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The effect of silver ion-implantation of stainless steel on bacterial adhesion and biofilm formation

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The effect of silver ion-implantation of stainless steel on bacterial adhesion and biofilm formation

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ABSTRACT

Biofilms comprise an assembly of microbial communities attached to a surface and enclosed in a polysaccharide matrix. Biofilms are a problem in dairy manufacturing plants where they cause biofouling of the stainless steel surface, resulting in product contamination and the need to shorten manufacturing runs for frequent cleaning. The equipment has to be thoroughly cleaned at regular intervals to remove biofilms. Clean-In-Place (CIP) procedures used in dairy manufacturing plants are not effective enough to remove all the biofilm cells as the extracellular polysaccharide (EPS), an integral part of a biofilm, reduces the penetration of cleaning agents. One possible alternative method to control biofilm growth involves surface modification of the stainless steel by implanting silver ions to prevent the attachment of viable bacteria that would otherwise form biofilms.

Stainless steel coupons, implanted with 1×10^{16} silver ions per cm^2, and control stainless steel coupons were tested for the attachment of *Streptococcus thermophilus* and *Pseudomonas fluorescens* in various media for up to 30 minutes. Biofilm formation and EPS production for up to 24 hours was studied on the silver-implanted and control coupons in whole milk, skimmed milk and whey. It was found that there was higher attachment (0.49 and 0.18 log CFU per cm^2 of *S. thermophilus* and *P. fluorescens*, respectively) on the stainless steel coupons than on the silver-implanted coupons in saline. In the presence of milk and whey, the difference in the attachment of bacteria on the two coupons reduced. Biofilm studies showed that the number of bacteria colonising both types of coupons was not statistically significantly different ($P > 0.05$). While the Live/Dead® BacLight™ Bacterial Viability stain showed that there were a few dead cells on the silver-implanted surfaces, scanning electron micrographs showed that the bacteria attached to a conditioning layer formed by the milk and whey proteins. Furthermore, both bacteria produced EPS, which, along with the conditioning film, might have masked the effect of silver ions from bacteria, resulting in similar numbers of bacteria present on the test and control coupons. Thus, due to the shielding effect of EPS and the conditioning film, the silver-implanted surfaces may be of limited practical value in the dairy industry.

LIST OF PRESENTATIONS

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LIST OF ABBREVIATIONS

%	percentage
℃	degree celsius
µl	microlitre
µg	microgram
Ag	silver
Al^{2+}	aluminium ion
Co^{2+}	cobalt ion
cm	centimetre
Cu^{2+}	copper ion
$C_6H_5K_3O_7.2H_2O$	tri-potassium citrate dihydrate
$CaCl_2.2H_2O$	calcium chloride dihydrate
CDC	centre for disease control
CIP	clean-in-place
CFU	colony forming units
DLC	diamond-like carbon
DNA	deoxyribonucleic acid
EPS	extracellular polysaccharide
g	acceleration due to gravity
g	gram
g/l	gram per litre
H_2SO_4	sulphuric acid
keV	kiloelectronvolt
KH_2PO_4	potassium dihydrogen phosphate
KOH	potassium hydroxide
L	litre
LPS	lipopolysaccharides

m	metre
M	molar
mbar	millibar
mm	millimetre
Mg	milligram
ml	millilitre
mM	millimolar
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
MoS_2^{2+}	molybdenum disulphide ion
N^+	nitrogen ion
Nm	nanometre
$Na_3C_3H_5O(CO_2)_3.2H_2O$	sodium citrate dihydrate
Ni	nickel
O^+	oxygen ion
O.D.	optical density
Pb^{2+}	lead ion
PTFE	polytetrafluoroethylene
R_a	average roughness
Rpm	revolutions per minute
SAS	statistical analysis system
SiF_3^+	silicon trifluoride ion
SEM	scanning electron microscopy
SS	stainless steel
TiC	titanium carbide
TSB	tryptic soy broth
UHT	ultra-high temperature
USFDA	United States food and drug administration

UV	ultraviolet
v/v	volume by volume
Zn^{2+}	zinc ion

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CHAPTER ONE: INTRODUCTION

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Biofilms can be defined as bacteria and their exopolysaccharide matrix growing on a surface (Donlan, 2002). Bacterial attachment is the first step in biofilm formation. Once the bacteria attach firmly to a surface, they multiply, produce extracellular polysaccharides (EPS) and form biofilms. Cells embedded in a biofilm have many advantages over the planktonic cells of the same species. Cells in a biofilm can easily acquire new genetic traits, including antibiotic resistant genes, due to their proximity to the other cells. EPS protect the cells from desiccation, provide nutrients and mechanical stability to the biofilm structure and help in restricting the penetration of biocidal agents into the biofilms (Donlan, 2002). Thus, the cells in a biofilm have more chances of survival in a harsh environment than the planktonic cells.

Bacteria can attach to the stainless steel surfaces in food manufacturing plants, including the dairy plants, and form biofilms. Biofilms formed in the dairy plants pose a threat to the smooth and continuous running of the plant (Flint et al., 1997 a). They result in reduced heat transfer efficiency, enhanced fluid frictional resistance, biofouling of the surface and product contamination. As a result, the plant has to be cleaned every few hours using the clean-in-place (CIP) procedure which usually involves an alkali wash, a water rinse, followed by an acid wash and a final water rinse (Bremer et al., 2006). The cleaning routines every few hours result in shortened production runs and increased operational costs (Brooks & Flint, 2008). Moreover, the CIP procedures are not effective in removing all the bacteria embedded in the biofilms as EPS restricts the penetration of cleaning agents into the biofilms (Brooks & Flint, 2008). Thus, it is better to prevent bacterial attachment and biofilm formation in the first place rather than treating it once the biofilm has formed.

An alternate method of controlling biofilms is to modify the surface of the stainless steel by ion implantation so that fewer bacteria attach to it or the viability of the cells is affected upon their attachment to the modified surface. Implantation of ions modifies the first few micrometres of the surface without changing any of its bulk properties (Rautray et al., 2010). Because the process is carried out in vacuum, it is highly controllable, repeatable and gives a clean finish.

Silver is a known antimicrobial agent and is used to treat wounds and infections (Ahearn et al., 1995). Implanting silver ions into the stainless steel surface may produce

an antibacterial surface which may result in the reduced bacterial attachment and or decreased bacterial viability and thus, may lead to diminished biofilm formation.

The first objective of this study was to determine the initial (30 minutes) attachment on stainless steel and silver-ion implanted stainless steel coupons. The second objective was to study the biofilm formation (24 hours) and EPS production by the bacteria in the presence of dairy substrates on the silver-implanted and the control stainless steel coupons. In general, the aim of this study was to contribute to the understanding of the effect of the silver-ion implantation on bacterial attachment and viability and subsequent biofilm formation. Information generated in this study will support future work on the selection and development of better food contact surfaces which may limit bacterial attachment or biofilm formation.

CHAPTER TWO: LITERATURE REVIEW

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2.1 BIOFILMS

2.1.1 Biofilms

Biofilms can be defined as complex and well-structured communities of microorganisms attached to a surface and enclosed in a self-produced matrix of EPS (Whitchurch et al., 2002). They form on both biotic and abiotic surfaces including living tissues, minimally processed fruits and vegetables, implanted medical devices and food processing equipment. The thickness of the film can range from few micrometers to several millimeters (Brooks & Flint, 2008). Biofilms are composed of microbial cells, enzymes, proteins, bacteriocins, EPS, low mass solutes and nucleic acids released due to cell lysis (Sutherland et al., 2004). Mixed species biofilms are generally more commonly found in nature as they are more stable than monospecies films (Brooks & Flint, 2008). Biofilms are a major concern in food processing industries, resulting, among other challenges mentioned earlier, in product contamination, disease transmission and reduced shelf life of food products (Chmielewski & Frank, 2003).

2.1.2 Formation of biofilms

Biofilm formation is a multistep process, often beginning with the conditioning of the surface and attachment of bacteria on the surface. As the bacteria multiply, the biofilm matures, thus, producing EPS and developing water channels. A few cells then bud off the biofilms and are released into the environment, where they may attach to a new place and start fresh biofilm.

2.1.2.1 Conditioning of the surface

Biofilm formation is often preceded by the development of a conditioning layer of organic material on a substrate on which the biofilm will eventually develop (Kumar & Anand, 1998). These conditioning films form when organic molecules are attracted to the substrate by a variety of interactions, of which charge plays a major role. Adsorption of organic molecules on the surface can occur within a few seconds of exposure (Chmielewski & Frank, 2003). The conditioning film alters the physico-chemical properties of the surface including the hydrophobicity, surface free energy and

electrostatic charges and also results in increased nutrient concentration on the substrate surface, all influencing microbial attachment (Dickson & Koohmaraie, 1989; Kumar & Anand, 1998; Jeong et al., 2009). However, the conditioning film is not absolutely necessary for attachment. Flint et al. (1997 b) reported the attachment of *Streptococcus thermophilus* and *Bacillus cereus* to clean stainless steel surface within 60 seconds, thus, showing that surface conditioning may not be essential for attachment.

2.1.2.2 Adhesion of cells

Adhesion of microbial cells to the conditioning layer is the second step in the formation of biofilms. Adherence of cells to the conditioning layer depends on a number of factors like nutrient availability, the growth phase of cells, transportation of planktonic cells and physicochemical properties of the bacterial cell surface (Van Loosdrecht et al., 1990; Kumar & Anand, 1998). Adhesion of bacterial cells is a two-step process. The first step is reversible adhesion. In this step, the interactions are weak long range forces such as van der Waals' forces, electrostatic forces and hydrophobic forces (Kumar & Anand, 1998). The bacterial cells are not tightly attached and can be easily removed by applying moderate sheer force. The second step is irreversible attachment. This involves short range forces like dipole-dipole forces, hydrogen, covalent and ionic bonds (Kumar & Anand, 1998). Bacterial appendages like flagella, pili and fimbriae form a bridge between the cell and the surface, which makes the bacteria irreversibly attached. Extracellular DNA, found in the EPS matrix of *Bacillus cereus*, was also shown to help with the attachment and biofilm formation of the cells on the surface (Vilain et al., 2009). The mutants lacking in purine biosynthesis genes were unable to form biofilms. Irreversible attachment requires a few hours of contact time (Chmielewski & Frank, 2003). To remove irreversibly attached bacteria, kinetic energy and detergents or enzymes are required. Once attached, the entire biofilm develops through the growth of the bacteria attached to the surface and through addition of more bacteria (Bos et al., 2000).

2.1.2.3 Formation of a microcolony

Irreversibly attached cells multiply to form microcolonies, which enlarge to form many layers of cells, thus, covering the surface. Microcolony formation is often accompanied by EPS production. EPS include hetero- and homopolysaccharides containing glucose, fructose, mannose, galactose, mannuronic acid and gluco-uronic acid complexes

(Poulsen, 1999). Attachment serves as one of the stimuli for EPS production, besides osmotic pressure, pH and temperature (Chmielewski & Frank, 2003). Cell-cell signaling and the production of EPS help in maintaining the microcolony (Simões et al., 2010).

2.1.2.4 Biofilm formation and maturation

Continuous attachment of cells over a period of time and their growth results in the formation of biofilms. Suitable growth conditions lead to the maturation of biofilms over time. Biofilm may be monolayered or multilayered. In mature biofilms, cells are not evenly distributed. Rather, microcolonies are embedded in EPS and interspersed with water channels (Chmielewski & Frank, 2003).

2.1.2.5 Detachment and dispersal of cells

Daughter cells are detached individually or are sloughed off from mature biofilms. Sloughing is the process in which large chunks of biomass detach from a biofilm at different intervals (Kumar & Anand, 1998). Detached cells are carried over to new places to start fresh biofilms. A number of factors have been implicated in the detachment of bacterial cells. These include the high growth rate of biofilms, shear forces, high levels of carbon inside the biofilm and the disruption of biofilm structure due to accumulation of insoluble gases and acid over time (Bryers, 1987; Wimpenny et al., 2000; Donlan, 2002; Chmielewski & Frank, 2003). The environmental stress results in the dispersal of cells which attach and form biofilms at new places. Bacteria tend to form a biofilm on a suitable surface as biofilm mode of growth confers many advantages on the cells.

2.1.3 Ecological advantages of biofilm formation

There are a number of advantages to microbial cells living in biofilms. EPS produced during biofilm formation has many protective functions. It helps in accumulating nutrients, sequestering metals and other toxic substances and impeding the access of biocidal agents to the cells (Carpentier & Cerf, 1993). Enhanced nutrient concentration at the site of growth helps the cells to proliferate. EPS also protects the cells against environmental stress like change in pH, desiccation and osmotic shifts (Davey & O'toole, 2000). As a structural element of biofilms, EPS provides mechanical stability, helps in the retention of extracellular enzymes and prevents loss of lysed cell components (Flemming, 2002).

Apart from the advantages conferred by EPS, there are additional ecological advantages for microbial cells in the biofilm. Cells embedded in the biofilm matrix are protected from shear forces due to decreased turbulence (Sutherland, 2001). Acquisition of new genetic traits is easier in biofilms as compared to planktonic cells. The biofilm is an ideal environment for bacterial conjugation as the shear rate in a biofilm is negligible and there is closer cell-to-cell contact (Donlan, 2002). The antibiotic resistance genes, located on plasmids, can easily pass from cell-to-cell, spreading the antimicrobial resistance in biofilm. Thus, due to all the above advantages, biofilms grow abundantly on abiotic surfaces, including food-contact surfaces.

2.2 BIOFILMS IN THE DAIRY INDUSTRY

Biofilms can form in the dairy industry due to the contamination from raw milk and the processing environment. While the milk is actually a sterile secretion, it gets contaminated on coming into contact with the udders of the cow or the milking equipment (Flint et al., 1997 a). The milk tankers, transporting milk over long distances, are another potential source of contamination of the raw milk where temperature variations in the different parts of the tankers provide ideal conditions of bacterial growth (Teh et al., 2011). In the manufacturing plant, the milk is pasteurised at 72 °C for 15 seconds to kill any harmful bacteria which may be present in the raw milk. However, if contamination still occurs in the final product, it may be due to the biofilm formation on and in milk processing equipment which may contaminate the pasteurised milk and its products.

2.2.1 Process biofilms

Process biofilms refer to the biofilms that are unique to dairy processing plants and are found on surfaces like heat exchangers through which the dairy products flow (Flint et al., 1997 a). In plate heat exchangers, generally single species of bacteria dominate as the high temperature kills heat-sensitive Gram negative bacteria and allows selective growth of only thermophilic species (Flint et al., 1997 a). Process biofilms mainly constitute bacteria, EPS, milk residues, proteins and calcium phosphate (Flint et al., 1997 a ; Mittelman, 1998). Milk proteins form a conditioning film to which bacteria adhere quickly and form a biofilm (Hood & Zottola, 1997 b). A variety of different species of bacteria, including spore formers and thermophilic bacteria, form biofilms in different parts of dairy manufacturing plant. *Streptococcus thermophilus, Anoxybacillus*

flavithermus, *Pseudomonas* spp., *Geobacillus* spp. and *Bacillus cereus* are major biofilm producers in dairy plants. The desire to increase the operating time of a dairy manufacturing plant, automation of the plant, the use of complex equipment and strict microbial requirements for manufactured dairy products have resulted in biofilms being a major nuisance in dairy plants (Bremer et al., 2006).

2.2.2 Effects of biofilms on dairy manufacturing plants

Biofilms in dairy manufacturing plants are the most common source of product contamination (Simões et al., 2010). Bacteria present on the processing equipment and detaching from biofilms can cause contamination of milk and milk products like cheese and milk powder. Undesirable bacteria present in biofilms can result in decreased product yields by fermentation and by the production of proteolytic and lipolytic enzymes resulting in reduced lactose, protein and milk fat as well as associated sensory defects (Simões et al., 2010; Tang et al., 2010). On biofilm maturation, some cells are released into the process fluid which contaminates downstream sections of the dairy plant. This may lead to microbial growth in some downstream areas of the dairy plant which might not have been conducive for biofilm growth otherwise (Bansal & Chen, 2006). Biotransfer potential i.e. ability of bacteria present before and after cleaning procedures to contaminate products during processing, also increases when bacteria are present in biofilms (Hood & Zottola, 1995). Biofilms also result in the biofouling of the equipment. Biofouling refers to the undesirable deposition of microorganisms and their decay products onto surfaces which are in direct contact with the flowing product (Kumar & Anand, 1998). Biofouling of plate heat exchangers results in reduced heat transfer efficiency (Mittelman, 1998; Azevedo et al., 2006). Biofilms formed in the heat exchanger can reduce the heat transfer efficiency by almost 200%. Similarly, they can result in increased fluid friction resistance by 200-300%, which further leads to high power consumption and maintenance costs (Bryers, 1987). Biofilms also catalyze chemical and biological reactions which cause metal corrosion in pipelines and result in equipment deterioration and blockages in pipes (Beech, 2004). As a result, the manufacturing run of the plant has to be shortened for cleaning, which further increases operational costs (Flint et al., 1997 a; Brooks & Flint, 2008). The consequences of biofilm development can be severe. The key to biofilm development is the attachment of bacteria to surfaces.

2.3. FACTORS AFFECTING BACTERIAL ATTACHMENT TO SURFACES

There are many factors affecting the attachment and subsequent growth of bacterial cells on the surface. Different properties of bacterial cells, liquids and surfaces influence attachment in different ways. Bacterial cell properties that affect the adhesion include number of cells, hydrophobicity, cell surface charge, growth phase and EPS production. The properties of the liquid in contact with the solid surface that affect adhesion are temperature, pH, suspended matter and colloids, shear forces, viscosity and surface tension. The surface parameters that influence adhesion are surface charge, roughness, chemical composition, surface tension and hydrophobicity (Flemming & Schaule, 1988). However, in this review, the main focus will be on the substratum and cell surface properties that can influence bacterial adhesion.

