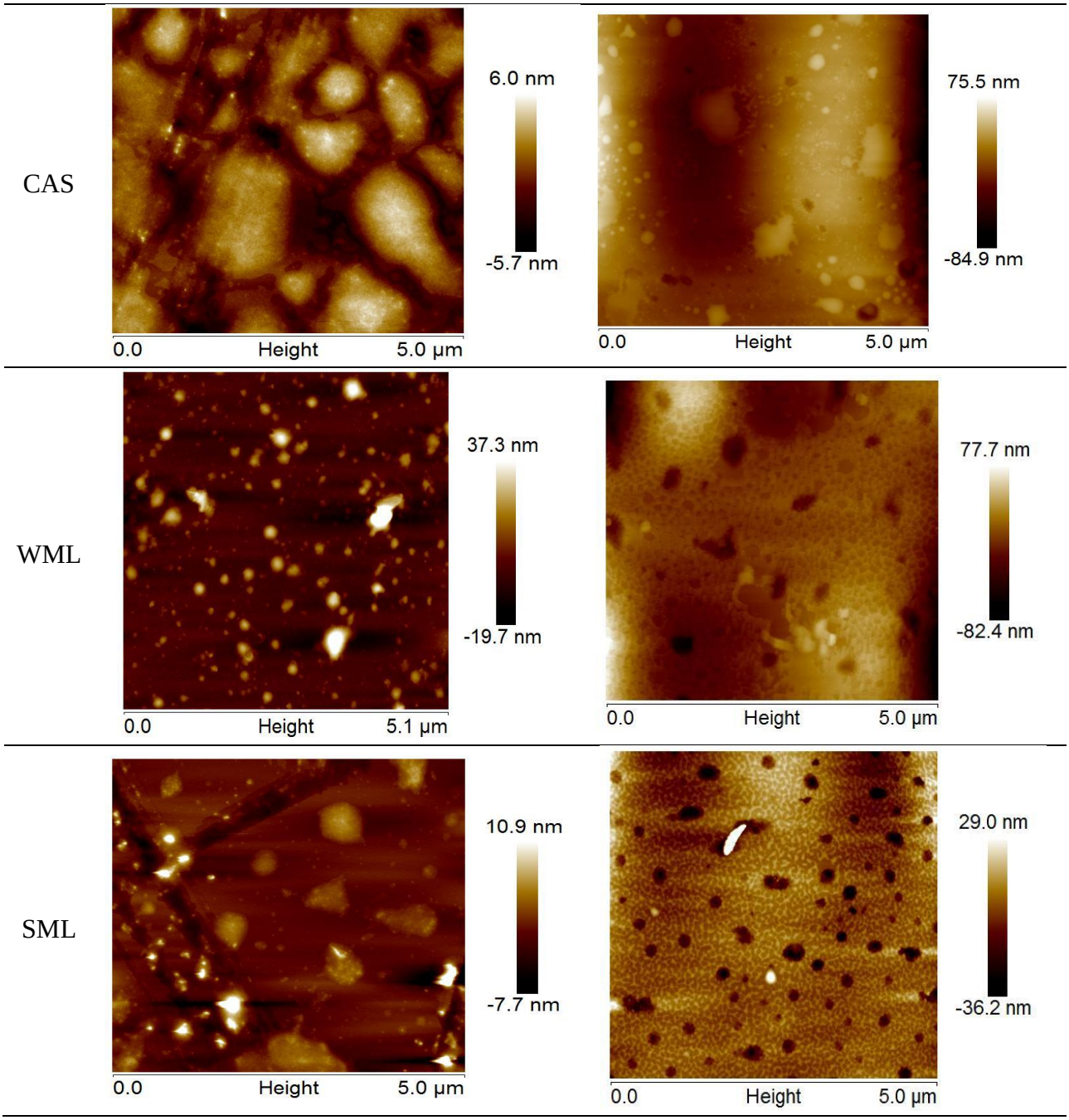


Figure 4.3: Tapping mode 2-dimensional AFM images of bare and flow conditioned mica and polystyrene surfaces

Table 4.5: AFM analysis

	R_q (nm)	R_{max} (nm)
Mica	0.11±0.03	0.32±0.04
Mica - BSA	0.81±0.26	2.31±0.42
Mica - TSB	0.45±0.08	3.09±0.72
Mica - CAS	0.25±0.43	4.83±0.08
Mica - WML	5.5±1.61	10.71±0.42
Mica - SML	2.32±0.04	5.73±1.12
PS	14.75±0.78	28.67±2.68
PS - BSA	5.91±0.47	6.13±0.80
PS - TSB	6.57±0.59	12.71±1.27
PS - CAS	9.73±5.05	14.44±3.85
PS- WML	9.39±0.80	8.81±0.53
PS - SML	12.01±5.05	7.81±0.94

4.5 Conclusions

. Flow is an important factor which affects the formation of conditioning film and the subsequent adhesion of bacteria onto surfaces. In this study the adsorption of conditioning film formed by protein solutions with a protein concentration of 33 mg/ ml were studied under flowing conditions. A modified Robbins device was used to obtain controlled laminar flow conditions and the amount of protein adsorbed onto mica and polystyrene was quantified and compared to the quantity obtained under static conditions. Approximately two times higher amount of protein was adsorbed under flow when compared to static conditions. Further, the surface energy parameters and hydrophobicity of conditioned surfaces were characterized by contact angle measurements. Dynamic light scattering measurements of conditioning solutions showed the particle sizes of the conditioning solutions were highly polydisperse, showing agglomeration of proteins in solution. The bulk solution concentration affects the adsorption by determining the aggregate state of the protein in solution. AFM images revealed the conditioning solutions adsorbed in varied pattern on mica and polystyrene surfaces under flow. This study provides some quantitative insights into adsorption of proteins under flow conditions. The set of characterization tools used in this study are appropriate to understand the protein adsorption under laminar flow in food industry environments and will be used in the future to investigate and understand the complex process of bacterial adhesion and biofilm formation on surfaces.