2.3.1 Hydrophobicity of the substratum

The degree of hydrophobicity of the substratum is often implicated as a major factor in bacterial adhesion to the surface (Chmielewski & Frank, 2003). However, there are conflicting reports as to whether highly hydrophobic or hydrophilic surfaces favour bacterial adhesion. It has been reported that bacteria attached in higher numbers to relatively hydrophobic surfaces, i.e. surfaces with low surface energy, like polytetrafluoroethylene (PTFE), stainless steel 316 and stainless steel 304 rather than glass (Teixeira et al., 2005). Similarly, Flint et al. (2000) reported that among the stainless steel samples, the highest number of bacteria attached to the most hydrophobic samples, while noting that other factors like substrate charge might have affected adhesion. It has been suggested that a more hydrophilic surface be used in dairy industry instead of stainless steel, which being hydrophobic attracts bacteria (Bower et al., 1996). On the contrary, more biofilm formation has been reported to occur on hydrophilic surfaces rather than the hydrophobic surfaces (Blackman & Frank, 1996; Hyde et al., 1997; Sinde & Carballo, 2000). Apart from these, Flint et al. (1997 b) reported no statistically significant relationship between surface hydrophobicity and the attachment of thermoduric streptococci. It is worth keeping in mind that all the researchers used different bacteria and different methods of determining the surface hydrophobicity and bacterial attachment which may have resulted in conflicting results. Moreover, surface hydrophobicity may not be the only factor affecting the bacterial attachment (Hood & Zottola, 1995; Brooks & Flint, 2008).

2.3.2 Topography of the surface

Surface topography is defined in terms of roughness, which is measured by average roughness (R_a). A European hygienic equipment design group has recommended a stainless steel finish of R_a 0.8 µm or less for food contact surfaces (Faille 2000). There is no fixed opinion on the effect of surface roughness on bacterial adhesion. Some studies have reported no correlation between surface roughness and bacterial adhesion (An et al., 1995; Boulangé-Petermann et al., 1997; Flint et al., 2000). On the other hand, a positive correlation was reported between R_a and bacterial attachment (Wirtanen et al., 1996; Coquet et al., 2002). Increased adhesion on the rough surfaces may be due to an increase in exposed surface area which protects the cells against the shear forces of liquid flow (Faille et al., 2000). However, according to Faille et al. (2000), surface topography cannot be fully characterised by a classical parameter like R_a. Other parameters like reduced peak height, reduced valley depth and core roughness depth also need to be taken into account while studying the effect of surface topography on bacterial adhesion.

Surface defects like scratches, scrapes and pitting may also play an important role in bacterial adhesion to the surface. Surface flaws were reported to result in increased bacterial adhesion (Holah & Thorpe, 1990; Jones et al., 1999). Conversely, it was reported that surface flaws did not lead to increased bacterial adhesion, which might be due to the smoothing of the microstructure which resulted in reduced bacterial adhesion (Flint et al., 2000).

2.3.3 Properties of the bacterial cell surface

The hydrophobicity of the cell membrane, production of EPS, zeta potential and cell membrane outgrowths have all been reported to influence the adhesion of bacterial cells to a surface (Boulangé-Petermann, 1996). Interaction between the hydrophobic cell surface and the hydrophobic substratum surfaces leads to high adhesion (Donlan, 2002). A mutant of *A. flavithermus*, with tenfold reduced capacity for attachment, was found to be less hydrophobic and having a lower negative surface charge than the parent strain (Palmer et al., 2010). Cell surface hydrophobicity is increased by the membrane protrusions like fimbriae (Donlan, 2002). Spores attach in higher numbers than vegetative cells because of their covering by hair-like structures and higher hydrophobicity as compared to vegetative cells. Attachment of spores to the surface is

also influenced by pH and the resulting electrostatic interactions and polymer conformation on the spore surface (Seale et al., 2010). Following their attachment, the spores may germinate to produce cells, which then multiply, produce EPS and finally, lead to biofilm formation (Bower et al., 1996).

Lipopolysaccharides (LPS) in the bacterial membrane have also been shown to affect adhesion. In *E. coli*, mutations in the DNA encoding LPS affected the biosynthesis of type I fimbriae and flagella decreasing bacterial adhesion (Genevaux et al., 1999). Cell surface proteins have also been shown to affect bacterial adhesion (Flint et al., 1997 b). Besides membrane proteins and protrusions, attachment can also be affected by the charge on the bacterial cell surface. Bacterial cell membranes are negatively charged and therefore, easily repelled from a negatively charged surface. However, there was no correlation between the magnitude of surface charge in thermophilic dairy streptococci and bacterial adhesion as the surface of the thermophilic streptococci is weakly negatively charged (Flint et al., 1997 b). Because microorganisms have different cell surface properties, the numbers and the force with which they attach to abiotic surfaces differ from one bacterial cell to the other, thus, making their control difficult.

2.4 TREATMENTS FOR CONTROLLING BIOFILMS

2.4.1 Cleaning

Biofilms are more resistant to cleaning agents than planktonic cells of the same species. Cells deeply embedded in the biofilm matrix receive less oxygen and nutrients than those closer to the surface. To tide over this state of apparent starvation, some cells alter their physiology and exhibit a low growth rate. In this quasi-dormant state, they resist the uptake of sanitizers and cleaning agents and thus, are less affected by cleaning agents than planktonic cells (Bower et al., 1996). The penetration of the cleaning agents into biofilms can be quenched by EPS through chemical reactions (Meyer, 2003). The result is reduced efficacy of the cleaning agents used to remove biofilms. Also, biofilm cells get more resistant to cleaning agents as they age. With time, the attached cells multiply, form colonies and produce multiple layers of cells within a biofilm. Sanitizers such as quaternary ammonium compounds were found to reach only the topmost layer of the biofilm cells and were ineffective on the underlying layers (Bower et al., 1996). Biofilms in the food industry contain a large amount of food residue and mineral content. These constituents also confer some protection to the cells within the biofilms

(Chmielewski & Frank, 2003). Thus, cleaning biofilms formed in the food processing equipment is a tough task.

Cleaning of the food contact surface normally begins with the physical scrubbing of the surface or circulating turbulent solutions through the equipment to remove bacteria by the shear force of the liquid. This is then followed by chemical cleaning agents. Hence, it is necessary that the surface must be free of any cracks or crevices which provide bacteria a safe harbour by shielding them from the cleaning agents (Bower et al., 1996).

The CIP procedures used in the dairy manufacturing plants involve an alkali and an acid step (Bremer, et al., 2006). The alkaline wash removes organic material like fats and carbohydrates, whereas, the acid step removes the minerals and traces of alkaline products. It also makes conditions unsuitable for microbial growth and thus, delays the growth of the remaining microbial cells. However, treatment of 316 stainless steel, with 2B finish, with caustic (2%, 75 °C, 30 min) and nitric acid (1.8%, 75 °C, 30 min) at Reynolds numbers greater than 2000 resulted in complete removal of *A. flavithermus* biofilms in a pilot plant study (Parkar et al., 2004). But a slight variation of the same treatment (1% NaOH, 65 °C, 30 min; 1% nitric acid, 65 °C, 30 min) did not result in the complete removal of a mixed species biofilm (Bremer et al., 2006). Mixed species biofilms are reported to be more resistant to cleaning agents than monospecies biofilms (Sharma & Anand, 2002; Tang et al., 2010). Routine CIP procedures may not be effective enough to remove all the cells in the dairy plant (Flint, 1998). Rosmaninho et al. (2007) found that even after a thorough cleaning procedure (CIP), involving disinfection, there was a considerable number of residual *B. cereus* and *B. subtilis* spores attaching to stainless steel.

Besides chemical cleaning agents, enzyme-based detergents can also be used to control biofilms. These “green chemicals” can be used as cleaning agents or can be used synergistically to improve the efficiency of the other cleaners (Simões et al., 2010). Enzyme-based cleaning agents like Pandion, Resinase A2X, Spezyme AA300 and Paradigm were shown to be effective against *P. aeruginosa* biofilm after 30 minutes exposure (Augustin et al., 2004). Only Resinase A2X and Paradigm were effective against the biofilm after 15 minutes. This supports the concept of slow penetration of cleaning agents into biofilms. In addition, the efficiency of the cleaning agents is often reduced in the presence of organic material such as milk. Parkar et al. (2004) reported

the cleaning efficiency of many enzyme-based cleaners on 18-hour old biofilm of *A. flavithermus* (B12-C^m). Paradigm, a proteolytic cleaner with surfactant activity, resulted in complete removal of biofilm from the stainless steel coupons. They concluded that the combination of a cleaning agent with proper chemical strength and right temperature can ensure a clean surface. Caustic (75 °C for 30 minutes) and acid (75 °C for 30 minutes) wash resulted in successful removal of *Bacillus* species biofilms in their study. It was also shown that QuatroZyme®, which is composed of mixed enzymes, performs slightly better than other single enzyme-based cleaners against the biofilms of *Klebsiella oxytoca* B006 (Tang et al., 2010). This is understandable as EPS is heterogeneous in nature and a mixture of enzymes is required to penetrate this heterogeneous complex and remove the biofilms (Simões et al., 2010). Even though the enzymatic cleaners are effective and safe to use, the high costs limit their use in the dairy manufacturing plants (Brooks & Flint, 2008). Hence, it is worthwhile exploring some alternative strategies for biofilm control.

2.4.2 Alternative control strategies

One of the few alternative biofilm control strategies is temperature cycling. The controlled temperature increase for a fixed time resulted in the control of growth of thermophilic streptococci like *S. thermophilus* (Knight et al., 2004). Increasing the temperature of the regeneration stage of a pilot plant pasteurizer to 55 °C for 10 minutes every hour resulted in the delay of growth of thermo-resistant streptococci by 6 hours. However, the increased costs of running the plant under these conditions may only be economically feasible under certain conditions (Brooks & Flint, 2008).

Many lytic bacteriophages may produce a polysaccharide degrading enzyme, polysaccharase, to degrade capsule and EPS and allow access to the bacterial cells (Brooks & Flint, 2008). Phages have been shown to control biofilms of microorganisms like *Enterobacter agglomerans*, *L. monocytogenes* and *P. aeruginosa* (Hibma et al., 1997; Hughes et al., 1998; Sillankorva et al., 2004). However, the use of phage cocktails may be necessary due to the development of phage resistance (Brooks & Flint, 2008). Moreover, a minimum number of host cells are required as a threshold before phage replication can occur (Hudson et al., 2005). There are also the chances of phages spreading virulence or antibiotic resistance genes from one bacterial cell to the other

(Hudson et al., 2005). Hence, the phages should be selected carefully for biofilm removal.

Inhibition of cell-to-cell signalling may influence biofilm removal (Simões et al., 2010). Bacteria use quorum sensing to control various activities within biofilms (Donlan, 2002). Thus, enhancing quorum sensing, involved in cell dispersion, or disrupting quorum sensing may provide an efficient method of combating biofilms. Another promising method for biofilm removal is turbulent two-phase flow, which has been shown to reduce biofilm levels by 6 log cycles during the cleaning of endoscopes (Benjamin & Labib, 2000). Cleaning may also be improved by ice pigging, which can be described as pushing crushed ice and water mixture through a pipe to clean the pipe walls. It has been shown to be useful in cleaning surfaces fouled with jam and fats (Quarini, 2002). However, many of the methods need to be adapted for commercial use.

2.4.3 Surface modification

Stainless steel is used in food manufacturing plants due to its strength, wear resistance, easy cleanability and efficiency in heat transfer (Brooks & Flint, 2008). In food manufacturing plants, stainless steel is prone to bacterial adhesion and subsequent biofilm formation. In commercial dairy plants, a standard alkali/acid wash can remove biofilms, provided a correct concentration of cleaning agents, temperature and contact time are applied (Parkar et al., 2004). However, it is not always feasible due to cracked seals, bends, joints and stagnant zones which harbour bacteria (Brooks & Flint, 2008). Hence, prevention of the formation of biofilms is an alternative control concept. One method to achieve this is to modify the surface of stainless steel in order to prevent bacterial adhesion. Surface modification consists of modifying the uppermost layers of a surface physically or chemically or coating the surface with a material that will change the properties of the surface (Chu et al., 2002). This has been achieved by implantation of ions, generation of diamond-like carbon surfaces, addition of PTFE to the surfaces, production of bioactive surfaces and coating with antimicrobial agents (Brooks & Flint, 2008).

2.4.3.1 Surface coatings

Different types of coatings are used on the surface to make it antibacterial. The coating may consist of PTFE, diamond-like carbon (DLC) coatings and silver-based coatings.

PTFE coatings are hydrophobic in nature and are used on stainless steel and other surfaces to prevent bacterial adhesion (Rosmaninho et al., 2007). A graded electroless Ni–P–PTFE coating on copper plates reduced bacterial adhesion by 87-92% (Zhao et al., 2004). Autocatalytic graded Ni–Cu–P–PTFE coatings and Ag-PTFE composite coatings also showed decreased bacterial adhesion and improved corrosion resistance (Zhao et al., 2005 a ; Zhao et al., 2005 b). It has also been shown that a Ni–P–PTFE surface promotes less organic build up and is easy to clean (Rosmaninho et al., 2007).

Besides PTFE coatings, DLC coatings, which consist of amorphous carbon having three-dimensional, tetragonal sp^3-bonds, are also used to modify the biomaterial surfaces (Dearnaley & Arps, 2005). Their biocompatibility and excellent wear and tear resistance has made them popular as medical implants (Hauert, 2003). DLC coatings, further doped by silicon and nickel, resulted in lower bacterial adhesion than pure DLC coatings (Liu et al., 2008).

2.4.3.1.1 Silver-based coatings

Silver has been used as an antimicrobial agent since times immemorial. It has been used as colloidal silver, silver nitrate, silver lactate, silver acetate and silver sulfadiazine to treat skin and eye infections, bladder irritation and burn wounds (Ahearn et al., 1995). In dentistry, it forms one of the components of an amalgam (Moretro & Langsrud, 2011). Besides medical uses, silver is also employed in water treatment plants. Many possible mechanisms have been suggested to explain the antimicrobial effect of silver. Silver acts via inactivation of enzymes in the electron transport chain by forming complexes with the sulfhydryl groups (Darouiche, 1999). It forms complexes with nitrogen bases in microbial DNA and thus, prevents DNA replication (Izatt et al., 1971). Silver has also been reported to disrupt the function of cell membranes by producing blebs and collapsing the proton motive force across the membrane (Ghandour et al., 1988; Hetrick & Schoenfisch, 2006). Furthermore, silver ions displace the metal ions like zinc, calcium and potassium which are vital for cell survival (Hetrick & Schoenfisch, 2006). Silver can be incorporated into surfaces in different ways. It can be added as pure silver, silver ions or as silver nanoparticles. It can be used with the help of carriers like zeolites and phosphate-based glasses, which slowly release ions into the environment.

Due to its antimicrobial properties, silver-based coatings have mainly been tried on medical implant materials. Biofilms form easily on implants once they are inserted in the body. Pure silver, colloidal silver, silver nitrate and silver zeolite have been used as antimicrobial coatings on biomaterials (Monteiro et al., 2009). Silver ions have been found to be effective against broad range of pathogens found at implant sites – *P. aeruginosa, E. coli, S. aureus* and *S. epidermis* (Hetrick & Schoenfisch, 2006). Silver-coated orthopaedic external fixation stainless steel pins were found to be neither cytotoxic nor genotoxic with human blood peripheral lymphocytes, thus, safe for use in medical implants (Bosetti et al., 2002). Further, the silver-coated pins were shown to be effective against *P. aeruginosa, E. coli* and *S. aureus* isolates from wound infections (Wassall et al., 1997). Similarly, silver ions incorporated into phosphate-based glass showed antibacterial activity against *Streptococcus sanguis* and *S. aureus* (Mulligan et al., 2003; Valappil et al., 2007). Stainless steel containing 0.2% or more silver by weight was shown to have 100% antimicrobial activity against *E. coli* and *S. aureus* after 24 hours (Huang et al., 2011).

Besides pure silver coating, silver has been used in other forms as well. Silver and zinc containing zeolite film with a thickness of 0.5 mm, when deposited on stainless steel, resulted in a 3 log reduction of *Bacillus* sp. vegetative cells in phosphate-buffered saline after 24 hours (Galeano et al., 2003). There was no reduction in the number of spores, however. Bright et al. (2002) tested the efficiency of silver-zinc containing zeolite coating on stainless steel against *S. aureus*. The bacterial suspension was placed on the coupon and incubated at 37°C for different times under controlled humidity conditions. A 2-3 log reduction was observed on the coated surfaces when compared with the control, uncoated stainless steel surfaces. Silver nanoparticles, when present in the medium, were found to inhibit growth and biofilm formation of *P. aeruginosa* and *S. epidermidis* (Kalishwaralal et al., 2010). It should be kept in mind that the above studies were done in laboratory conditions where growth variables were strictly controlled. The results may vary outside the laboratory.

There are numerous reports on the bacterial resistance of surfaces implanted with silver ions and coatings of silver on medical grade stainless steel and other biomaterials (Ahearn et al., 1995; Bosetti et al., 2002; Furno et al., 2004). Most work has focused on modifying the surface to prevent implant-related infections. Little work has been done on silver-based coatings in the food industry and especially the dairy industry.

Silver has been used for some food contact surfaces, with a few commercial products now available with a silver coating (Moretro & Langsrud, 2011). Silver coatings have been used in refrigerators, chopping boards, knives and conveyor belt systems, where they are claimed to provide a germ-free environment. In these cases, mostly nanosilver and silver zeolite was used. To date, 17 food contact surfaces, containing silver, mostly in the form of zeolites and phosphate-based glasses, have been given approval by U.S. Food and Drug Administration (USFDA, 2012). However, there have been some concerns regarding the toxicity and environmental effects of nanosilver, with some groups suggesting that the U.S. Environmental Protection Agency (EPA) classify nanosilver as a new pesticide.

Kampmann et al. (2008) tested the inner lining of refrigerators with a coating of zirconium phosphate resin containing silver using a few spoilage and pathogenic bacteria. A suspension of bacterial cells was placed on the surfaces and incubated for various lengths of time to determine the antibacterial activity of silver. It was found that with *Lactobacillus delbrueckii,* there was no reduction after 24 hours at 5°C. After 72 and 144 hours of incubation, there was a reduction of 1.4 and 1.1 $\log_{10}$ units, respectively. In the case of *P. fluorescens*, there was a 0.2 $\log_{10}$ unit reduction after 24 hours, which rose to 5-6 $\log_{10}$ units after 72 or more hours. When food was placed in direct contact with a silver-coated lined refrigerator for 6-11 days, it was found that there was a lower bacterial growth than in the control refrigerator, with approximately a one log reduction in the number of *Listeria monocytogenes* in under-cooked ham after 8 days and pork sausage after 11 days. There was no impact on the sensory characteristics of most of the foods.

Cowan et al. (2003) tested the efficiency of a silver-zinc coating containing zeolite against food pathogens. A bacterial suspension in buffer was applied on the coated stainless steel coupons and incubated at 37°C. There was a 99-100% reduction in the numbers of *S. aureus* and *E. coli*, respectively, on coated surfaces after 6-24 hours.

Chaitiemwong et al. (2010) investigated the survivability of *L. monocytogenes* on a conveyor belt with and without antimicrobial agents. The antimicrobial agents included silver zeolite, aluminium oxide, calcium oxide, magnesium oxide, zinc pyrithione and oxybisphenoxarsine. These were implanted in the polyester fabric along with thermoplastic polyurethane. There was a 0.5-1.5 log reduction in the number of bacteria

after inoculating the conveyor belts with the bacterial suspension in peptone water and incubating them at 10 and 25°C for 6 hours under controlled relative humidity conditions. However, there was a high loss of antibacterial effect when food debris was introduced on the conveyor belts, suggesting that it might not work very well in the presence of organic matter. Thus, silver-based coatings can result in decreased bacterial adhesion. However, the antimicrobial effect in the presence of food components needs further evaluation.

2.4.3.2 Ion Implantation

Ion implantation is the process in which ions, accelerated at 20-200 keV, hit the surface to become implanted in the first few micrometers of the surface (Cui & Luo, 1999). Because the ions can only penetrate the uppermost layers of the surface, the surface properties change, whereas, the bulk properties remain the same (Chu et al., 2002). There are no changes in the dimensions of the surface and the implantation process gives a clean finish since it is conducted in a vacuum (Conrad et al., 1987). In addition, the process is highly reproducible and easily controllable (Cui & Luo, 1999). The lifetime of the ion-implanted surfaces is longer than the coated surfaces as the ions have actually penetrated the surface and are not released from the surface while in use. Coated surfaces, on the other hand, result in a change in the bulk properties of the surface, with the risks of getting chipped or damaged.

Surface implantation with SiF_3^+-ions resulted in reduced attachment of *S. epidermis* and *S. aureus* (Zhao et al., 2008). SiF_3^+-implanted surfaces were more effective in preventing bacterial attachment than N^+- and O^+-implanted stainless steel surfaces. Low surface roughness and low total surface energy of SiF_3^+-implanted surfaces as compared to N^+- and O^+-implanted surfaces resulted in low bacterial adhesion. In another study, TiC-implanted stainless steel surfaces had the lowest number of thermophilic bacilli attached compared with the control as well as MoS_2^{2+} and SiF_3^+-implanted stainless steel surfaces (Rosmaninho et al., 2007). However, the dose and implantation energy used in both studies was different. Cu^{2+}-implanted stainless steel samples showed over 99% antibacterial activity against *E. coli* and 40-60% antibacterial activity against *S. aureus* (Dan et al., 2005). Annealed Cu^{2+}-implanted samples also showed good corrosion resistance. Besides the conventional method of ion implantation, other

methods such as plasma source ion implantation, ion beam assisted deposition and ion beam sputtering deposition are also used (Wang & Zreiqat, 2010).

2.5 CONCLUSIONS

Biofilms are a source of bacterial contamination in many environments, including the contamination of food in food manufacturing plants. The penetration of cleaning agents and sanitizers into biofilms is limited due to the presence of EPS, making it difficult to control biofilms using standard cleaning techniques. An alternative approach is to prevent biofilm formation, specifically bacterial attachment. This can be done by modifying the substratum so that fewer bacteria attach. Surface modification of materials such as stainless steel, commonly used in food manufacturing plants and medical implants, can result in lower bacterial adhesion. Implantation of ions like SiF_3^+, TiC and Cu^{2+} on the stainless steel surface has shown promising results. Also, silver is known to have antimicrobial activity, making the prospect of silver-implanted surfaces in food manufacture a possible strategy to limit biofilm development and reduce product contamination.

2.6 AIM OF THIS STUDY

The aim of this study is to test the effect of silver-ion implanted stainless steel coupons on bacterial viability and biofilm formation.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Bacterial isolates

3.2 Growth and maintenance of isolates

3.3 Ion implantation

3.4 Adhesion studies

3.4.1 CDC Biofilm reactor

3.4.2 Preparation of bacterial suspension

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3.4.4 Exposure of coupons to suspension

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3.6 Microscopy

3.6.1 Epifluorescence microscopy

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CHAPTER THREE: MATERIALS AND METHODS

3.1 Bacterial isolates

Two bacterial isolates were used for testing the resistance of silver-ion implanted stainless steel coupons to bacterial attachment. *Streptococcus thermophilus* H, isolated from pasteurized milk in a cheese manufacturing plant in New Zealand, and *Pseudomonas fluorescens* C224, isolated from a raw milk tanker in New Zealand, were used for this study. These bacteria were selected because of their potential to form biofilms and cause contamination in dairy products (Flint, 1998; Teh et al., 2011).

3.2 Growth and maintenance of isolates

M17 broth (Merck, New Zealand), prepared according to the manufacturer's instructions, was used for the growth of the *S. thermophilus* H culture. For the enumeration of bacteria present on coupons, the broth was supplemented with 15% agar-agar (Merck, New Zealand). TSB medium (Difco, France) was used for culturing *P. fluorescens* cells. All media were autoclaved at 121 °C for 15 minutes before use. The mother cultures of *S. thermophilus* and *P. fluorescens* were stored at 4 °C on M17 and TSB slopes, respectively.

3.3 Ion implantation

Ion implantation is a method of introducing foreign atoms into a solid matrix. The amount of atoms introduced is highly controlled and repeatable. For this experiment, stainless steel (316L with a 2B surface finish) coupons (BioSurface Technologies, Inc., USA) with 1.27 cm diameter were used. The coupons were implanted with the dose of 1×10^{16} silver ions per cm^2. The implantation of ions was carried out at the Institute of Geological and Nuclear Sciences, Lower Hutt, New Zealand.

Prior to implantation, the coupons were passivated to obtain a clean surface. Passivation involved washing the coupons to remove any dirt, soaking the coupons in 50 % nitric acid for 30 minutes at 70 °C and rinsing the coupons thoroughly (Bremer et al., 2001).

A silver ion source was present in the ion implanter to generate charged atoms of the selected species using high voltage (5-90 keV). A magnetic field was then used to select the silver ion species from other charged states. The exit of the magnetic field was at 90 degrees to the entrance (Figure 1.1). Thus, by tuning the magnetic field, the ions were

mass separated at the exit port. The mass selected silver ion beam was then steered and focused towards the target coupon using electrostatic lens and other steering elements. The coupon was fixed on a coupon holder using a carbon tape (Figure 1.2). The ion beam was then raster scanned over the coupon surface to generate a homogenous surface with a dose of 1×10^{16} silver ions per cm^2. Because the ions had kinetic energy, on reaching the coupons, they penetrated the surface and came to rest within the stainless steel surface, thus, implanting themselves within it. The whole system was enclosed in high vacuum (10^{-7} mbar). This was essential in order for silver ions to travel from their source to the target stainless steel coupons, a distance of about 3m and to ensure a clean implantation on the coupon.

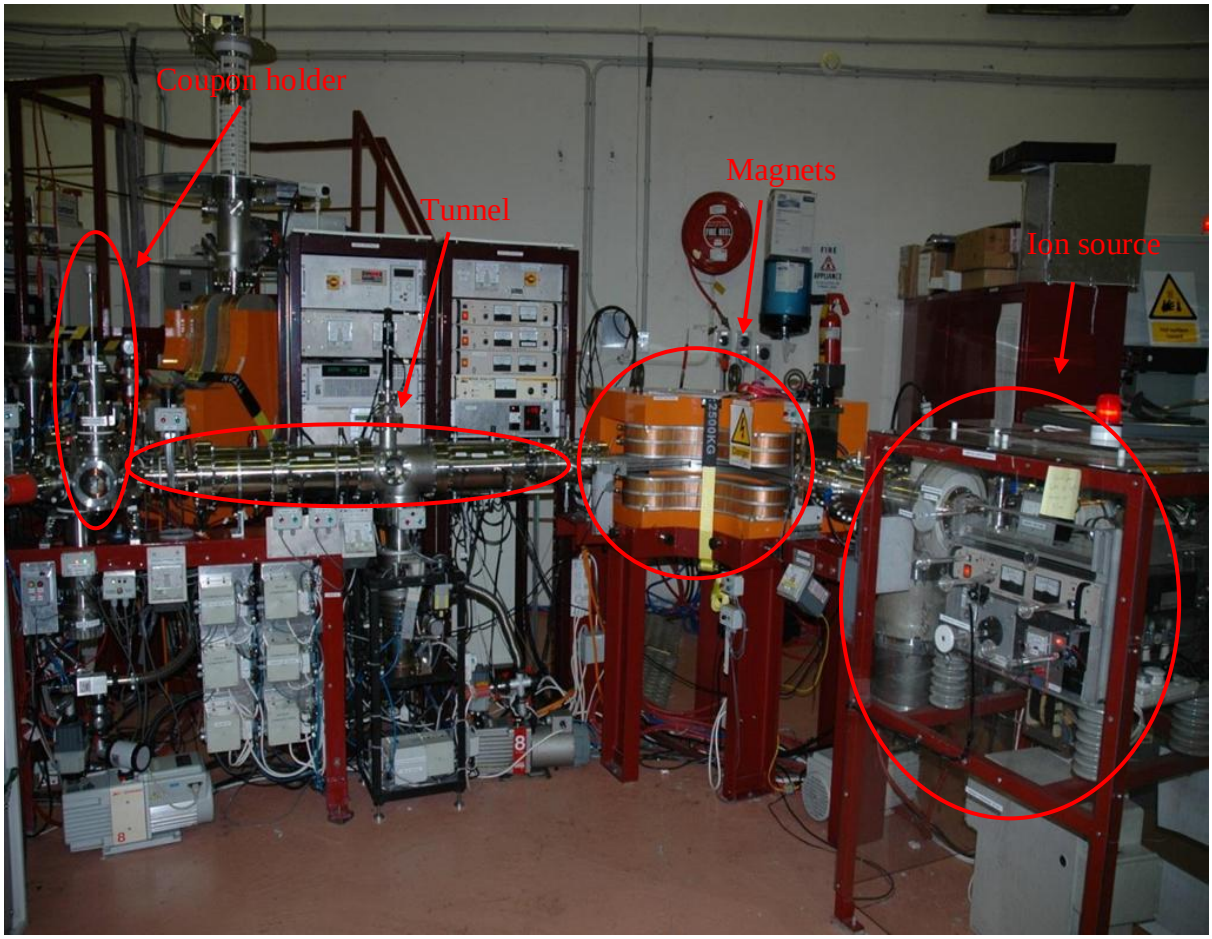

Figure 1.1: An ion implanter. The distance between the ion source and the target coupon is about 3 meters.

Figure 1.2: The coupon holder with the coupon fixed on it using a carbon tape.

3.4 Adhesion studies

3.4.1 CDC biofilm reactor

CDC biofilm reactor (BioSurface Technologies, Inc., USA) was used for the experiments. It consists of a 1000 ml Berzelius Pyrex beaker with an effluent spout at 400 ml (Goeres et al., 2005). The polyethylene lid has three holes for media inlet, air exchange and inoculation port and eight rod holes (Figure 1.3). Eight polypropylene rods can fit into the lid, each of which bears three removable coupons. In this study, stainless steel coupons were used as control and silver-ion implanted stainless steel coupons were used as test coupons. A baffled stir bar, which is driven by a magnet, is used for constant mixing of the fluid in the reactor. The glass vessel is placed on a digitally controlled hotplate/stirrer (VWR International, USA) which provides the required temperature and the constant rotation of the stir bar at the designated speed. The CDC biofilm reactor unit is a very well tested method for growing and sampling biofilm formation in different media (Goeres et al., 2005; Tang et al., 2010). The biofilm reactor along with coupons and medium was autoclaved at 121 °C for 15 minutes before use.

Figure 1.3: CDC biofilm reactor used for the experiments in the study.

3.4.2 Preparation of bacterial suspension

A loopfull of bacterial culture was inoculated into 10 ml broth and incubated. *S. thermophilus* was inoculated in M17 broth and incubated at 37 °C for 18 hours, whereas, *P. fluorescens* was inoculated in TSB broth and incubated at 25 °C for 18 hours. After incubation, the cells were in their log phase of growth and harvested by centrifuging the culture at 3000 *g* for 10 minutes. The supernatant was discarded and the pellet was suspended in 0.1% peptone water (Merck, New Zealand) to reach a density of 10^8 cells ml^{-1} as determined by the standard plate counting method on M17 agar and TSB agar for *S. thermophilus* and *P. fluorescens*, respectively. This suspension was used to inoculate the media in the CDC reactor for allowing the attachment and subsequent biofilm formation on control and test coupons.

3.4.3 Media used

Different media were used for studying attachment on the coupons. Saline, consisting of 0.75 % NaCl (Biolab Limited, Australia), was used for studying attachment along with M17, TSB and whey. UHT treated standard milk (Anchor blue™, Fonterra Brands Ltd., New Zealand) and skimmed milk (Anchor trim™, Fonterra Brands Ltd., New Zealand) were also used. Whey was prepared by mixing 5% whey protein concentrate (Fonterra Co-operative Group Ltd., Auckland, New Zealand) with 6.1% sterilized lactose (Fonterra Co-operative Group Limited, New Zealand) and 6.1% artificial whey permeate in sterile deionised water to make 1L (Tang et al., 2010). The whey permeate included the mixing of the following minerals in sterile deionised water to make 1 L - 52.7 mL of 2mol L^{-1} KOH (BDH, England), 24.29 g $Na_3C_3H_5O(CO_2)_3.2H_2O$ (Merck, Germany), 4.99 g $C_6H_5K_3O_7.2H_2O$ (UNIVAR, New Zealand), 3.67 g $CaCl_2.2H_2O$ (Biolab Limited, Australia), 5.85 g $MgCl_2.6H_2O$ (Fisher Scientific Limited, UK), 23.36 g KH_2PO_4 (Fisher Scientific Limited, UK) and 17.1mL of 3 mol L^{-1} H_2SO_4 (J. T. Baker, USA). All media except standard and skimmed milk and whey were autoclaved at 121 °C for 15 minutes prior to use. The reconstituted whey was autoclaved at 110 °C for 5 minutes. Since UHT treated milk was used, it was already sterile and was added directly to the autoclaved reactor under sterile conditions.

3.4.4 Exposure of coupons to suspension

The silver-ion implanted stainless steel coupons acted as test coupons and plain stainless steel coupons were used as controls. The sterile reactor containing control and test coupons and growth medium was inoculated with 1 mL of the bacterial suspension containing about 1×10^8 cells per ml. The reactor was run for up to 24 hours with coupons being sampled at 30 minutes for attachment studies and at 6, 12, 18 and 24 hours for biofilm studies. The reactor was operated in batch mode at 180 rpm at a temperature of 37 °C for *S. thermophilus* and 25 °C for *P. fluorescens*. The coupons, after designated periods of exposure, were removed from the rods aseptically using a sterile screwdriver.

The coupons were then washed with three changes of sterile deionised water to remove loosely attached bacteria. In order to determine the bacteria attached to the ion-implanted side, the bacteria attached on non-implanted side of the coupons were killed by swabbing with 1% formalin, followed by 75% ethanol and lastly, by sterile distilled water to remove any residual formalin or ethanol. The coupons were then air-dried in a sterile Petri dish in laminar flow chamber for approximately 2-3 minutes.

3.4.5 Plating and enumeration

The air-dried coupons were placed in a 20 ml bottle containing 15 g glass beads (4.5 mm diameter) (BDH, England) and 10 ml peptone water. The cells attached to the coupons were removed by vortex-mixing for 2 minutes. Serial dilutions were made in 0.5% peptone water and plated on M17 and TSB agar for *S. thermophilus* and *P. fluorescens*, respectively. The plates were incubated for 24 hours at 37 °C and 25 °C for *S. thermophilus* and *P. fluorescens*, respectively. After incubation, the number of colonies present on a plate was counted with the help of a colony counter (Suntex, Taipei). Each dilution was plated twice. The coupons were disinfected and washed after use. The attachment experiments were repeated on three separate occasions with two coupons (test and control) removed at each sampling time.