PREFACE TO CHAPTER 5

Due to the adsorption of organic molecules, conditioning films are usually formed on solid surfaces in aqueous environments, which can significantly impact bacterial initial adhesion and biofilm formation through altering surface tension, charge, roughness, and hydrophobicity of the surface. Bacterial adhesion to conditioning films is also influenced by characteristics of the conditioning films, bacterial cells and environmental conditions. Previous studies have shown that the conditioning film formed under static and laminar flow conditions can alter the surface properties. Therefore, in this chapter, the study was focused on effectively using surfaces that have been conditioned either under static or laminar flow settings and studying the attachment of *Listeria innocua* and *Listeria monocytogenes* on these preconditioned surfaces.

One manuscript will be prepared from this Chapter.

Nikhil Hiremath, Louise Deschênes, Hosahalli Ramaswamy, and Mohammed. Reza Zareifard. Adhesion of *Listeria innocua* and *Listeria monocytogenes* on mica and polystyrene pre-conditioned under static and laminar flow settings (in preparation)

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. Louise Deschênes, and Dr. H. S. Ramaswamy

Chapter 5. THE ADHESION OF *LISTERIA INNOCUA* AND *LISTERIA MONOCYTOGENES* ON MICA AND POLYSTYRENE SURFACES PRECONDITIONED UNDER STATIC AND FLOW SETTINGS

5.1 Abstract

Biofilm or biofouling formation on the food processing equipment has been recognized as a widespread problem. Studies have shown that macromolecules such as proteins play a significant role in biofouling of equipment surfaces by formation of a conditioning film upon which subsequent bacterial adhesion occurs. In the food industry, surfaces contacting food or food residues during handling, washing, processing etc could change bacterial attachment behavior. In order to simulate and elucidate this process, mica and polystyrene coupons were conditioned with solutions of bovine serum albumin, tryptic soya broth, sodium caseinate, whole milk and skim milk under static and flow conditions and exposed to *L.innocua* cells for 24 hours. Additional experiments were also carried out with *L. monocytogenes* on statically conditioned surfaces. Inhibitory effects of bovine serum albumin, tryptic soya broth and sodium caseinate solutions on *L. innocua* and *L. monocytogenes* were noticed. Results also indicated that the adhesion of *L.innocua* cells to statically conditioned surfaces were significantly lower than flow conditioned surfaces. *L. innocua* attached in higher numbers than *L. monocytogenes* to mica and polystyrene surfaces that were statically conditioned with sodium caseinate, whole milk and skim milk solutions. AFM images showed that the adhesion of cells to be patchy and clustered on both conditioned and bare (control) surfaces.

5.2 Introduction

Bacterial adhesion and biofilm formation on solid surfaces are considered a threat to food safety and are known to cause foodborne illness/outbreaks (Kumar & Anand, 1998; Shi & Zhu, 2009; Srey et al., 2013). This complex process has been researched over the past several decades and currently the progress of biofilm formation that has been recognized to include: (1) pre-conditioning of surface by macromolecules present in the bulk liquid; (2) transport of free-floating cells from the bulk liquid to the conditioned surface; (3) reversible adsorption or attachment of cells on to the surface bio-film, (4) irreversible attachment and production of EPS

(exo-polymeric substances), (5) cell-cell adhesion, proliferation communication and formation of three dimensional structures, (6) maturation of the bio-film containing additional polymer matrix, which stabilizes the bio-film against fluctuations, and (7) detachment of cells (Bryers, 2008; Donlan, 2002; Sauer et al., 2007; Simões et al., 2010).

The process of pre-conditioning of surface by macromolecules from the bulk fluid generally occurs within a short time when a pristine surface comes in contact with a solution containing macromolecules (Carpentier & Cerf, 1993; Chmielewski & Frank, 2003). Among the different macromolecules, the conditioning film formed by milk protein adsorption on bacterial adherence has been widely studied (Al-Makhlafi et al., 1994; Barnes et al., 1999; Helke et al., 1993; Hood & Zottola, 1997) as proteins are considered to be one of the most difficult macromolecules to be cleaned from a food contact surface (Schmidt, 1997). In addition to milk proteins, milk (Nguyen et al., 2010; Parkar et al., 2001), fish protein (Meyer et al., 2013), tryptic soy broth (Robitaille et al., 2014), aqueous cod muscle extract (Bernbom et al., 2009), mussel cooking juice (Herrera et al., 2007; Saa et al., 2009), and other complex food soils (Gram et al., 2007; Whitehead et al., 2010) on bacterial adherence has been investigated. Most of these studies have reported antibacterial effects of milk and animal proteins and milk. However, contradictory observations have also been noted and mainly attributed to the different laboratory conditions, different bacterial strains, surfaces and differing macromolecules or food soils that have been used to create a conditioning film (Palmer et al., 2007).

In relation to protein concentrations in bulk solution, most studies commonly used final proteins concentrations of 1 mg/ml (usually suspended in buffer) to pre-condition the surfaces before performing the bacterial adhesion experiments. However, Whitehead et al. (2010) used protein (or complex food soil) with concentrations of 100 mg/ ml although the proteins (or complex food soils) and bacterial cells were mixed before application on the surface rather than pre-conditioning to study the effect of proteins on bacterial adherence. Though, these studies have provided valuable insights about the influence of conditioning films on bacterial adhesion, a broader perspective to understand this complex phenomenon is necessary, especially under the common situations used in food handling and processing conditions.