3.5 Cleaning

After each use, the coupons were disinfected by soaking them in ethanol for 10 minutes to kill any bacteria still attached to the coupons. After disinfection, the coupons were soaked in laboratory detergent Pyroneg (Johnson Diversey, New Zealand) and lightly

scrubbed using a soft toothbrush. The coupons were then rinsed in distilled water. This was then followed by a washing process that closely resembled the clean-in-place procedures used in the dairy manufacturing plants. The coupons were treated with 2% sodium hydroxide (BDH, England) at 75 ℃ for 30 minutes, and then rinsed in deionised water for 15 minutes. Then they were acid treated with 1.8 % nitric acid (J. T. Baker, USA) at 75 ℃ for 30 minutes, rinsed thoroughly in deionised water and sterilized by autoclaving at 121 ℃ for 15 minutes (Parkar, et al., 2004).

3.6 Microscopy

3.6.1 Epifluorescence microscopy

To determine the cellular integrity of the cells attached, the coupons were stained with Live/Dead® BacLight™ Bacterial Viability stain kit (Molecular Probes, The Netherlands). The kit contains SYTO® 9 green-fluorescent stain and red-fluorescent stain propidium iodide. SYTO® 9 generally labels all bacteria green, when used alone. However, when used in combination with propidium iodide, it stains only live bacteria with intact membranes as propidium iodide penetrates bacteria with damaged membranes and causes a reduction in SYTO® 9 fluorescence. The dead bacteria appear red and can be distinguished easily from live bacteria that fluoresce green.

The coupons were exposed to bacteria and rinsed in sterile distilled water for three times as previously described. The dye was then diluted according to the manufacturer's instructions and coupons were flooded with 300 µl of the dye (Lindsay et al., 2002). They were incubated in the dark for 10 minutes and washed with 10 ml of sterile distilled water afterwards. They were then visualized under an epifluorescence microscope (BX53, Olympus, USA) (50% laser intensity, 488/563 excitation wavelengths, 100 × oil objective). The images were captured using a camera (XC50, Olympus, USA).

3.6.2 Scanning electron microscopy

Scanning electron microscopy (SEM) was also used for observing the biofilms formed on the coupons and to determine if there were any changes in the appearance of the stainless steel surface following ion implantation and if so, how this would impact on the presence of biofilm. The cells were grown on the coupons for 24 hours as previously described. After rinsing the coupons with sterile deionised water, they were soaked in

4% glutaraldehyde (BDH, England) and left overnight at room temperature (Lindsay & Von Holy, 1999). The coupons were then sequentially dehydrated with 30, 40, 50, 60, 70, 80, 90, and 100 % ethanol. They were air dried and sputter coated and viewed under SEM. To look at the change in the surface appearance after implantation, the coupons were viewed at a tilt of 65°.

3.7 EPS quantification

To determine the amount of EPS produced by the bacteria when attached to the coupons, the following protocol was used, modified from the original method given by Dall and Herndon (1989) – The bacteria attached to the coupons were removed by vortex mixing with beads in 10 ml peptone water as described above. Peptone water (10 ml), containing bacteria dislodged from the coupons, was again vortexed at 3000 x *g* for 10 minutes to remove the cells. The pellet was discarded and 1 ml of supernatant was added drop wise to 8 ml absolute alcohol and left overnight at 4 °C to precipitate the polysaccharides. It was then centrifuged at 10,000 x *g* for 20 minutes. The supernatant was discarded and the pellet was re-suspended in 1 ml distilled water by vortex-mixing. Seven ml sulphuric acid (77% v/v) (J.T. Baker, USA) was added slowly to the re-suspended pellet suspension. Finally, 1 ml of 1% cold L-tryptophan (BDH, England) was added to the pellet suspension and thoroughly mixed using a vortex machine. It was then dispensed into a glass test tube and heated at 100 °C for 20 minutes and then cooled to room temperature. It was again thoroughly mixed and the absorbance was read at 500 nm using a spectrophotometer. One ml distilled water mixed with seven ml sulphuric acid (77% v/v) and 1% cold L-tryptophan, heated at 100 °C for 20 minutes, was used as a reference. EPS, in the presence of acid, degrades to produce a furan, which then reacts with the aromatic amino acid tryptophan to produce a coloured product which can be quantified using a UV/ visible spectrophotometer. Dextran (Sigma-Aldrich, USA) was used to generate a standard curve (10 – 200 µg/ml). The results were expressed as average EPS produced by number of bacteria attached per cm^2.

3.9 Statistical analysis

Tukey-Kramer tests were carried out using SAS (SAS Institute Inc., USA) to check if there were any significant differences ($P < 0.05$) in terms of bacterial attachment on the ion-implanted coupons and the control coupons in different media.

CHAPTER FOUR: RESULTS

4.1 Attachment studies

4.2 Biofilm studies

4.2.1 Biofilm formation in whole milk

4.2.2 Biofilm formation in skimmed milk

4.2.3 Biofilm formation in whey

4.3 EPS quantification

4.3.1 Dextran standard curve

4.3.2 EPS production in whole milk

4.3.3 EPS production in skimmed milk

4.3.4 EPS production in whey

4.4 Microscopy

4.4.1 Epifluorescence microscopy

4.4.2 Scanning electron microscopy

CHAPTER FOUR: RESULTS

4.1 Attachment studies

The attachment of *S. thermophilus* and *P. fluorescens* in different media on the silver-ion implanted and the stainless steel coupons was determined as described in section 3.4. The results are shown in Figure 4.1. For *S. thermophilus*, in saline, the attachment on the silver-ion implanted coupons was statistically significantly less ($P < 0.05$) (2.63 log CFU cm^{-2}) than that obtained on the stainless steel coupons (3.12 log CFU cm^{-2}) (Figure 4.1 A). A similar pattern was observed in the M17 medium, where there was a difference of about 0.5 log CFU cm^{-2} between the silver-ion implanted and stainless steel coupons. However, with the whole milk, skimmed milk and whey, the attachment on the control and the test coupons was more or less similar, with the difference being statistically insignificant ($P > 0.05$). Specifically, there was a difference of about 0.04 log CFU cm^{-2} and 0.05 log CFU cm^{-2} between the silver-implanted coupons and the stainless steel coupons with the attachment being higher on the stainless steel coupons in the presence of whole milk and whey, respectively. In the case of skimmed milk, the attachment on the stainless steel coupons was 4.02 log CFU cm^{-2}, whereas, on the silver-implanted coupons, 4.08 log CFU cm^{-2} were attached.

P. fluorescens in saline had an attachment of 2.93 log CFU cm^{-2} on the stainless steel, which was significantly higher ($P < 0.05$) than that observed on the silver-implanted coupons (2.75 log CFU cm^{-2}) (Figure 4.1 B). In TSB broth, the attachment was higher than in saline with 3.2 and 3.42 log CFU cm^{-2} on the silver-implanted and stainless steel coupons, respectively. The bacteria still attached in higher numbers to the stainless steel coupons than on the silver-implanted coupons when whole milk, skimmed milk and whey were used. However, the difference, in terms of attachment, was markedly reduced and it varied from 0.1 log CFU cm^{-2} in whey to 0.4 log CFU cm^{-2} in skimmed milk. In the case of whole milk and whey, the difference in terms of bacterial attachment on the silver-ion implanted coupons and the control coupons was not statistically significant ($P > 0.05$).

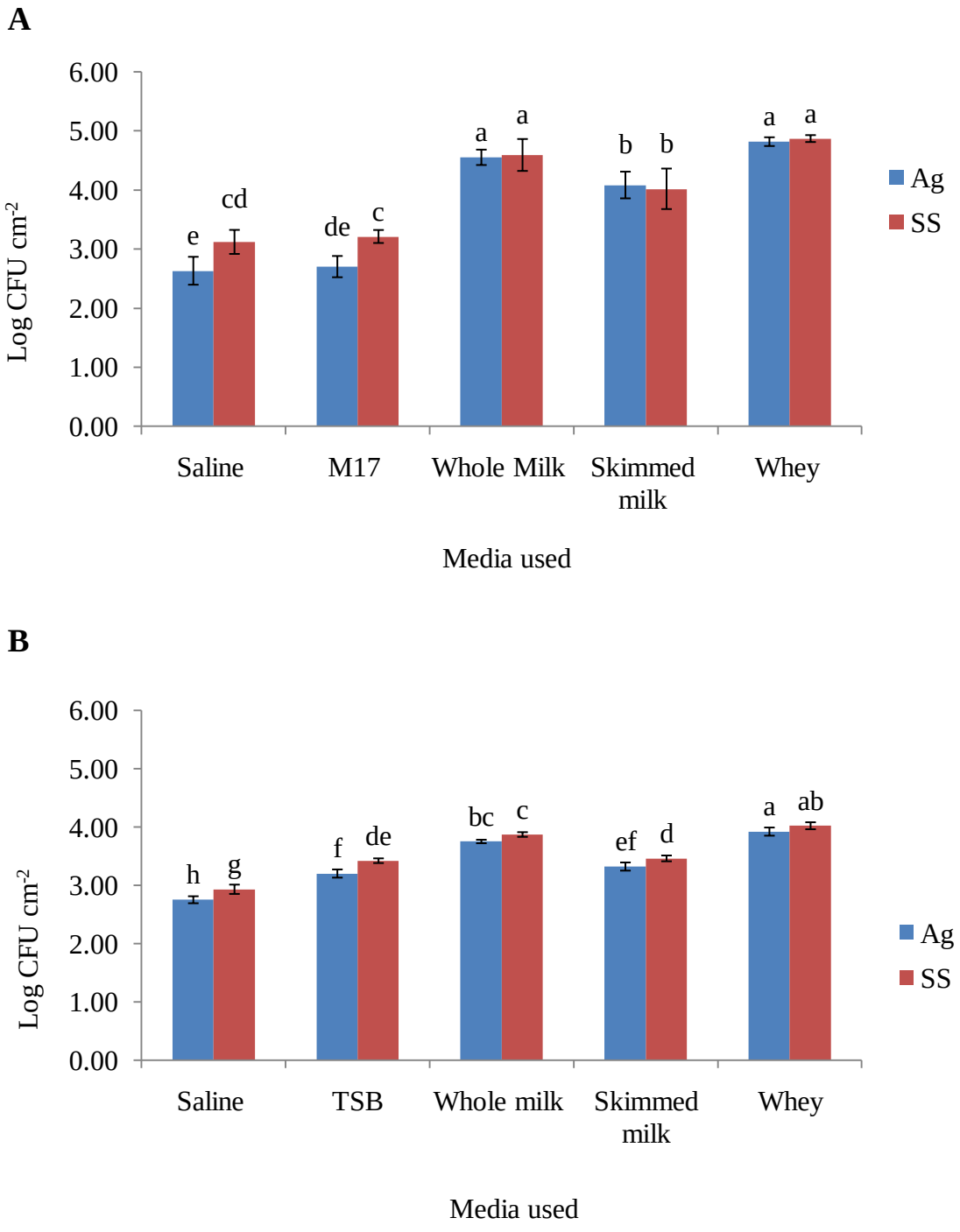

Figure 4.1: Attachment of *S. thermophilus* (A) and *P. fluorescens* (B) on silver-implanted (Ag) and stainless steel coupons (SS) in different media after exposure of the coupons for 30 minutes. Means with the different letters are significantly difference from each other ($P < 0.05$).

4.2 Biofilm studies

4.2.1 Biofilm formation in whole milk

The number of cells present during biofilm formation of *S. thermophilus* in whole milk on the control and the test coupons was determined as described in section 3.4. The results are shown in Figure 4.2 A. During biofilm formation, there was no statistical difference ($P > 0.05$) in the number of cells present on the silver-ion implanted and the stainless steel coupons at any given time interval ($P > 0.05$). After six hours, the number of cells on the silver-implanted coupons was 4.89 log CFU cm^{-2}, while, on the stainless steel, was 4.98 log CFU cm^{-2}. The total number of cells on the coupons increased with the time. However, the difference between the stainless steel and the silver-implanted coupons decreased each time the coupons were sampled. After 12 hours, the stainless steel coupons had 0.09 log CFU cm^{-2} higher than the silver-ion implanted coupons. This difference was lowered to 0.04 log CFU cm^{-2} after 18 hours and further reduced to 0.03 log CFU cm^{-2} after 24 hours.

The same trend was observed in the case of *P. fluorescens* (Figure 4.2 B). After 6 hours, the stainless steel coupons had 0.09 log CFU cm^{-2} more than the silver-ion implanted coupons. The difference was reduced after 12 hours when the test and control coupons had 5.06 and 5.14 log CFU cm^{-2}, respectively. After 18 hours, stainless steel coupons had 5.65 log CFU cm^{-2}, whereas, the silver-implanted coupons had 5.60 log CFU cm^{-2}. The number of cells on the control and test coupons increased to 5.78 and 5.75 log CFU cm^{-2} after 24 hours, although the difference in the number of cells on each type of coupon decreased.

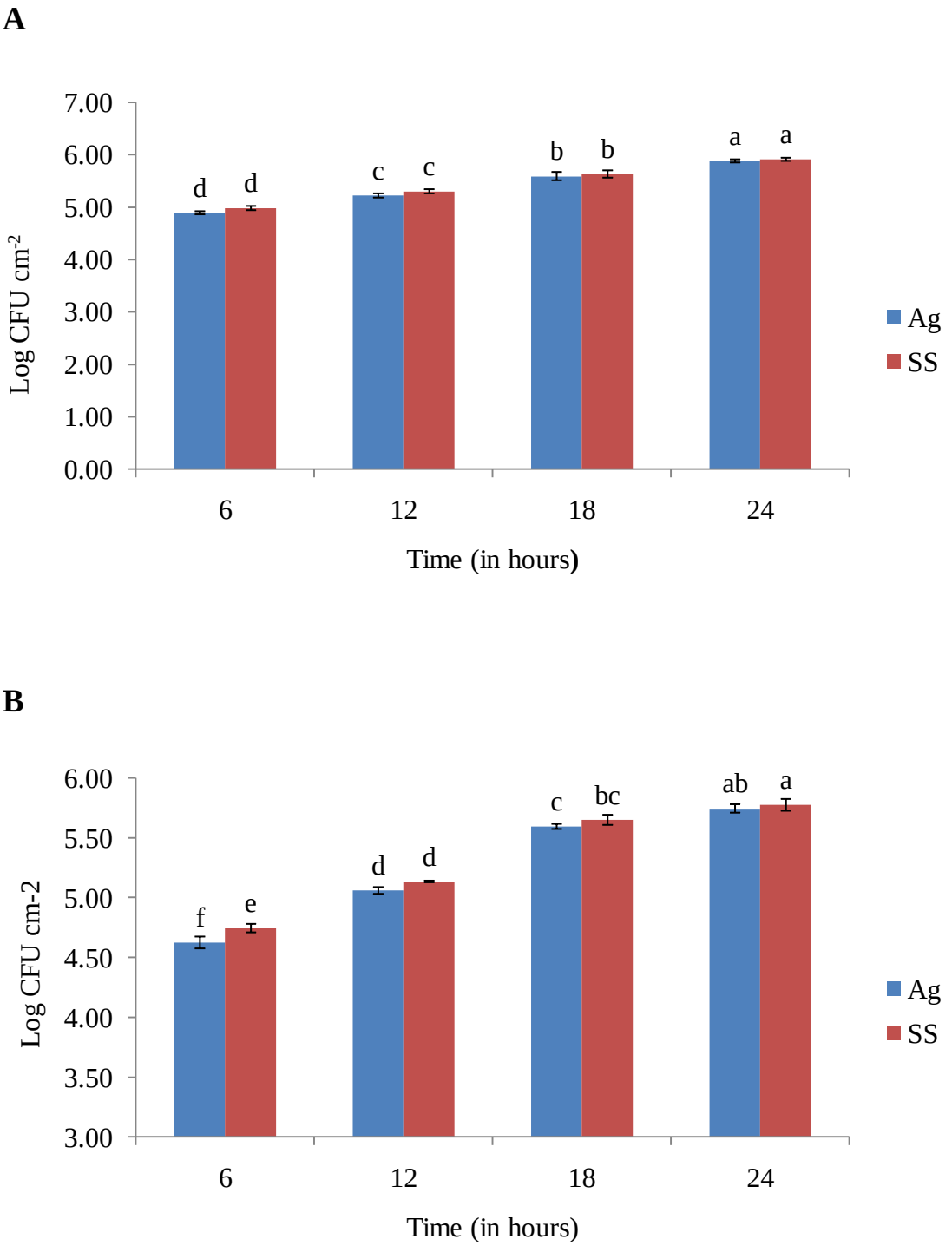

Figure 4.2: The number of cells of *S. thermophilus* (A) and *P. fluorescens* (B) on the silver-implanted (Ag) and stainless steel coupons (SS) during biofilm formation in whole milk at different time intervals. Means with the different letters are significantly difference from each other ($P < 0.05$).

4.2.2 Biofilm formation in skimmed milk

The number of cells present during biofilm formation of *S. thermophilus* in skimmed milk on the control and the test coupons was determined as described in section 3.4. The results are presented in Figure 4.3 A. After 6 hours, the silver-implanted coupons had 4.88 log CFU cm^{-2}, whereas, the stainless steel coupons had 4.93 log CFU cm^{-2}. The same pattern was observed after 12 and 18 hours when the attachment and growth on the stainless steel coupons was slightly higher than that on the silver-implanted coupons. After 24 hours, the silver-ion implanted coupons had 6.09 log CFU cm^{-2} compared to the stainless steel coupons which had 6.08 log CFU cm^{-2}.

When *P. fluorescens* was used as the test organism in skimmed milk, the pattern was similar to that observed with *S. thermophilus.* (Figure 4.3 B). There was very little difference in terms of cells colonising the test and the control coupons and this was not statistically significant ($P > 0.05$). After 6 hours, the silver-implanted coupons had 4.52 log CFU cm^{-2}, whereas, the stainless steel coupons had 4.61 log CFU cm^{-2}. After 12 and 18 hours, the stainless steel coupons still had a higher number of cells (0.08 and 0.07 log CFU cm^{-2}, respectively) than the silver-implanted coupons. After 24 hours, there was even less difference between the number of cells attached to the both types of coupon, with the stainless steel and silver-ion implanted coupons having 5.79 log CFU cm^{-2} and 5.77 log CFU cm^{-2}, respectively.