The main purpose of this work was to test the influence of pre-adsorbed conditioning film formed with relatively high concentrations of proteins (33 mg/ml), dissolved in water, on adhesion of *Listeria monocytgenes* (a pathogen) and *Listeria innocua* (a non-pathogen) onto mica (hydrophilic) and polystyrene (hydrophobic) surfaces. This particular protein concentration was selected to mimic protein concentrations commonly found in milk. The pre-adsorbed conditioning films were formed with milk proteins- bovine serum albumin (BSA) and sodium caseinate (CAS), microbial culture media - tryptic soya broth (TSB). In addition, the influence of pre-adsorbed whole milk (WML) and skim milk (SML) on the adhesion of the two *Listeria* spp. was also investigated as milk represents "actual" conditioning fluids systems in the dairy industries.

5.3 Materials and methods

5.3.1 Bacterial strains and growth media.

L. innocua #156 was obtained from the Atlantic Food and Horticulture Research Centre's culture collection. This strain had been isolated from strawberry and was selected due to its capability to produce biofilms on polystyrene surfaces (Robitaille et al., 2014). *L. monocytogenes* (ATCC 19114) were obtained from the Food Research and Development Centre's (FRDC) strain collection. All the strains were stored in glycerol at -80 °C. Whenever required, stock strains were thawed, and subcultured twice (at 1% vol/vol) in TSB (Difco, Fisher Scientific, Ottawa, Ontario, Canada), and incubated at 37°C for 24 h. Cells were centrifuged at 3,500 g for 15 min at 4°C, washed once in phosphate buffered saline (PBS pH 7.4; 2mM KCl; 5mM Na_2PO_4M; 1.8mM KH_2PO_4; 0.13 M NaCl) and resuspended in PBS to obtain an initial count of 10^8CFU/mL (inoculum). The inoculum was sonicated for 10 s to remove any microbial aggregates. Peptone water (Difco, Fisher Scientific, Ottawa, Ontario, Canada) 0.1% was used as dilution medium.

5.3.2 Surfaces and conditioning solutions

Mica sheets were obtained from FRDC, Ste Hyacinthe, Quebec, Canada and polystyrene weighing dishes were purchased from Fisher scientific, Canada. Mica sheets were cleaved to reveal clean surfaces. Cleaved mica and polystyrene surfaces were cut into rectangular coupons (3.8cm x 1.9cm) with a total surface area of 14.4 cm^2. Polystyrene coupons were cleaned by

sonication in methanol for 10 min followed by sonication in water for 10 min. To remove any remaining traces of methanol, polystyrene surfaces were thoroughly re-rinsed with abundant quantities of water and dried under nitrogen.

Five types of conditioning solutions were used in the study

i. BSA (Fraction V, Cold-Ethanol Precipitated, purity > 98%, Fisher Scientific Canada),
ii. CAS (92.2% protein Nealanders International Inc. Dorval, Quebec, Canada),
iii. TSB (Difco, Fisher Scientific Canada),
iv. WML, 3.25% fat (Quebon brand, purchased from local supermarket) and
v. SML, 0% fat (Quebon brand, purchased from local supermarket).

BSA, CAS and TSB were dissolved in analytical grade ultra-pure water (18.2 MΩ•cm at 25°C) to obtain the desired concentration (33 mg/ml). TSB solution was autoclaved. WML and SML were irradiated (25kGy) to obtain sterile milk. All the solutions were stored in refrigerator and thawed to room temperature before each adsorption experiment. Solutions were used within two days of preparation and new solutions were prepared for each batch of experiments.

5.3.3 Pre-conditioning under static settings

Pre-conditioning of mica and polystyrene coupons under static settings was done by completely immersing the coupons in 25 ml of conditioning solution in polypropylene tubes (Fischer scientific, Canada) followed by incubation for 1 h at room temperature (25^{o}C).

5.3.4 Pre-conditioning under laminar flow settings

Surfaces were also pre-conditioned under continuous flow (laminar) conditions. This was done using a modified Robbins device (MRD) (Tyler Research Corporation, Edmonton, Alberta, Canada) which is a commonly used experimental flow model to study bacterial adhesion and bio-film formation in the laboratory. Five coupons of each mica and polystyrene surface were mounted on previously cleaned supports (polystyrene supports for mica and glass supports for mica) and inserted into MRD (refer chapter 4 Figure 4.1). The condition solutions were circulated by means of peristaltic pump through food grade piping system. Before each experimental run, the MRD was cleaned with 1000 ppm sodium hypochlorite (pH adjusted to 12

using 0.1M sodium hydroxide solution). This was followed by rinsing with abundant quantities of water, autoclaving, overnight drying followed by irradiation.

5.3.5 Processing and preparation of pre-conditioned surfaces

Processing of static and continuous flow pre-conditioned coupons were carried out by withdrawing them after a predetermined contact time and rinsing them with water (with wash bottle) on both sides to remove any loosely bound protein. This was followed by re-rinsing twice (sequentially) by dipping in 40ml volumes of water (in polypropylene tubes) for about 10 seconds in each tube to remove any residual loosely bound protein. The rinsed coupons were then placed in sterile empty tube and dried by blowing nitrogen gas for 10 min to remove excess water remaining on the surface. Finally, the pre-conditioned surfaces were irradiated (25 kGy) to obtain sterile surfaces and stored in sterile containers under refrigerated conditions until use.

Clean mica and polystyrene surfaces (bare or control) were also irradiated and were used as a reference for comparison.

5.3.6 Bacterial adhesion and enumeration of attached cells

Bacterial adhesion and enumeration of attached cells were carried out in a laminar flow hood. All materials used were sterilized by autoclaving prior to the experiments. Bacterial adhesions process was carried out under static conditions by immersing the pre-conditioned surfaces in bacterial suspensions (in PBS) for 24 h. The adhesion tests were performed with both static and continuous flow preconditioned surfaces for *L. innocua* and static pre-conditioned surfaces for *L. monocytogenes* respectively.

At the end of the 24 h, the coupons were removed with a forceps and immersed in 25 ml PBS to rinse any loosely bound cells. The rinsed coupons were transferred into tubes containing PBS and glass beads. The tubes with the glass beads were sonicated for 10 min and vortexed for 1min to dislodge the attached cells from the coupons. This procedure of shaking with beads was used as it has shown that the cells as well as residues of extracellular polymeric substances (EPS) could be removed more efficiently from solid surfaces (Lindsay & von Holy, 1997). After dislodging the cells into PBS, the suspensions corresponding to each individual coupon were

serially diluted in 0.1% peptone water and plated on tryptic soy agar. The plates were incubated at 37°C for 24 h prior to colony enumeration. All experiments were carried out in duplicate and repeated two times.

5.3.7 Atomic force microscopy

AFM was used to image the adhesion of *L. innocua* and *L.monocytogenes* before and after conditioning mica and polystyrene surfaces. Only a few selected images are shown. AFM experiments were conducted using a Brucker -Multimode® 8 Nanoscope (Brucker, USA) which was operated at room temperature. The images were collected using non-contact tapping mode with an Arrow™ Non-contact / Tapping™ mode - Reflex coating (NCR) probe (NanoWorld®, Switzerland). The probe consisted of a rectangular cantilever having a length and width of 160 µm and 45 µm respectively . The spring constant of these cantilevers was 42N/m, and their resonance frequency was 285 kHz. The surfaces were imaged in different scan sizes (3.5 X 3.5 to 20 X 20 µm). The image processing was carried out using the NanoScope Analysis software Version 1.40.

5.3.8 SAS analysis

Statistical analysis of all data groups was performed using SAS software Version 9.4 (SAS Institute Inc., Cary, NC). The data were analysed using the GLM (general linear model) procedure for analysis of variance. The Duncan's multiple ranges test was also used to separate means (only for influence of time and temperature). Statistical significance ($P < 0.05$) was used to perform the Duncan's multiple range tests.

5.4 Results and Discussions

5.4.1 Adhesion of *L. innocua* to mica and polystyrene surfaces preconditioned under static and continuous flow settings

The amount of proteins attached (using the same conditioning solutions and protein concentrations used in this study) onto mica and polystyrene surfaces under static and laminar flow settings were previously quantified (detailed in Table 4.2). The results showed that higher amount of proteins were adsorbed from all conditioning solutions on both mica and polystyrene

surfaces under laminar flow settings than under static settings. In addition higher amount of protein was also adsorbed on mica in comparison with polystyrene surfaces. This was the main reason, to test adhesion studies with *Listeria innocua* using surfaces that were conditioned under static and laminar flow settings.

Surfaces obtained from the conditioning process under (either under static or flow) were immersed into a *L. innocua* suspended in PBS for 24 h. The results obtained for the adhesion of *Listeria innocua* on static or laminar flow conditioned surfaces are shown in Figure 5.1 (a) and Figure 5.1 (b), respectively.

L. innocua attached in similar numbers ($p>0.05$). to both control mica and polystyrene surfaces. However, in comparison with the control (, adhesion of *Listeria innocua* on mica surfaces pre-conditioned by TSB, CAS, WML and SML (except BSA) under static conditions showed lower adherence values (Figure 5.1 a) and the observed adherence values were significantly different as seen from Table 5.1. Statically conditioned polystyrene surfaces also showed similar trends in comparison with the control polystyrene surfaces (Table 5.1).

Laminar flow conditioned surfaces showed lower adhesion values of *Listeria innocua* for surfaces that were conditioned by BSA, TSB and CAS whereas WML and SML conditioned surfaces showed similar number of adherent bacteria in comparison to control surfaces. (Figure 5.1 b).

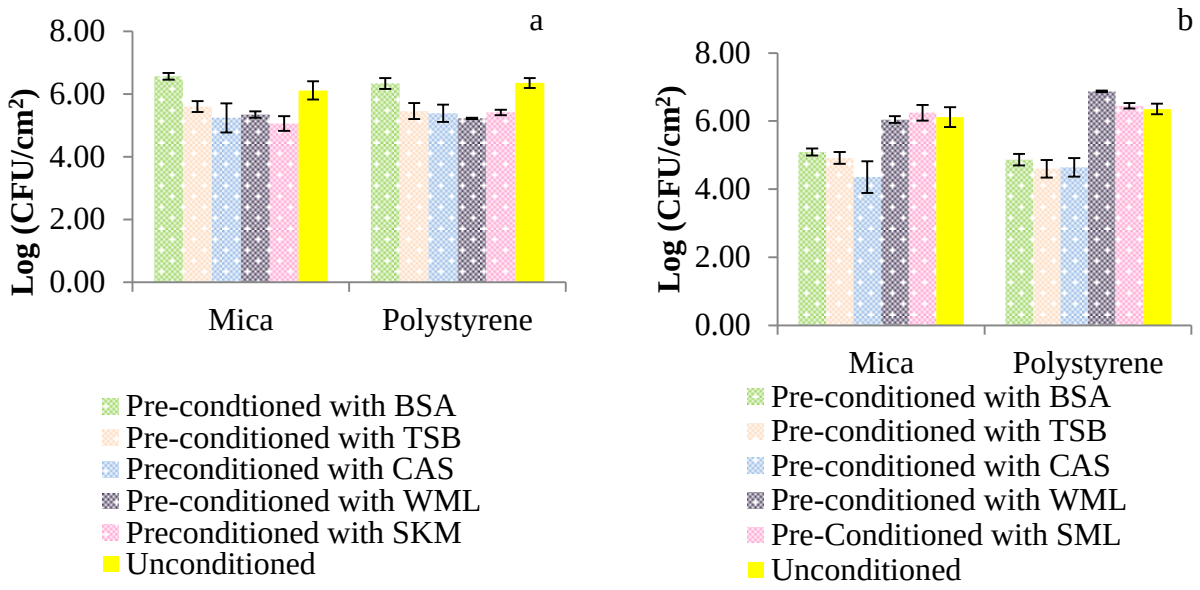


Figure 5.1: Adherent cell counts of *L. innocua* on mica and polystyrene which were pre-conditioned under static (a) and laminar flow (b) settings.