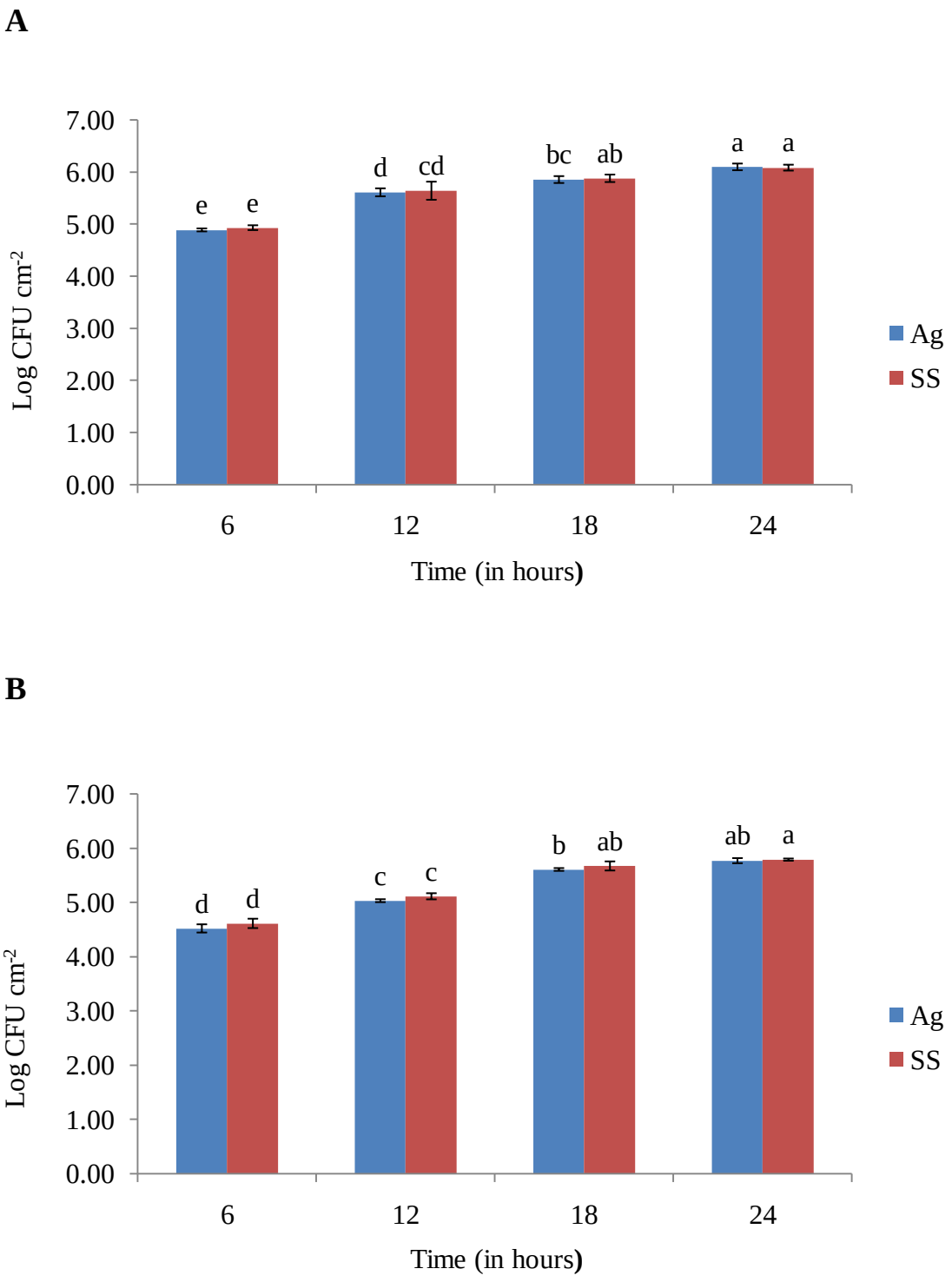

Figure 4.3: The number of cells of *S. thermophilus* (A) and *P. fluorescens* (B) on the silver-implanted (Ag) and stainless steel coupons (SS) during biofilm formation in skimmed milk at different time intervals. Means with the different letters are significantly difference from each other ($P < 0.05$).

4.2.3 Biofilm formation in whey

The number of cells attaching to the silver-implanted and stainless steel coupons in whey was determined as described in section 3.4. The results are shown in Figure 4.4. For *S. thermophilus*, a higher number of cells were present on the coupons in whey as compared to the whole and skimmed milk (Figure 4.4 A). After 6 hours, the number of cells present on the silver-implanted coupons and stainless steel coupons was 5.98 log CFU cm^{-2}, and 6.02 log CFU cm^{-2}, respectively. The same trend was observed after 12 and 18 hours, with the cells present on the stainless steel coupons in marginally higher numbers than on the silver-implanted coupons. However, after 24 hours, the silver-implanted coupons had 7.20 log CFU cm^{-2} compared to 7.17 log CFU cm^{-2} on the stainless steel coupons.

With *P. fluorescens* in whey, a similar trend to *S. thermophilus* was observed where the stainless steel coupons exhibited slightly higher number of cells than the silver-implanted coupons after 6-18 hours (Figure 4.4. B). However, after 24 hours, there were marginally more cells present on the silver-implanted coupons than on the stainless steel coupons. The number of cells present on the silver-implanted coupons was 4.89 log CFU cm^{-2} after 6 hours. This continued to increase with the time and after 24 hours, 5.78 log CFU cm^{-2} cells were present on the silver-implanted coupons. For the stainless steel coupons, there were 4.95 log CFU cm^{-2} after 6 hours, slowly increasing to 5.77 log CFU cm^{-2} after 24 hours.

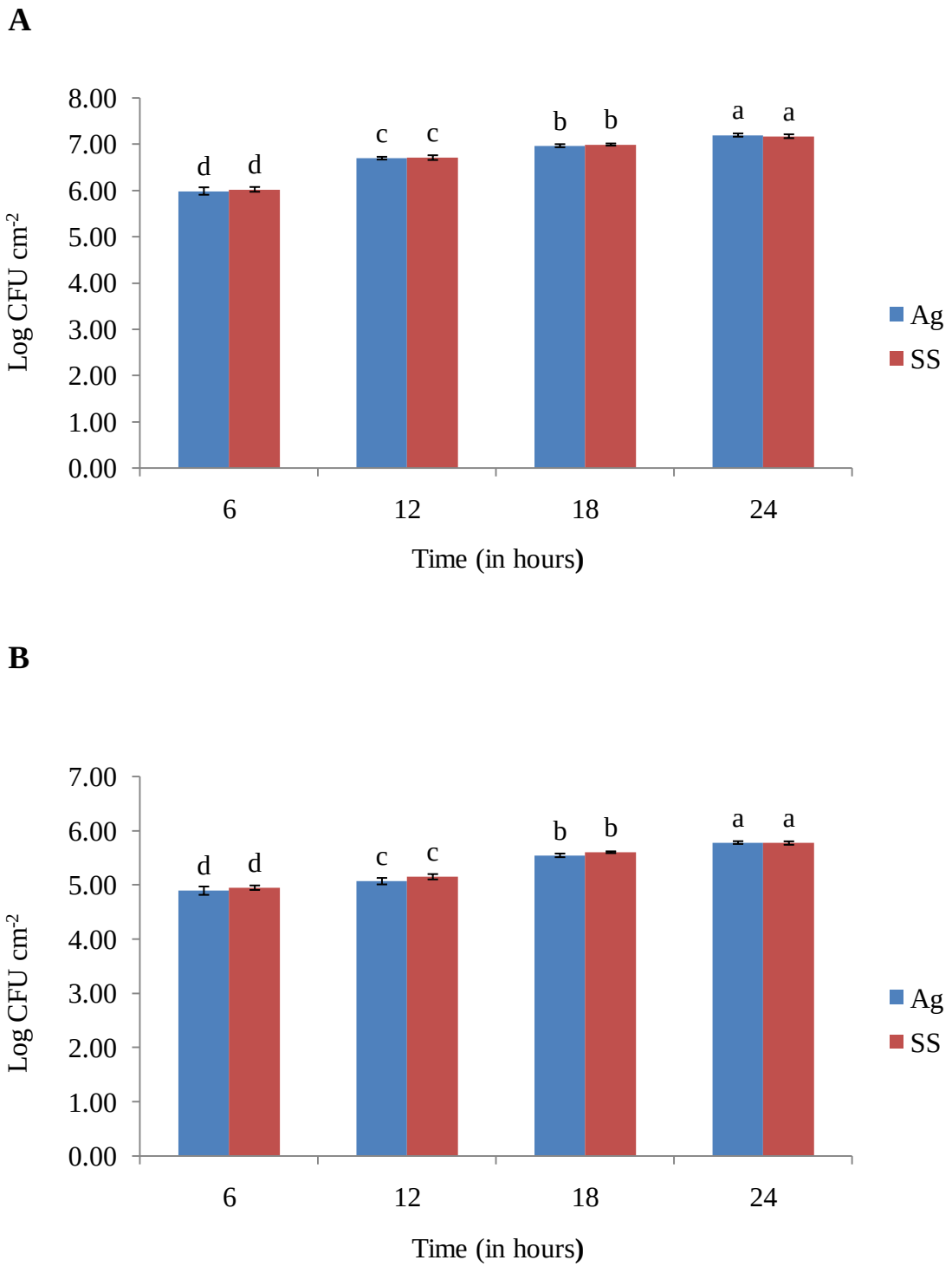

Figure 4.4: The number of cells of *S. thermophilus* (A) and *P. fluorescens* (B) on the silver-implanted (Ag) and stainless steel coupons (SS) during biofilm formation in whey at different time intervals. Means with the different letters are significantly difference from each other ($P < 0.05$).

4.3 EPS quantification

4.3.1 Dextran standard curve

The calibration curve for the quantification of EPS was generated using dextran as described in section 3.7. The results are shown in Figure 4.5. The R-square value for the curve was 0.9628 and the equation was: $y = 396.09x - 17.324$.

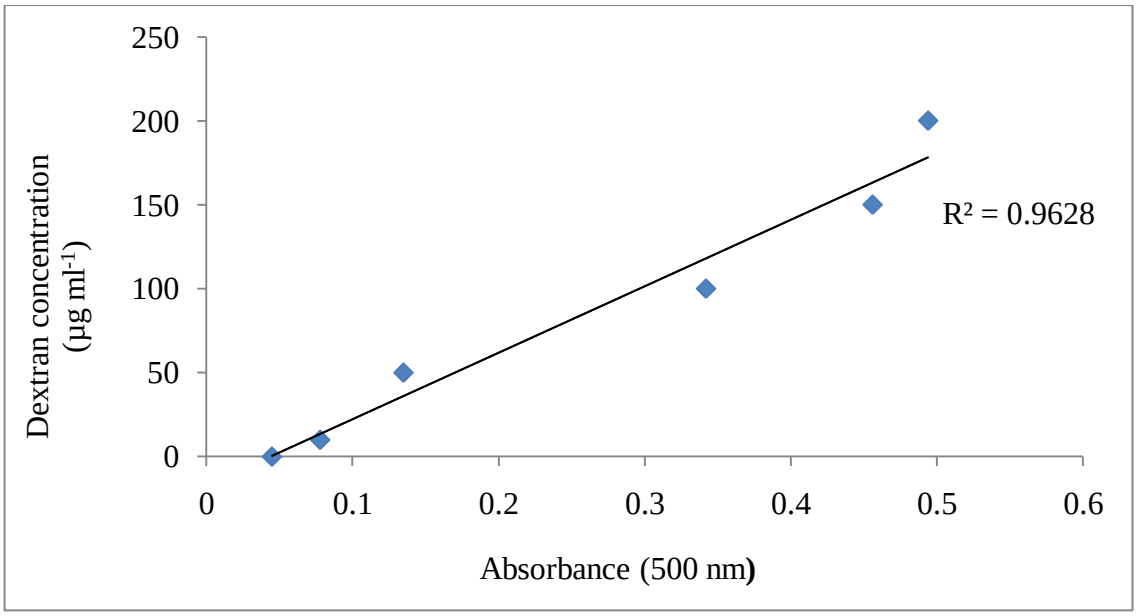

Figure 4.5: Calibration curve for the detection of EPS.

4.3.2 EPS production in whole milk

The EPS produced by the bacterial cells attached to the control and the test coupons in the whole milk was measured as described in section 3.7. The amount of EPS produced by *S. thermophilus* is shown in Figure 4.6 A. After 6 hours, the bacteria on the silver-ion implanted samples produced 580.30 µg EPS per cm^2, whereas, the bacteria on the stainless steel coupons produced 596.04 µg EPS per cm^2. The amount of EPS produced increased with time as more bacteria grew on the coupons. After 24 hours, 769.79 µg EPS was produced per cm^2 on the silver-implanted coupons, whereas, for the stainless steel coupons the amount was 821.65 µg EPS per cm^2. The difference in the amount of EPS produced on the test and the control coupons was statistically significant only after 18 hours ($P > 0.05$).

Similarly, when *P. fluorescens* was used as a test organism, the amount of EPS produced by bacteria increased with time, with the bacteria on the stainless steel coupons producing more EPS than those on the silver-implanted coupons (Figure 4.6 B). There was a statistically significant difference ($P < 0.05$) in terms of the amount of the EPS produced by bacteria on the test and the control coupons, after 6, 12 and 24 hours (104.74, 76.12 and 102.30 µg per cm^2, respectively). After 18 hours, the bacteria on the stainless steel coupons produced 1085.36 µg EPS per cm^2, as compared 1259.04 µg per cm^2 produced on the silver-implanted coupons. The difference between the two was, however, not statistically significant ($P > 0.05$).

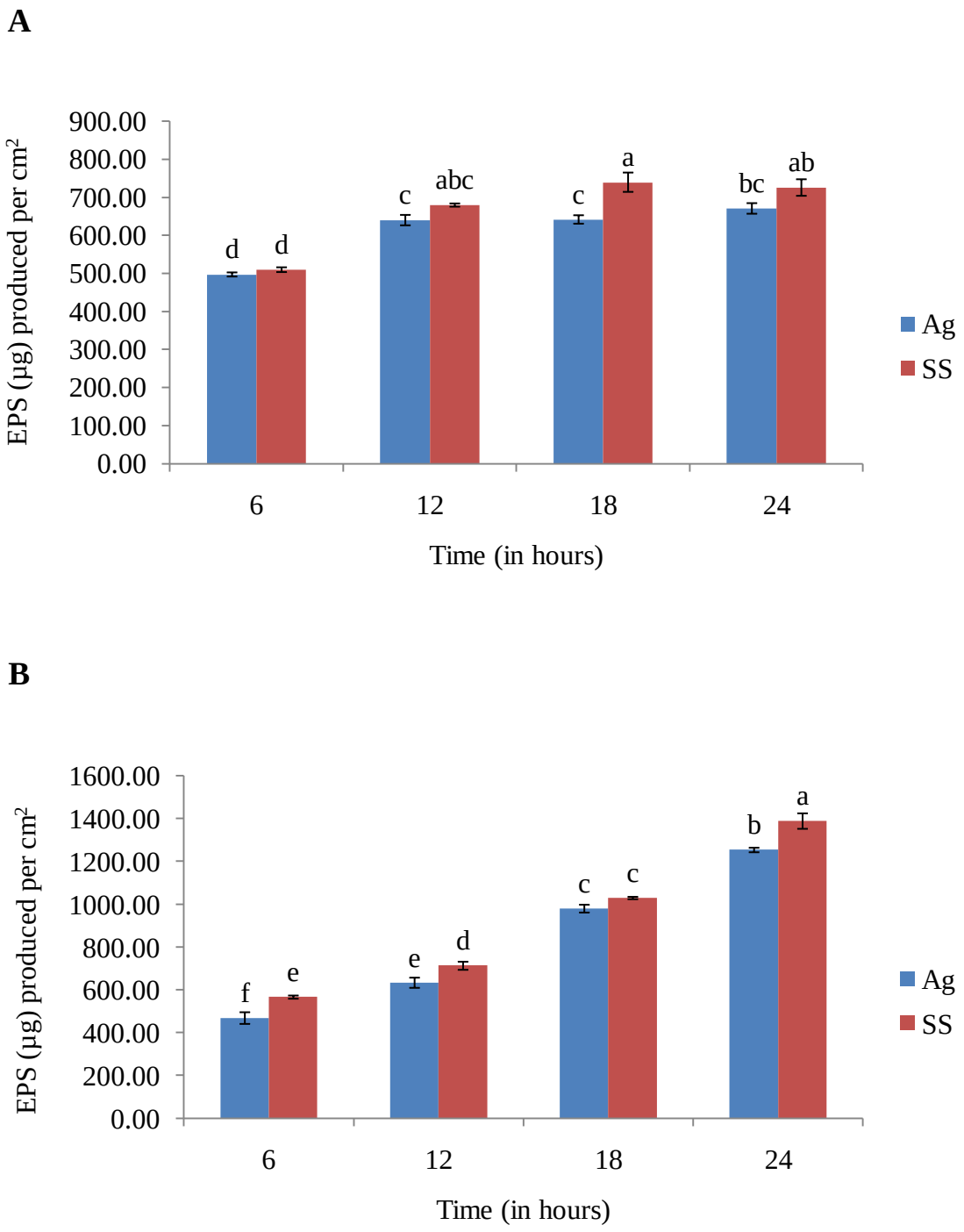

Figure 4.6: Amount of EPS produced per cm^2 by *S. thermophilus* (A) and *P. fluorescens* (B) in whole milk, on silver-implanted (Ag) and stainless steel (SS) coupons at different time intervals. Means with the different letters are significantly difference from each other ($P < 0.05$).