Table 5.1: Comparison of the adhesion of *L. innocua* on static or laminar flow conditioned surfaces with control surfaces using Duncan's grouping of means

		Mean amount of *L.innocua* adhered (Log CFU/cm^2)	
Surface	**Conditioning Solution**	**Flow**	**Static**
Mica	BSA	5.09 B	6.56 A
	TSB	4.92 B	5.60 C
	CAS	4.36 C	5.24 D
	WML	6.05 A	5.35 D
	SML	6.25 A	5.06 D
	Control	6.12 A	6.12 B
PS	BSA	4.87 C	6.33 A
	TSB	4.60 C	5.45 B
	CAS	4.64 C	5.38 B
	WML	6.87 B	5.29 B
	SML	6.45 B	5.41 B
	Control	6.36 B	6.36 A

Means with the same letter are not significantly different

The ANOVA analysis comparing the number *Listeria innocua* cells adhered to mica and polystyrene surfaces that were conditioned either under static or flow with each respective conditioning solution is shown in Table 5.2. It can be observed that, surfaces that were preconditioned with BSA, TSB and CAS under laminar flow settings showed lower adhesion values in comparison to surfaces that were preconditioned with the same solutions under static settings ($p<0.05$). This trend was not observed in case of polystyrene conditioned under flow with whole milk and skim milk solutions which showed higher adherence values ($p<0.05$).

Table 5.2: Comparison of the adhesion of *Listeria innocua* on static or laminar flow conditioned surfaces with ANOVA

Surface	Conditioning Solution	Mean amount of microbes adhered (Log CFU/cm^2) ± standard deviations		ANOVA analysis
		Flow	Static	
Mica	BSA	5.09±0.41	6.57±0.11	***
	TSB	4.92±0.26	5.60±0.17	***
	CAS	4.36±0.50	5.24±0.47	**
	WML	6.05±0.25	5.35±0.10	***
	SML	6.25±0.25	5.06±0.23	**
PS	BSA	4.87±0.57	6.34±0.17	**
	TSB	4.60±0.21	5.46±0.26	***
	CAS	4.64±0.21	5.39±0.27	**
	WML	6.87±0.05	5.23±0.02	***
	SML	6.45±0.05	5.41±0.08	**

*** = $p<0.001$, ** = $p<0.05$, ns= not significant ($p>0.05$)

5.4.2 Adhesion of *L. monocytogenes* to surfaces preconditioned under static settings

Figure 5.2 shows the number of *Listeria monocytogenes* cells attached to mica and polystyrene surfaced that were preconditioned with various solutions under static settings. Again, similar numbers of attached bacteria was observed as *Listeria innocua* on control surfaces ($P>0.05$). However, in comparison to control mica and polystyrene surfaces, no difference ($P>0.05$) was seen in the number of adherent bacteria on pre-conditioned surfaces (Table 5.3).

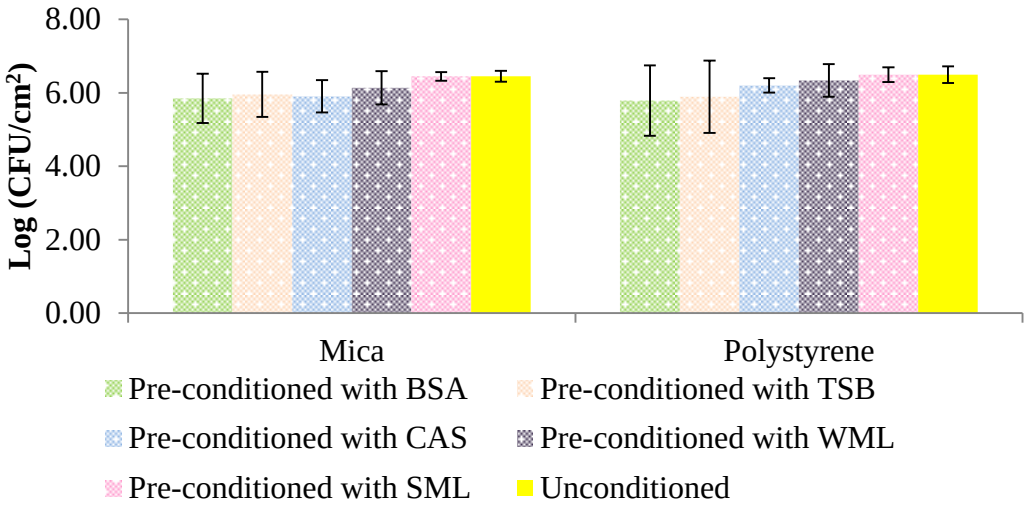


Figure 5.2: Adherent cell counts of *L. monocytogenes* on mica and polystyrene which were pre-conditioned under static settings.