4.3.3 EPS production in skimmed milk

The amount of EPS produced by *S. thermophilus* cells growing on the control and the test coupons in skimmed milk was determined as described in section 3.7. The results are shown in Figure 4.7 A. Although a larger amount of EPS was produced by the bacteria when grown on the stainless steel coupons as compared to the silver-implanted coupons, the difference was not statistically significant ($P > 0.05$). After 6 hours, the bacteria growing on the silver-implanted coupons produced 642.90 µg EPS per cm^2, which was about 5.50 µg per cm^2 less than produced by the bacteria on the stainless steel coupons. The same trend was evident after 12 and 18 hours. However, after 24 hours, the EPS produced by the bacteria on the silver-implanted coupons was slightly higher (3.14 µg per cm^2) than produced by the bacteria on the stainless steel coupons.

P. fluorescens, in skimmed milk, showed the same trend as with *S. thermophilus* with the bacteria on the stainless steel coupons producing a higher amount of EPS than that produced by the bacteria on the silver-implanted coupons, but again, the difference was not statistically significant ($P > 0.05$) (Figure 4.7 B). After 6 hours, the difference in the amount of EPS produced by the bacteria on the control and the test coupons was 85.45 µg per cm^2. It further decreased with the time, and after 12 and 18 hours, was 25.93 and 8.96 µg per cm^2. Finally, after 24 hours, the amount of EPS produced on the control and test coupons was almost the same at nearly 906 µg per cm^2.

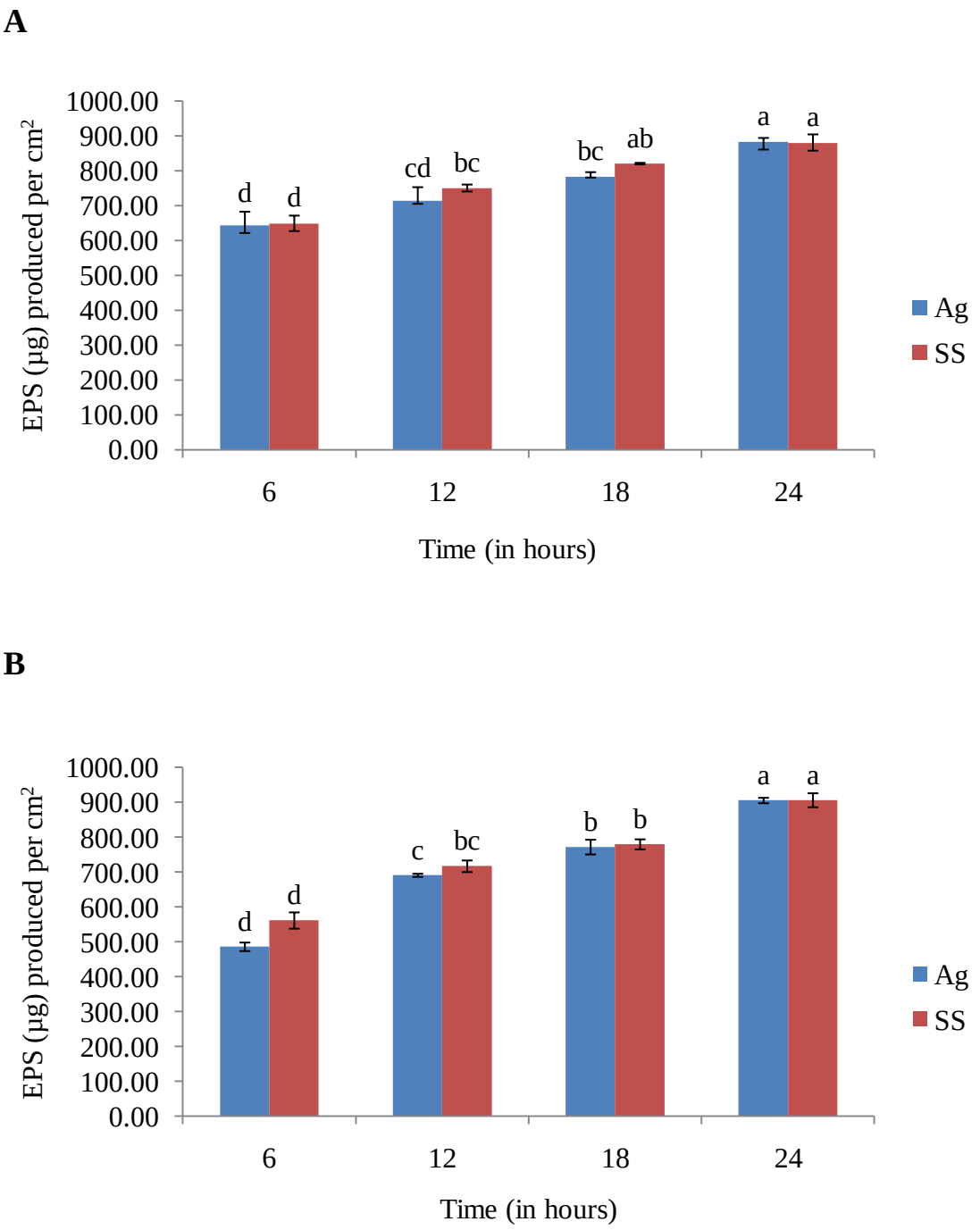

Figure 4.7: Amount of EPS per cm^2 produced by *S. thermophilus* (A) and *P. fluorescens* (B) in skimmed milk, on silver-implanted (Ag) and stainless steel (SS) coupons at different time intervals. Means with the different letters are significantly difference from each other ($P < 0.05$).

4.3.4 EPS production in whey

The amount of EPS produced by *S. thermophilus* cells while growing on silver-ion implanted coupons and stainless steel coupons in whey was determined as described in section 3.7. The results are presented in Figure 4.8 A. The amount of EPS produced by bacteria on the silver-implanted coupons was 855.87 µg per cm^2 as compared to 862.95 µg EPS per cm^2 on the stainless steel coupons after 6 hours. The amount of EPS produced increased after 12 hours with on the stainless steel coupons having higher EPS compared to the silver-implanted coupons. After 18 hours, the silver-implanted coupons had more EPS than the stainless steel coupons. The difference, however, was not statistically significant ($P > 0.05$). After 24 hours, the stainless steel coupons had more EPS than the silver-ion implanted coupons. The difference was 3.92 µg EPS per cm^2 but it was not statistically significant ($P > 0.05$).

For *P. fluorescens*, the amount of EPS on the silver-ion implanted coupons was less than that on the stainless steel coupons after 6 - 18 hours of incubation (Figure 4.8 B). After 6 hours, the amount of EPS on the silver-ion implanted coupons was 546.23 µg per cm^2, which increased to 615.40 µg per cm^2 after 12 hours and 729.35 µg per cm^2 after 18 hours. In contrast, with the stainless steel samples, the amount of EPS was 566.67 µg per cm^2 after 6 hours, 649.97 µg per cm^2 after 12 hours and 750.57 µg per cm^2 after 18 hours. The difference between the two coupons at any of the time intervals was not statistically significant ($P > 0.05$). After 24 hours, the amount of EPS on the silver-ion implanted coupons (965.12 µg per cm^2) was slightly higher than on the stainless steel coupons (959.62 µg per cm^2).

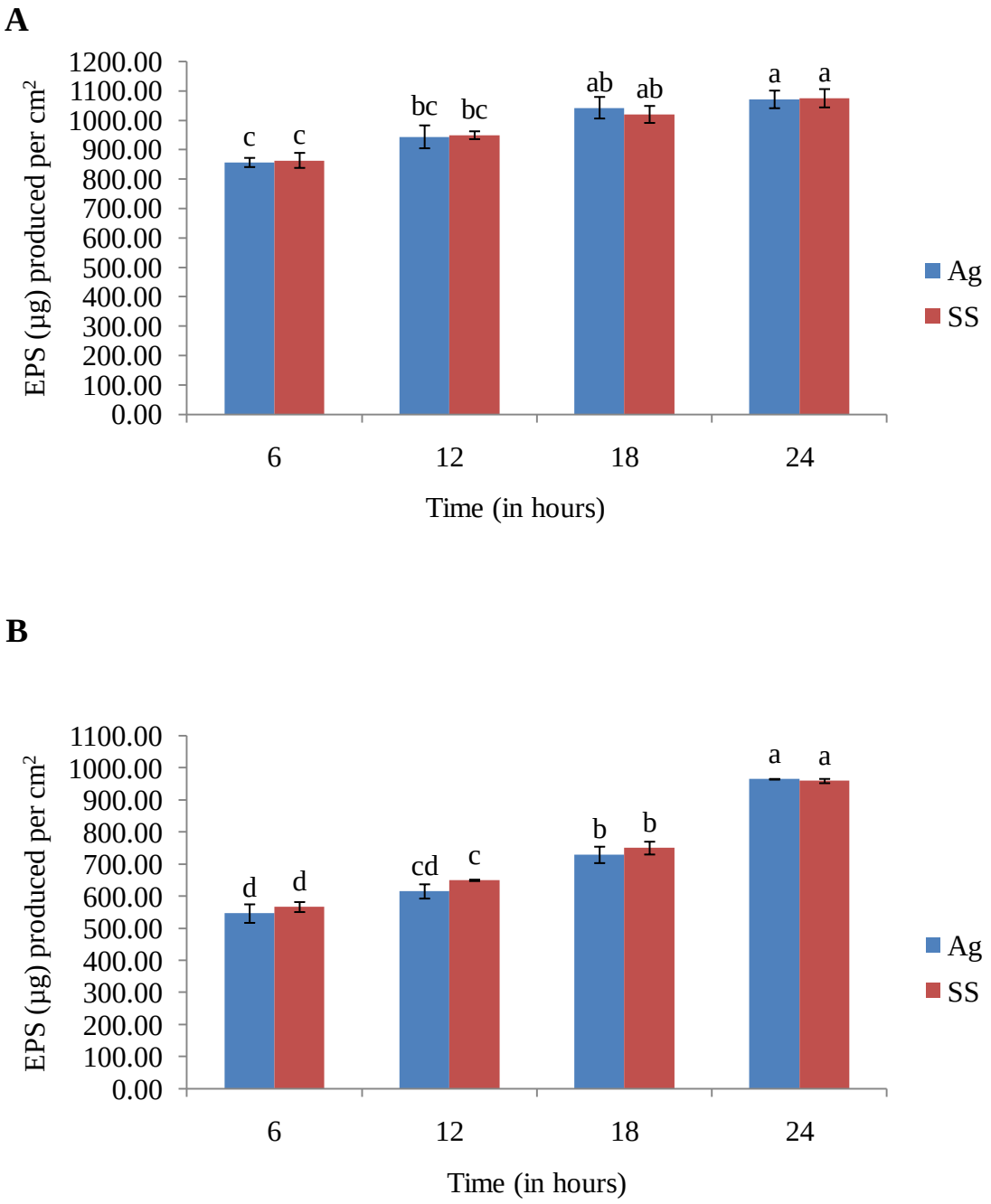

Figure 4.8: Amount of EPS per cm^2 produced by *S. thermophilus* (A) and *P. fluorescens* (B), in the whey, on silver-implanted (Ag) and stainless steel (SS) coupons at different time intervals. Means with the different letters are significantly difference from each other ($P < 0.05$).

4.4 Microscopy

4.4.1 Epifluorescent microscopy

Figure 4.9 shows the images of *S. thermophilus* biofilm, growing on the stainless steel and the silver-implanted coupons, stained with the Live/Dead® BacLight™ Bacterial Viability stain (Molecular Probes, The Netherlands) as described in section 3.6.1. For the silver implanted coupons, it can be seen that there are some areas of bacteria which fluoresced red, implying that the bacteria are dead and some areas with green and red cells, which indicate that there are viable bacteria, which fluoresced green, and dead cells, which fluoresced red. A few yellow coloured cells indicate injury to the cell walls (Boulos et al. 1999). On the other hand, the bacteria growing on the stainless steel coupons fluoresced green, showing that they are alive. No large patterns of red-stained cells were observed. The same was observed for *P. fluorescens* biofilms (Figure 4.10).

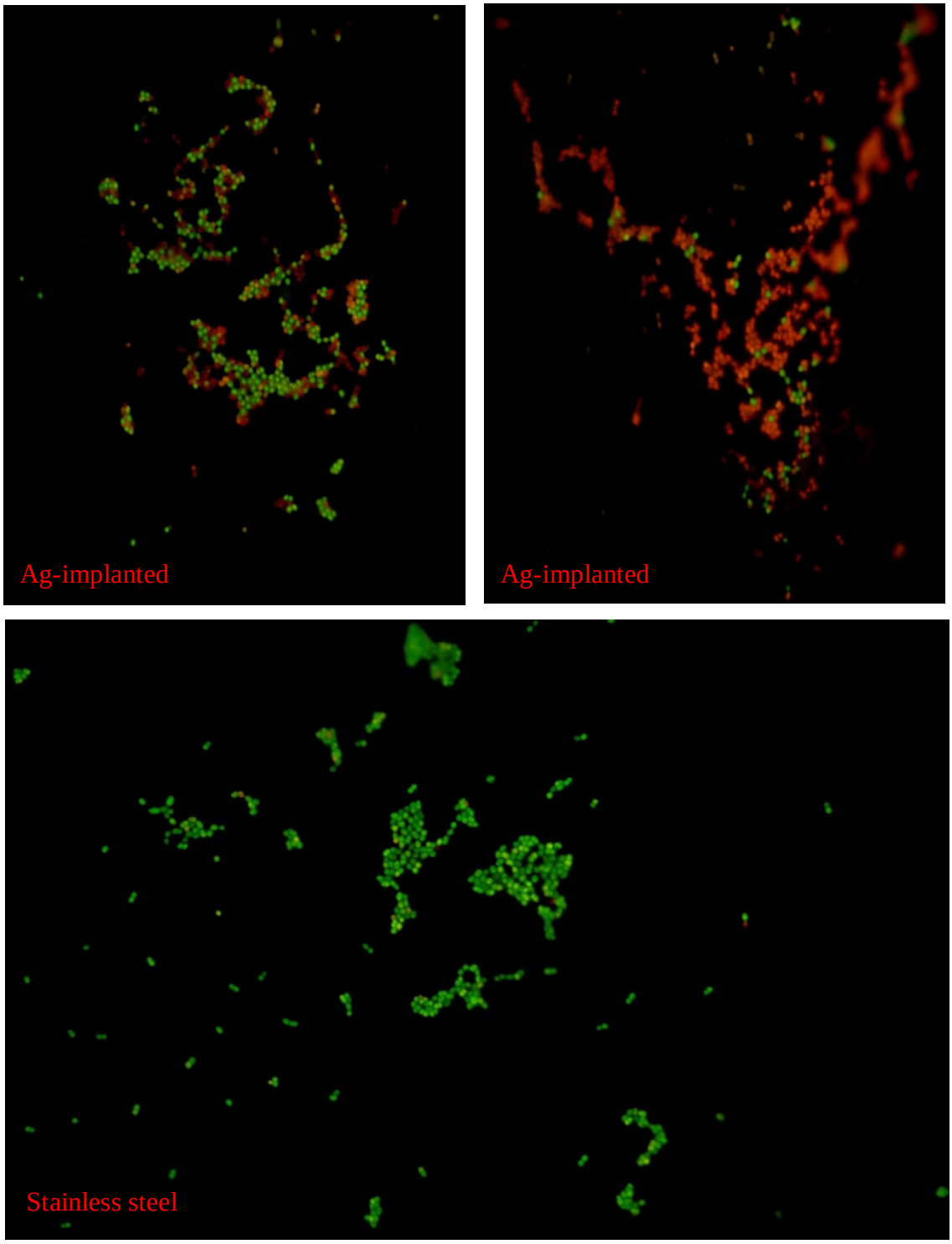

Figure 4.9: Images of silver-implanted (Ag-implanted) and stainless steel coupons, exposed to *S. thermophilus* for 24 hours in the whole milk, stained with the Live/Dead® BacLight™ Bacterial Viability stain. Magnification = 100 X.

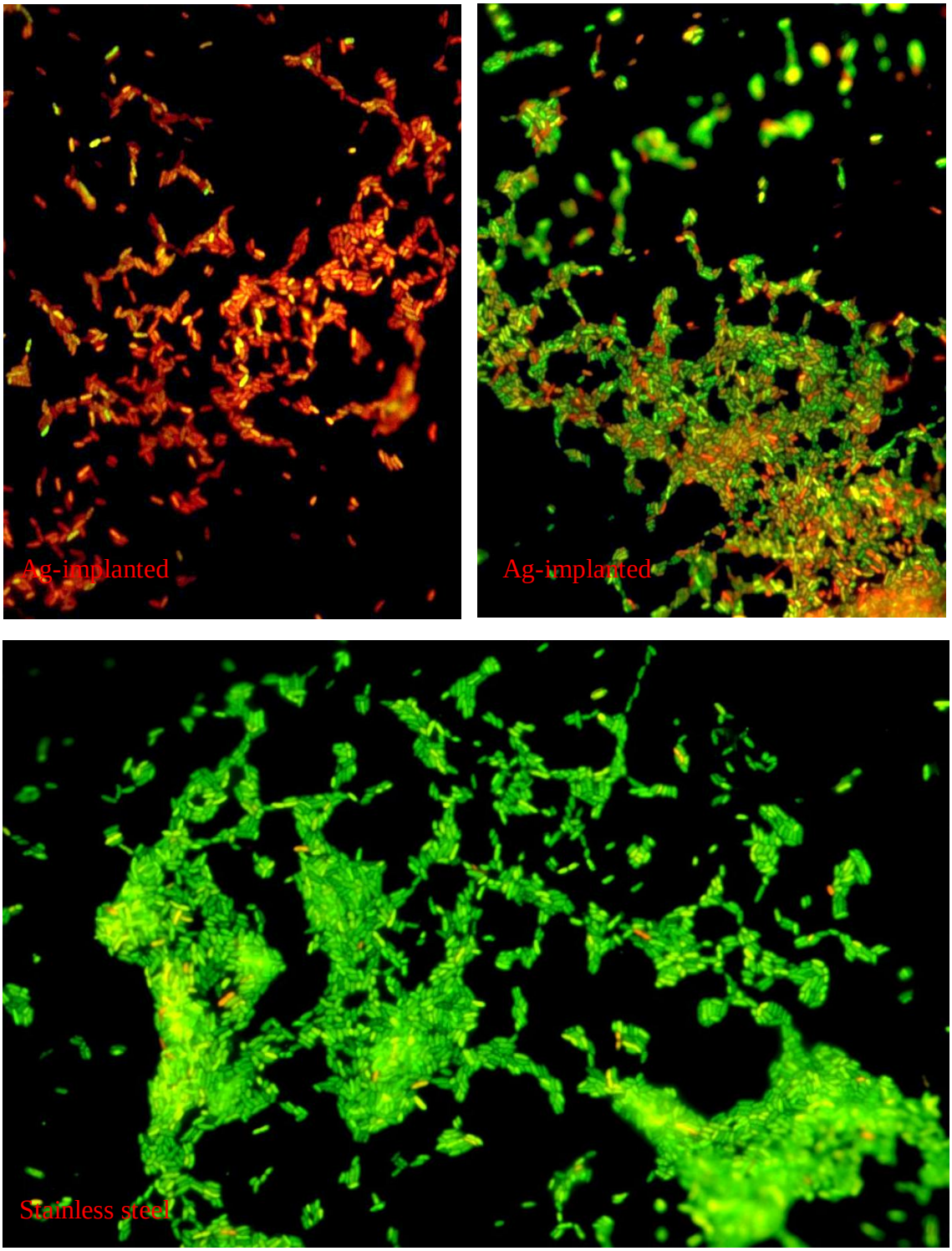

Figure 4.10: Images of silver-implanted (Ag-implanted) and stainless steel coupons, exposed to *P. fluorescens* for 24 hours in the whole milk, stained with the Live/Dead® BacLight™ Bacterial Viability stain. Magnification = 100 X.

4.4.2 Scanning electron microscopy

Figure 4.11 shows the bacteria growing on the silver-ion implanted coupons and the stainless steel coupons under the electron microscope as described in section 3.6.2. Both *S. thermophilus* and *P. fluorescens* can be seen attached to both the types of coupons. The images also show the coupon surface at a tilt of 65°. No particularly significant difference is visible to the eye in terms of the surface structure and topography of the silver-ion implanted coupon and the stainless steel coupon.

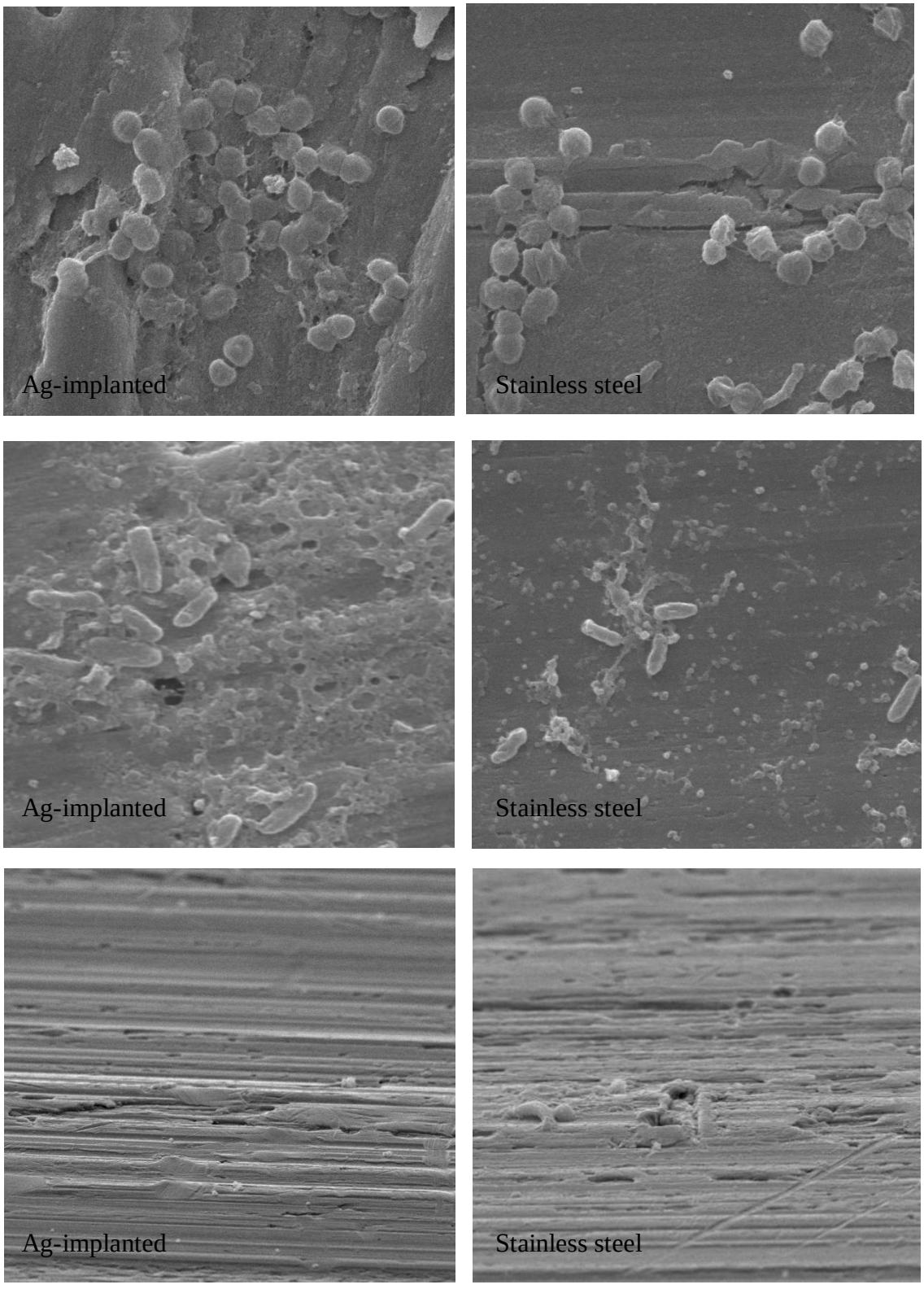

Figure 4.11: Images of stainless steel (right) and silver-implanted (left) coupons with biofilms of *S. thermophilus* (top) and *P. fluorescens* (middle) after 24 hours. The bottom two images show the surfaces of stainless steel (right) and silver-implanted (left) coupons when observed under SEM at a tilt of 65°.

CHAPTER FIVE: DISCUSSION

5.1 Discussion

5.2 Future work

5.3 Conclusions

CHAPTER FIVE: DISCUSSION

5.1 Discussion

Biofilms are a major problem in food processing industries including the dairy industry. They can result in contamination of products when the cleaning process in a manufacturing plant is ineffective (Sharma & Anand, 2002). The routine CIP procedures used in food manufacturing plants are not always effective enough to remove all attached bacterial cells (Flint, 1998). Bacterial attachment is the initial and the most important stage in biofilm formation (Palmer et al., 2007). Stainless steel is commonly used in food manufacturing plants because of its heat transfer efficiency, corrosion resistance and strength (Brooks & Flint, 2008). However, it also has a tendency to attract bacteria that can develop into biofilms. Surface modification is an alternative method for the control of biofilms and involves changing the surface properties of a material by physical or chemical methods to stop bacteria from attaching to the surface. Implanting ions on the stainless steel surfaces is an effective way of reducing bacterial attachment (Zhao et al., 2008). This study showed the effect of silver-ion implanted stainless steel surfaces on the cell viability and biofilm formation of *S. thermophilus* and *P. fluorescens*.

In this study, the silver ions were implanted onto stainless steel coupons and the number of bacteria attaching to the coupons, after exposure to the bacterial suspension in different media for 30 minutes, was determined by removing the cells by vortex mixing and plating the dislodged cells on agar. For both *S. thermophilus* and *P. fluorescens*, it was found that the difference in the number of bacteria attached to the silver-implanted coupons and the control stainless steel coupons was higher when saline and M17 were used for incubation as compared to whole milk, skimmed milk and whey. This may be due to the conditioning layer formed on the coupon surface by milk and whey proteins, preventing direct contact of the bacteria with the silver ions implanted on the surface. A conditioning layer is formed on a surface within 5 to 10 seconds on coming into direct contact with milk (Mittelman, 1998). It appears that the conditioning film could have influenced attachment. To investigate this further, biofilm formation on the silver-implanted and the stainless steel surfaces was studied in the presence of whole milk, skimmed milk and whey.

Monitoring biofilm formation of both *S. thermophilus* and *P. fluorescens* on the silver-implanted and stainless steel coupons showed similar trends in the presence of whole milk, skimmed milk and whey. There was a marginal difference in the number of bacteria present on the stainless steel coupons and the silver-implanted coupons. But after 24 hours of incubation, the number was more or less the same on both types of coupons. SEM images showed that some of the bacteria were trapped in the conditioning layer and thus, unable to come in direct contact with the silver ions implanted on the surface (Appendix). Due to the protective effect of the conditioning layer, silver ions couldn't completely exhibit their antibacterial activity which resulted in similar attachment on both types of coupons. However, there are conflicting opinions on the role of conditioning film in the attachment of bacteria.

Barnes et al. (1999) reported that the milk proteins formed a conditioning layer and prevented attachment of bacteria to the pre-conditioned surface. Presence of proteins such as fibrinogen, albumin and gelatin inhibited attachment of *Pseudomonas* on polystyrene (Fletcher, 1976). Preconditioning stainless steel and buna-N rubber with milk was also shown to inhibit attachment of *Listeria* (Wong & Amy, 1998). Both *L. monocytogenes* and *Salmonella typhimurium* attached to stainless steel in fewer numbers in the presence of individual milk components such as casein and β-lacto globulin (Helke et al., 1993). Coating of skimmed milk proteins on stainless steel also reduced the attachment of thermophilic bacilli to the surface (Parkar et al., 2001). On the other hand, it was suggested that the presence of a conditioning film of proteins may result in higher adhesion because the attached proteins could act as source of nutrients for the bacteria (Jeong & Frank, 1994). Stainless steel and rubber surfaces had higher attachment of bacteria when treated with lactose and whey proteins (Speers & Gilmour, 1985). Skimmed milk resulted in enhanced attachment of *P. fluorescens* after 1 hour on stainless steel as compared to other media like TSB and meat juice (Hood & Zottola, 1997 a). In this study, it was found that the bacteria attached to the organic molecules forming the conditioning layer, which in turn protected bacteria from coming in direct contact with the silver-implanted surface. Thus, milk and whey proteins did not inhibit attachment of these bacteria.

Epifluorescence micrographs of the silver-implanted coupons on which biofilms had formed, showed that there were a few areas containing dead (red coloured) cells only, and a few areas containing dead (red coloured), alive (green coloured) and injured

(yellow coloured) cells on the same coupon (Figure 4.9 & 4.10). The coupon surface was homogenously implanted with silver ions. However, it appears that there were certain sites which bacteria preferred for attachment and certain sites where there were more dead cells than others. This may be due to the non-uniform conditioning layer formed on the surface which protected bacteria from the silver ions only at a few sites. The images confirmed that the slightly reduced attachment of viable cells on silver-implanted coupons was due to the antibacterial action of silver ions. Varying amounts of propidium iodide enter the cells depending on the damage to the cell wall. Thus, yellow coloured cells have had their walls partially damaged as compared to the red coloured cells which had fully damaged cell walls (Boulos et al., 1999).

Although few cells had partially damaged cell walls, they might have recovered and formed colonies on the agar plates after being dislodged from the coupons and plated. Therefore, the differences between the number of alive cells present on the silver-implanted and stainless steel coupons, as seen under the microscope, is not reflected on the plate counts. Also, once the bacteria are dead, they form a layer which acts as a physical barrier, which the silver ions must overcome to come in direct contact with other bacterial cells (Mulligan et al., 2003). Silver is a potent antimicrobial agent which normally results in the killing of cells. So the cells might have been injured due to the restricted access of the silver ions to bacteria or due to the shielding effect of EPS.

When EPS was quantified, it was found that both *S. thermophilus* and *P. fluorescens* produced EPS. However, EPS produced on the stainless steel coupons was found to be slightly higher than that produced on the silver-implanted coupons. Cells produce EPS during biofilm formation where it provides mechanical stability to the biofilm structure and protects the cells from harmful environmental conditions. EPS production can be one of the reasons why cells embedded in biofilms are more resistant to antimicrobial agents than the planktonic cells. EPS can also form a conditioning film on the surface (Allison & Sutherland, 1987). Thus, the conditioning film formed by the EPS could have acted as a physical barrier between the silver ions and the cells. The protection conferred by the EPS on biofilm cells against biocidal agents has been well documented. There was a decreased penetration of the antimicrobial agents in the biofilms of *Klebsiella pneumoniae* and *P. aeruginosa* (De Beer et al., 1994; Huang et al., 1995). The inhibitory concentration of the silver ions against the bacteria embedded in biofilms was 10-100 times higher than that required for the planktonic cells of *P.*

aeruginosa (Bjarnsholt et al., 2007). The negatively charged EPS can restrict permeation of the silver ions by binding to the positively charged silver ions (Lewis, 2001). However, according to Silvestry-Rodriguez et al. (2008), with high silver concentration (more than 100 µg/litre) or long exposure times, the biofilm capacity to absorb the silver cations can be exceeded and then silver ions can be effective against the biofilms. The authors mentioned this in a study on the effect of silver ions on biofilm development in water distribution systems. The results in dairy manufacturing plants could be different due to the presence of the milk product-based conditioning layer. It would be interesting, however, to determine the optimum dose of the silver ions required to penetrate and kill the cells in biofilms in the presence of milk and whey proteins. Therefore, it can be concluded that the EPS production may be one of the reasons why there was no significant difference between the number of bacteria present on the silver-implanted and stainless steel coupons.

Other possible reasons for the poor action of silver ions could be the physiological state of the cells in the biofilm or the presence of subpopulations with resistant phenotypes (Hall-Stoodley et al., 2004). The physiological state of the cells embedded deep in the biofilms can change as they face nutrient limitation (Brown & Gilbert, 1993). Nutrient deprivation forces cells to slow their growth rate and alter their metabolism and physiological responses. Due to this semi-dormant state, cells are resistant to the uptake of antimicrobial agents including the silver ions. Furthermore, the existence of subpopulations with the resistant phenotype, known as persisters, could also be one of the reasons for the poor effect of the silver ions on bacteria. Persister cells are not resistant to antimicrobials but they have a very high tolerance for them. The fraction of persister cells present in biofilms is higher than that present in planktonic cells (Spoering & Lewis, 2001). In a typical medical infection, persister cells constitute 0.1-10 % of biofilms (Percival et al., 2011). Harrison et al. (2005) showed that a small percentage of persister cells existed in *P. aeruginosa* biofilms which were tolerant to high concentrations of metal cations such as Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{2+} And Pb^{2+}. Although, the study did not test the resistance for silver cations, there is a likelihood that in our present study, there might be a percentage of persistent cells tolerant to high amount of silver ions. Hence, a few cells embedded in the biofilms grown on the coupons might be resistant to the silver ions.

There are various proposed mechanisms for the antimicrobial action of silver (Section 2.4.3.1.1). However, it is thought that the antibacterial action of silver is such that the chances of bacteria developing resistance to the silver-based antimicrobials due to regular exposure are very low (Clement & Jarrett, 1994). Bacteria are less likely to develop resistance to the chemical-based antimicrobials as compared to antibiotics (Duncan, 2011). Silver is also very easy to incorporate into materials. Thus, there have been many instances where the resistance of bacteria to silver incorporated into food contact surfaces has been investigated.

Silver can be incorporated in different forms into materials such as silver ions, silver zeolites, silver nanoparticles etc. Silver nanoparticles have been investigated for increasing the shelf life of certain foods. Silver nanoparticles increase the shelf life of the food products in terms of physical, microbial and nutritional aspects (An et al., 2008; Mohammed et al., 2009; Duncan, 2011). However, the toxicity and environmental safety of silver nanoparticles has not been clearly established, with some groups hoping to get silver nanoparticles classified as a new pesticide (Moretro & Langsrud, 2011). Clearly there is controversy and silver nanoparticles shouldn't be used as an additive in the food packaging films and other food contact surfaces unless declared safe for long term use.