Table 5.3: Comparison of the adhesion of *Listeria monocytogenes* on statically conditioned surfaces with control surfaces using Duncan's grouping of means

		Mean amount of *L.monocytogenes* adhered (Log CFU/cm^2)
Surface	**Conditioning Solution**	**Static**
Mica	BSA	5.85 B
	TSB	5.96 AB
	CAS	5.91 B
	WML	6.14 AB
	SML	6.45 AB
	Control	6.28 AB
PS	BSA	5.78 B
	TSB	5.89 AB
	CAS	6.20 AB
	WML	6.34 AB
	SML	6.50 A
	Control	6.42 AB

Means with the same letter are not significantly different.

Comparisons of the number of *L.innocua* and *L.monocytogenes* cells adhered to mica and polystyrene conditioned surfaces under static conditions with ANOVA analysis are shown in Table 5.4. It can be observed that, the surfaces that were preconditioned with CAS WML and SML showed higher number (P<0.05) of *L. monocytogenes* adhered onto surfaces.

Table 5.4: Comparison of the adhesion of *L. innocua* and *L. monocytogenes* on statically conditioned surfaces with ANOVA

		Mean amount of microbes adhered under static conditions (Log CFU/cm^2) ± standard deviations		
Surface	**Conditioning Solution**	***L.innocua***	***L.monocytogenes***	**ANOVA analysis**
Mica	BSA	6.57±0.11	5.85±0.67	**
	TSB	5.60±0.17	5.96±0.61	ns
	CAS	5.24±0.47	5.91±0.44	**
	WML	5.35±0.10	6.14±0.45	**
	SML	5.06±0.23	6.45±0.12	***
	Unconditioned	6.12±0.29	6.28±0.15	ns
PS	BSA	6.34±0.17	5.78±0.96	ns
	TSB	5.46±0.26	5.89±0.99	ns
	CAS	5.39±0.27	6.20±0.20	***
	WML	5.23±0.02	6.34±0.45	***
	SML	5.41±0.08	6.50±0.20	***
	Unconditioned	6.36±0.15	6.42±0.23	ns

*** = p<0.001, ** = p<0.05, ns= not significant (p>0.05)

The effect of pre-conditioning of milk and milk proteins on bacterial adhesion on surfaces (commonly under static conditions) has been systematically tested. Varied observations are obtained depending upon the experimental conditions that have been used in these studies. Milk and individual milk proteins (α-casein, β-casein, κ-casein, and α-lactalbumin) were found to reduce bacterial adherence on stainless steel surfaces in some studies (Barnes et al., 1999; Helke et al., 1993). Al-Makhlafi et al. (1994) observed inhibition of *L. monocytogenes* pre-reconditioned with BSA and β-LG, while on the other hand, an enhancement in attachment was reported on hydrophobic surface on BSA pre-conditioned hydrophilic surface. Robitaille et al. (2014) also found antibacterial effect of milk proteins on the adhesion of *L.innocua* on

polystyrene, however they observed that the inhibitory effects of the milk protein conditioning films was short-lived. Nguyen et al. (2010) showed the stainless steel surfaces pre-conditioned with acidified skim milk gave lower adherence values after 30 min of exposure; however, the numbers increased after 12 h. In addition they reported that a thicker layer of milk had a stronger ability in reducing bacterial attachment which was mainly attributed to the casein in milk. Our results from previous experiments showed that higher amount of proteins was adsorbed onto both mica and polystyrene surfaces under laminar flow. Consequently, higher quantity of proteins may show a stronger ability in reducing bacterial attachment. However, this was observed only with surfaces pre-conditioned with BSA, TSB and CAS on *L. innocua* strains. Our results also showed reduction in the number of adhered *L. innocua* on surfaces pre-conditioned with TSB, CAS, WML and SML under static settings. Electrostatic repulsion between the surface of *L.innocua* and mica or polystyrene may also possibly explain the reduced amounts of bacterial adherence as both the bacteria and preconditioned solid surfaces were negatively charged. The inhibition of *Psuedomonas aeruginosa* and *Staphylococcus aureus* attachment to stainless steel and gold caused due to electrostatic repulsion of negatively charged surfaces has also been observed (Birkenhauer & Neethirajan, 2014).

The reduction in the number of *Listeria innocua* attachment was not observed with pre-conditioned surfaces with BSA under static conditions and for pre-conditioned surfaces with WML and SML under flow settings. The difference in the properties of conditioning layers can reflect differences in the type and/or conformation of the adsorbed proteins and hence can affect it's adhesion.

The adhesion of *L. monocytogenes* on preconditioned mica and polystyrene surfaces showed no difference in attached numbers in comparison with respective bare surfaces indicating that preconditioning mica and polystyrene did not affect the adhesion dynamics of L. monocytogenes However, the number of *L. monocytogenes* attaching to both mica and polystyrene surfaces was significantly higher than the *L. innocua* for surfaces preconditioned with CAS, WML and SML under static conditions showing differential attachment dynamics between *L. monocytogenes* and *L.innocua* strains. Similar observations have also been noticed in the adhesion behaviour of pure culture of *L. monocytogenes* and *L. innocua* onto stainless steel and aluminium surfaces where higher number of *L.monocytogenes* attached to both surfaces in

comparison to *L. innocua* cells, however, on bare (not conditioned) surface (Koo et al., 2014). It has been reported that the presence of PrfA (a virulence regulator) which plays a significant role in biofilm formation in *L. monocytogenes* but not in *L. innocuas* may reflect the differential adhesion dynamics of these two closely related species (Zhou et al., 2011). In addition other factors such as differences in cell surface properties and growth characteristics of *Listeria monocytogenes* and *Listeria innocua* (Meylheuc et al., 2002) may have resulted in the difference of adhesion behaviour on surfaces.

5.4.3 AFM images

Results discussed above reveal that conditioning film formed under static and flow conditions affect the adherence of *Listeria* to mica and polystyrene surfaces. We further used AFM to image the distribution of cells on conditioned and on clean or bare (control) surfaces. AFM images of *L.innocua* adhesion on polystyrene without any conditioning film (a), *L. monocytogenes* adhesion on polystyrene without any conditioning film (b) and *L. monocytogenes* adhesion on mica after preconditioning with BSA under static conditions (c) are shown in Figure 5.3. The preconditioning was carried out for 1 h and bacterial adhesion for 24 h at room temperature conditions. Observations under the AFM microscope showed that both bacteria had the capacity to adhere to both on both control and conditioned surfaces. The adhesion of cells was seen to be patchy seem to adhere in clusters or aggregates on both conditioned and unconditioned surfaces. We also observed that the crevices, scratches in the polystyrene coupons harbored considerable amount of bacteria, but we could also identify many individual cells and micro-colonies on the entire surface. The attachment pattern observed in the present report were similar to the observations made by Di Bonaventura et al. (2008) and Moltz and Martin (2005) where they observed that *L. monocytogenes* biofilm formed aggregates of cells which were bound by extracellular polymeric substances or held together in a meshlike webbing on glass, stainless steel and polystyrene surfaces. Clustered or aggregated attachments patterns have also been observed in case of *E.coli* and *P. fluorescens* attachement on stainless steel surfaces (Bagge et al., 2001; Whitehead et al., 2010).

Overall our results showed the conditioning film formed from higher bulk solution concentrations under static and laminar flow conditions might change the dynamics of bacterial

adhesion. However, more research is needed to better define the influence of these conditions on physical and chemical properties that directly influence the interaction of bacterial cells with surfaces.

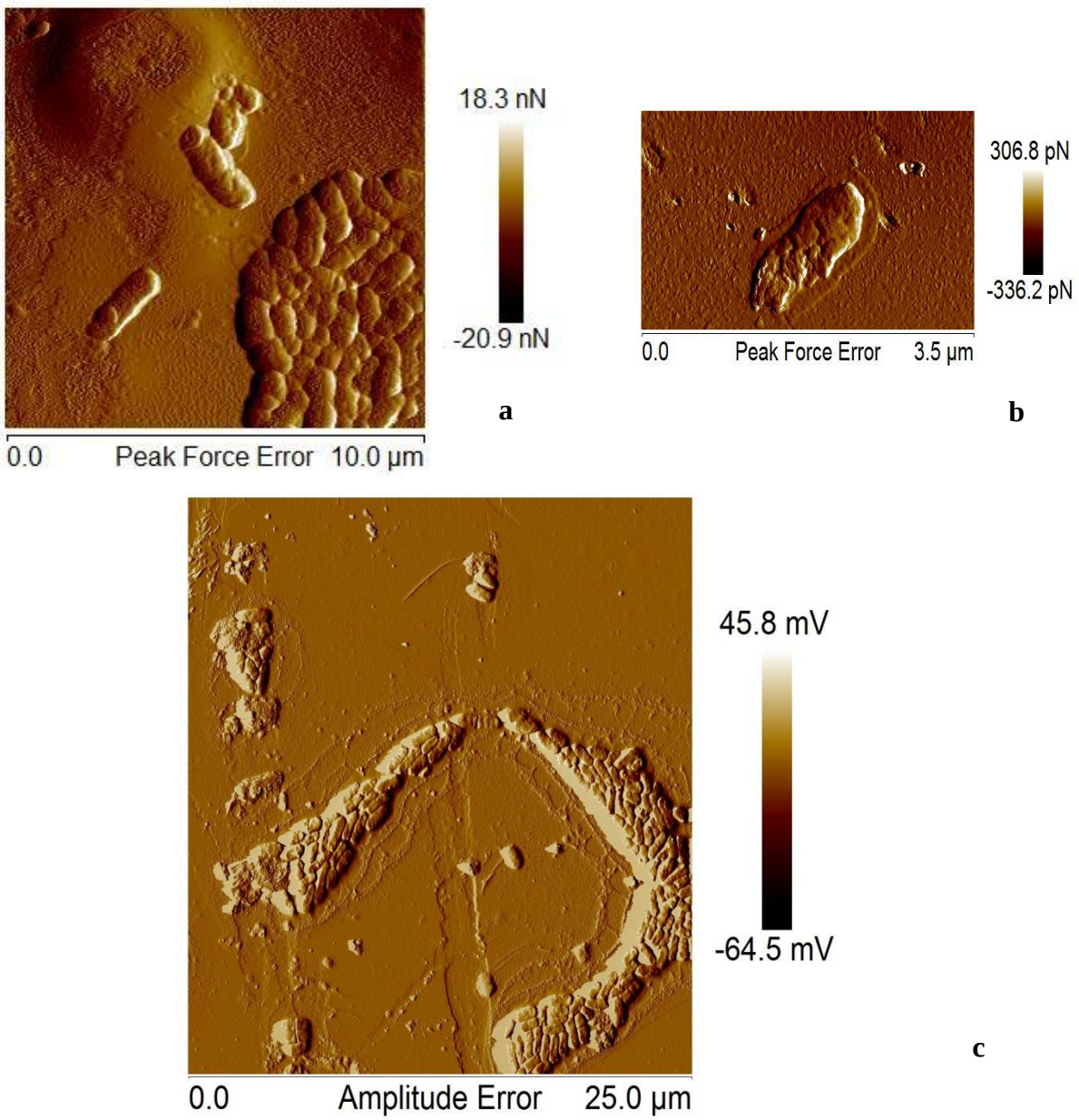


Figure 5.3: AFM images *L. innocua* and *L. monocytogens* adhering on mica and polystyrene coupons

a) *L.innocua* adhesion on polystyrene without any conditioning film; b) *L. momocytogenes* adhesion on polystyrene without any conditioning film; c) *L. monocytogenes* adhesion on mica after preconditioning with BSA under static conditions

5.4.4 Conclusions

Bacterial biofilm formation is a complex process and depends on prevailing environmental conditions. In food industry settings, the presence of conditioning film is known to affect bacterial attachment to contact surfaces. Conditioning films become the new interacting surfaces for attached bacteria and affect their removal during cleaning. The results of this study indicated that pre-conditioning of surfaces either under static or laminar flow settings resulted in different adhesion dynamics of *L. innocua* and *L. monocytogenes* strains. In particular, the observation that *L. innocua* adhered less to surfaces that were conditioned under flow suggests that pre-conditioning under flow changes the adhesion conditions of bacteria onto a surface. Further research exploring the interaction between conditioned surfaces with bacteria may shed even more light on the reported phenomena.

Chapter 6. GENERAL CONCLUSIONS, CONTRIBUTION TO THE KNOWLEDGE AND RECOMMENDATIONS

GENERAL CONCLUSIONS

Surface conditioning is the process of covering a surface by a film of molecules from the bulk fluid and considered as the foremost step before bacteria can attach to the surface. Surface conditioning can modify the physicochemical properties of the surface consequently changing the ability to attract bacterial cells. The nature of the conditioning films may be quite different depending on the kind of environment the surface is exposed to. In food industry, surface conditioning by macromolecules such as proteins can cause fouling of food contact surfaces and along with bacterial adhesion can be detrimental to the food industry. Hence it is necessary to understand the adsorption behaviour of proteins in food industry environments using model hydrophilic and hydrophobic surfaces. The amount of protein adsorbed to model surfaces was quantified and physicochemical surface thermodynamic properties of surfaces were characterized. The amount of protein adsorbed was predicted by applying two thermodynamic models by calculating the total free energy of interaction. Although the models showed favorable adhesion of all proteins on polystyrene and most proteins of mica surface it could not accurately predict the amount of protein adsorbed onto mica and polystyrene surfaces.

Previous conditioning experiments were performed under static conditions. However, it is well known that several factors affect the process of protein adsorption and subsequently the bacterial adhesion and biofilm formation to surfaces. Flow is an important unit operation in the food industry and considered to be an important factor that affects protein adsorption to surfaces. A modified Robbins device was used to obtain laminar flow conditions. The amount of protein adsorbed under flow was quantified and surface conditioned under flow were characterized. The amount of protein adsorbed under flow were found to be significantly higher than the under static conditions. Flow conditioned surfaces showed changes in the surface thermodynamic characteristics when compared to statically conditioned surfaces.

A large number of factors contribute for the adhesion processes leading to bacterial adhesion and biofilm formation. The effect of pre-conditioning surfaces under static, on the adhesion of two bacteria *L. innocua* (non-pathogenic) and *L. monocytogenes* (pathogenic) was tested. In addition the effect of pre-conditioning surfaces under flow, on the adhesion *L. innocua* was also examined. *L. innocua* adhered in higher numbers on both the surfaces that were statically conditioned with whole and skim milk in comparison with *L. monocytogenes* conditioned with the same soltuion under static settings. The attachment of *L.innocua* on flow conditioned surfaces (with BSA, TSB and CAS) was lower than on statically conditioned surfaces with the same solutions. However, opposite was found with surfaces that were conditioned with whole and skim milk solutions. This shows that the adhesion dynamics of bacteria can change with respect to the environment.

CONTRIBUTION TO THE KNOWLEDGE

It is well recognized that the pre-conditioning of surfaces is the first step in the process of initial bacterial attachment to the surface. In addition it is well known the formation of conditioning film (such as proteins) on surfaces has a significant effect on the surface physicochemical properties and bacterial attachment to surfaces. No quantification studies and characterization were done using high protein solution concentrations as these conditions mainly represent food industry situations. In this study the surface bound protein was quantified after adsorption from solutions with high protein concentrations. In addition surface thermodynamic parameters were characterized after adsorption from solutions with high protein concentrations high protein solution concentrations.

Several studies have applied the thermodynamic models to evaluate bacterial adhesion to surfaces in food industry environments, however with conflicting opinions. A few studies have applied these models to study protein adsorption to surfaces mainly in marine and waste water treatment industry. Our study showed that although the models showed favorable adhesion of proteins onto surfaces it could not precisely predict the amount of protein adsorbed onto surfaces.

No work has been done before to compare static and flow adsorption behaviour of proteins on surfaces from solutions with high protein concentrations. Our study showed higher

amount of protein absorbed under flow in comparison with static conditions and changes to the surface thermodynamic properties of flow conditioned surfaces were also observed.

Although the effect of preconditioning on bacterial adhesion has been well studied, few studies have been reported in the literature to study the bacterial adherence to surfaces conditioned than have been conditioned under flow especially in food industry environments. It can be said now that flow conditioned surfaces can change the dynamics of bacterial adhesion and subsequent formation of biofilms.

RECOMMENDATIONS FOR FUTURE RESEARCH

This research has demonstrated several important findings. Meanwhile, it also showed some ideas of interest for future research and development, which could be summarized as follows:

Investigating the adsorption of other types of macromolecules such as carbohydrates and fats by using other biochemical methods to quantify and characterize surface bound macromolecules.

Using stainless steel and plastics as a model surfaces to study the adsorption behaviour of real foods such as oils, honey etc.

Quantifying and characterizing surface bound macromolecules after adsorption under turbulent flow settings.

Investigating the adhesion of other pathogenic bacteria such as (*E. coli)* to conditioned and bare surfaces under static, laminar and turbulent flow settings.

Quantifying the interactions between bacteria and conditioned surfaces using the thermodynamic and XDLVO theories.

Exploring remedial methods for cleaning and disinfection of surfaces that have conditioned and contaminated under food industry settings.

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