Surfaces treated with silver ions or silver-containing zeolites have been tested for their antibacterial activity in food-based systems. Ilg et al. (2011) tested polypropylene sheets containing 1% silver, in a zirconium phosphate based ceramic ion exchange resin, against *P. fluorescens* at 35°C for 24 hours in the presence of protein rich foods such as turkey meat, egg white and yoghurt. They found no significant difference ($P > 0.05$) between the number of bacteria present on the reference material and the test material. The other non-protein components such as saccharose, glucose, thistle and soy oil had no effect on the antimicrobial nature of the test material. The inhibition of the effect of silver was thought to be due to the binding of silver ions by the functional groups of the food proteins. In another study, it was shown that the antimicrobial activity of a silver-implanted polymer catheter surface against *Candida albicans* was totally eliminated by the addition of 5 % horse serum (Kampf et al., 1998). Silver initially reacts with the thiol groups present in enzymes and thus, may not be an effective antimicrobial when present in nutrient rich media with lysine, sulphates, sulphides and other sulphur-containing amino acids present (Appendini & Hotchkiss, 2002). Our study also found

out that the antimicrobial activity of the silver ions is inhibited in the presence of whole milk as well as skimmed milk. The whole milk contained 3.2 g protein and 3.3 g fat per 100 ml. Skimmed milk, on the other hand, contained 3.7 g protein and 0.1 g fat per 100 ml. This shows that the presence of proteins is a major factor affecting the antimicrobial activity of silver ions. Similarly, another study showed that the presence of lipids did not affect the antimicrobial nature of the silver containing polypropylene sheets (Ilg & Kreyenschmidt, 2011). Yet another study reported a reduction in the inhibitory effect of silver zeolite and other antimicrobial agents, added to a conveyor belt material made up of polyurethane, by 1.2 – 2.5 log CFU/100 cm^2 when food debris, including meat and fish, was introduced on the conveyor belt (Chaitiemwong, et al., 2010). This suggests that the addition of silver ions to food contact surfaces in direct contact with protein rich foods may be of limited use in industry.

In contrast, it has also been reported that the proteins did not interfere with the antimicrobial activity of the silver ions when the inner lining of a refrigerator, with a coating of zirconium phosphate resin containing silver, was tested against a few spoilage and pathogenic bacteria (Kampmann et al., 2008). The study reported a reduction of 1.4 log_{10} units in the case of *Lactobacillus delbrueckii* after incubation at 5°C for 72 hours as compared to the control polystyrene surface, whereas, for *P. fluorescens*, it was 5.4 log_{10} units. When food items were stored in silver-lined refrigerators, it was found that there was less bacterial growth in the food compared to those placed in control refrigerators after 6-11 days of storage. There was, on average, 1 log CFU cm^{-2} difference between the food items such as under-cooked ham, sliced cheese and pork sausage placed in the silver-lined refrigerator as compared to that placed in the control refrigerators after 6-11 days. There was no impact on the sensory characteristics of the foods except under-cooked ham, which had better sensory characteristics when stored in the silver-lined refrigerator as compared to that stored in the control.

However, even without the presence of any proteinaceous substance from food, a study reported no difference between the silver-implanted polyethylene surfaces and the control polyethylene surfaces (Berrang et al., 2010). The surfaces were suspended in *L. monocytogenes* and *P. putida* cell suspensions for 2 hours before rinsing and incubating in dilute brain heart infusion broth for 24 hours at 25°C. The surfaces were also dried for 24 hours after exposure and incubated in the broth for 24 hours at 25°C. It was

found that there was no difference, in terms of the number of bacteria present, between the two surfaces when they were sampled immediately after exposure or when they were sampled after drying for 24 hours. But the dose of silver atoms implanted in this study was not reported. The results might have been due to the low silver dose as the antimicrobial effect of silver ions is directly dependent on the concentration of the ions.

In our study, the effect of silver ions on Gram positive *S. thermophilus* and Gram negative *P. fluorescens* was almost the same, with both bacteria present on the silver-implanted coupons in almost the same number as that on the stainless steel coupons. The same findings were reported by Berrang et al. (2010), where they found no difference between the antimicrobial activity of silver on Gram positive *L. monocytogenes* and Gram negative *P. putida*. However, a few studies have reported higher antimicrobial action of silver against Gram negative bacteria than against Gram positive bacteria due to the thicker peptidoglycan layer in Gram positive bacteria which makes them less susceptible to antimicrobial compounds (Huang et al., 1995; Kampmann et al., 2008; Ilg et al., 2011). On the other hand, Huang et al. (2011) reported higher antimicrobial activity of silver against Gram positive *Staphylococcus aureus* than against Gram negative *E. coli*. They attributed higher resistance of *E. coli* to the tightly packed lipopolysaccharide layer in the outer membrane which provided an extra barrier to the biocidal molecules. The similar response between the Gram positive and Gram negative bacteria in the present study may be the result of the similarity between the effect of the thick peptidoglycan layer in the Gram positive bacterium and the effect of the EPS produced by the Gram negative bacterium.

In summary, the silver ions implanted into a surface can kill microbial cells. However, the surface has to be free of any conditioning film or protein layer. Our study found that there was no significant difference ($P > 0.05$) between the number of bacteria present on the silver-implanted coupons and the stainless steel coupons in the presence of skimmed milk, whole milk and whey. This might have been due to the conditioning layer formed by the milk proteins or due to the binding of proteins with the silver ions, which resulted in very low number of free silver ions to produce efficient antimicrobial activity. The effect of silver ions was, however, similar on Gram negative and Gram positive bacteria.

5.2 Future work

Our study showed that the effect of silver ions on bacterial viability was low in the presence of milk and whey. Similarly, another study showed that there was no effect of silver ions (50 ppb) on viability of *S. epidermidis* (Chaw et al., 2005). But they found that the binding of silver ions to the proteins and polysaccharides lead to the destabilization of biofilm structure when observed under SEM. It should be determined if the silver ions can destabilize the structure of a dairy biofilm grown over a couple of days as the biofilms in dairy plants not only contaminate the final product but are hard to clean. Thus, it is important to see if there are any structural differences in the biofilms due to the presence of silver ions and if it leads to easy cleaning of biofilms from the surface.

Antimicrobial activity of the silver ions is reduced in the presence of EPS and proteinaceous materials due to the binding of silver ions to EPS and proteins. This reduces the availability of silver ions to act on the bacterial cells. It would be useful to know how much silver is required to function as an antimicrobial agent. In our study, the coupons were implanted with 1×10^{16} silver ions per cm^2. However, in reality the effect of the silver ions was quenched by EPS and proteins. Higher silver ion concentrations may overcome this quenching

It would be interesting to know the effect of the silver ion-implantation on other food contact surfaces which do not come in direct contact with proteinaceous materials like milk, meat or fish. Surfaces coming in contact with fruits and vegetables should be tested to see if silver-implantation leads to less contamination.

5.3 Conclusions

This study was conducted to determine the effect of the silver-implanted stainless steel coupons on the attachment and biofilm formation of bacteria of importance to the dairy industry. It was found that in the presence of whole milk, skimmed milk and whey, the number of bacteria present on the silver-implanted coupons and the stainless steel coupons was not significantly different ($P > 0.05$). The epifluorescence micrographs showed that there were some dead bacteria present on the silver-implanted coupons, as well as a few injured and alive cells. Thus, the silver ions had antimicrobial activity but not enough to reduce the number of bacteria present on the test surfaces by one log or more. The poor antibacterial activity of silver could be due to the conditioning layer formed by the proteins which prevented direct contact between the bacteria and the silver ions. It could also be attributed to the binding of silver ions by the EPS or proteins, which resulted in fewer silver ions available to act on the bacterial cells. The silver-implanted surfaces may be of limited practical value in the industry as the presence of milk proteins greatly reduces the antibacterial activity of the silver ions.

REFERENCES

Ahearn, D., May, L., & Gabriel, M. (1995). Adherence of organisms to silver-coated surfaces. *Journal of Industrial Microbiology and Biotechnology, 15*(4), 372-376.

Allison, D. G., & Sutherland, I. W. (1987). The role of exopolysaccharides in adhesion of freshwater bacteria. *Journal of General Microbiology, 133*(5), 1319-1327.

An, J., Zhang, M., Wang, S., & Tang, J. (2008). Physical, chemical and microbiological changes in stored green asparagus spears as affected by coating of silver nanoparticles-PVP. *Food Science and Technology, 41*(6), 1100-1107.

An, Y., Friedman, R., Draughn, R., Smith, E., Nicholson, J., & John, J. (1995). Rapid quantification of staphylococci adhered to titanium surfaces using image analyzed epifluorescence microscopy. *Journal of Microbiological Methods, 24*(1), 29-40.

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science & Emerging Technologies, 3*(2), 113-126.

Augustin, M., Ali-Vehmas, T., & Atroshi, F. (2004). Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *Journal of Pharmacy and Pharmaceutical Science, 7*, 55-64.

Azevedo, N. F., Pinto, A. R., Reis, N. M., Vieira, M. J., & Keevil, C. W. (2006). Shear stress, temperature, and inoculation concentration influence the adhesion of water-stressed *Helicobacter pylori* to stainless steel 304 and polypropylene. *Applied and Environmental Microbiology, 72*(4), 2936-2941.

Bansal, B., & Chen, X. (2006). A critical review of milk fouling in heat exchangers. *Comprehensive Reviews in Food Science and Food Safety, 5*(2), 27-33.

Barnes, L. M., Lo, M., Adams, M., & Chamberlain, A. (1999). Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. *Applied and Environmental Microbiology, 65*(10), 4543-4548.

Beech, I. (2004). Corrosion of technical materials in the presence of biofilms - current understanding and state-of-the art methods of study. *International Biodeterioration & Biodegradation, 53*(3), 177-183.

Benjamin, S., & Labib, M. (2000). Turbulent two phase flow; A novel method for cleaning and high grade disinfection of flexible endoscopies and internal channels. *The American Journal of Gastroenterology, 95*(9), 2492.

Berrang, M. E., Frank, J. F., & Meinersmann, R. J. (2010). *Listeria monocytogenes* biofilm formation on silver ion impregnated cutting boards. *Food Protection Trends, 30*(3), 168-171.

Bjarnsholt, T., Kirketerp-Moller, K., Kristiansen, S., Phipps, R., Nielsen, A. K., Jensen, P. O., . . . Givskov, M. (2007). Silver against *Pseudomonas aeruginosa* biofilms. *Apmis, 115*(8), 921-928.

Blackman, I. C., & Frank, J. F. (1996). Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *Journal of Food Protection, 59*(8), 827-831.

Bos, R., Mei, H., Gold, J., & Busscher, H. (2000). Retention of bacteria on a substratum surface with micro-patterned hydrophobicity. *FEMS Microbiology Letters, 189*(2), 311-315.

Bosetti, M., Mass , A., Tobin, E., & Cannas, M. (2002). Silver coated materials for external fixation devices: *in vitro* biocompatibility and genotoxicity. *Biomaterials, 23*(3), 887-892.

Boulangé-Petermann, L. (1996). Processes of bioadhesion on stainless steel surfaces and cleanability: A review with special reference to the food industry. *Biofouling, 10*(4), 275-300.

Boulangé-Petermann, L., Rault, J., & Bellon-Fontaine, M. (1997). Adhesion of *Streptococcus thermophilus* to stainless steel with different surface topography and roughness. *Biofouling, 11*(3), 201-216.

Boulos, L., Prévost, M., Barbeau, B., Coallier, J., & Desjardins, R. (1999). Live/Dead BacLight™: Application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiological Methods, 37*(1), 77-86.

Bower, C., McGuire, J., & Daeschel, M. (1996). The adhesion and detachment of bacteria and spores on food-contact surfaces. *Trends in Food Science & Technology, 7*(5), 152-157.

Bremer, P., Fillery, S., & McQuillan, A. (2006). Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology, 106*(3), 254-262.

Bremer, P. J., Monk, I., & Osborne, C. M. (2001). Survival of *Listeria monocytogenes* attached to stainless steel surfaces in the presence or absence of *Flavobacterium* spp. *Journal of Food Protection, 64*(9), 1369-1376.

Bright, K., Gerba, C., & Rusin, P. (2002). Rapid reduction of *Staphylococcus aureus* populations on stainless steel surfaces by zeolite ceramic coatings containing silver and zinc ions. *Journal of Hospital Infection, 52*(4), 307-309.

Brooks, J., & Flint, S. (2008). Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science & Technology, 43*(12), 2163-2176.

Brown, M., & Gilbert, P. (1993). Sensitivity of biofilms to antimicrobial agents. *Journal of Applied Microbiology, 74*, 87S-97S.

Bryers, J. (1987). Biologically active surfaces: processes governing the formation and persistence of biofilms. *Biotechnology Progress, 3*(2), 57-68.

Carpentier, B., & Cerf, O. (1993). Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Microbiology, 75*(6), 499-511.

Chaitiemwong, N., Hazeleger, W., & Beumer, R. (2010). Survival of *Listeria monocytogenes* on a conveyor belt material with or without antimicrobial additives. *International Journal of Food Microbiology, 142*(1-2), 260-263.

Chaw, K., Manimaran, M., & Tay, F. E. H. (2005). Role of silver ions in destabilization of intermolecular adhesion forces measured by atomic force microscopy in *Staphylococcus epidermidis* biofilms. *Antimicrobial Agents and Chemotherapy, 49*(12), 4853.

Chmielewski, R., & Frank, J. (2003). Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety, 2*(1), 22-32.

Chu, P., Chen, J., Wang, L., & Huang, N. (2002). Plasma-surface modification of biomaterials. *Materials Science and Engineering: R: Reports, 36*(5-6), 143-206.

Clement, J. L., & Jarrett, P. S. (1994). Antibacterial silver. *Met Based Drugs, 1*(5-6), 467-482.

Conrad, J., Radtke, J., Dodd, R., Worzala, F., & Tran, N. (1987). Plasma source ion implantation technique for surface modification of materials. *Journal of Applied Physics, 62*(11), 4591-4596.

Coquet, L., Cosette, P., Junter, G., Beucher, E., Saiter, J., & Jouenne, T. (2002). Adhesion of *Yersinia ruckeri* to fish farm materials: Influence of cell and material surface properties. *Colloids and Surfaces B: Biointerfaces, 26*(4), 373-378.

Cowan, M. M., Abshire, K. Z., Houk, S. L., & Evans, S. M. (2003). Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. *Journal of Industrial Microbiology & Biotechnology, 30*(2), 102-106.

Cui, F., & Luo, Z. (1999). Biomaterials modification by ion-beam processing. *Surface and Coatings Technology, 112*(1-3), 278-285.

Dall, L., & Herndon, B. (1989). Quantitative assay of glycocalyx produced by viridans group streptococci that cause endocarditis. *Journal of Clinical Microbiology, 27*(9), 2039.

Dan, Z. G., Ni, H. W., Xu, B. F., Xiong, J., & Xiong, P. Y. (2005). Microstructure and antibacterial properties of AISI 420 stainless steel implanted by copper ions. *Thin Solid Films, 492*(1-2), 93-100.

Darouiche, R. (1999). Anti-infective efficacy of silver-coated medical prostheses. *Clinical Infectious Diseases, 29*(6), 1371-1377.

Davey, M., & O'toole, G. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews, 64*(4), 847.

De Beer, D., Srinivasan, R., & Stewart, P. S. (1994). Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology, 60*(12), 4339-4344.

Dearnaley, G., & Arps, J. (2005). Biomedical applications of diamond-like carbon (DLC) coatings: A review. *Surface and Coatings Technology, 200*(7), 2518-2524.

Dickson, J., & Koohmaraie, M. (1989). Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Applied and Environmental Microbiology, 55*(4), 832.

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases, 8*(9), 881.

Duncan, T. V. (2011). Applications of nanotechnology in food packaging and food safety: Barrier materials, antimicrobials and sensors. *Journal of Colloid and Interface Science*. *363*(1), 1.

Faille, C., Membre, J., Ussier, J., Bellon-Fontaine, M., Carpentier, B., Laroche, M., & Benezech, T. (2000). Influence of physicochemical properties on the hygienic status of stainless steel with various finishes. *Biofouling, 15*(4), 261-274.

Flemming, H. C. (2002). Biofouling in water systems–cases, causes and countermeasures. *Applied Microbiology and Biotechnology, 59*(6), 629-640.

Flemming, H. C., & Schaule, G. (1988). Biofouling on membranes-a microbiological approach. *Desalination, 70*(1-3), 95-119.

Fletcher, M. (1976). The effects of proteins on bacterial attachment to polystyrene. *Journal of General Microbiology, 94*(2), 400.

Flint, S. (1998). *Formation and control of biofilms of thermo-resistant streptococci on stainless steel.* Doctor of Philosophy, Massey University, Palmerston North.

Flint, S., Bremer, P., & Brooks, J. (1997 a). Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling, 11*(1), 81-97.

Flint, S., Brooks, J., & Bremer, P. (1997 b). The influence of cell surface properties of thermophilic streptococci on attachment to stainless steel. *Journal of Applied Microbiology, 83*(4), 508-517.

Flint, S., Brooks, J., & Bremer, P. (2000). Properties of the stainless steel substrate, influencing the adhesion of thermo-resistant streptococci. *Journal of Food Engineering, 43*(4), 235-242.

Furno, F., Morley, K., Wong, B., Sharp, B., Arnold, P., Howdle, S., Bayston, R., Brown, P., Winship, P., & Reid, H. (2004). Silver nanoparticles and polymeric medical devices: A new approach to prevention of infection? *Journal of Antimicrobial Chemotherapy, 54*(6), 1019.

Galeano, B., Korff, E., & Nicholson, W. (2003). Inactivation of vegetative cells, but not spores, of *Bacillus anthracis, B. cereus,* and *B. subtilis* on stainless steel surfaces coated with an antimicrobial silver-and zinc-containing zeolite formulation. *Applied and Environmental Microbiology, 69*(7), 4329.

Genevaux, P., Bauda, P., DuBow, M., & Oudega, B. (1999). Identification of Tn*10* insertions in the *rfaG, rfaP*, and *galU* genes involved in lipopolysaccharide core biosynthesis that affect *Escherichia coli* adhesion. *Archives of Microbiology, 172*(1), 1-8.

Ghandour, W., Hubbard, J., Deistung, J., Hughes, M., & Poole, R. (1988). The uptake of silver ions by *Escherichia coli* K12: Toxic effects and interaction with copper ions. *Applied Microbiology and Biotechnology, 28*(6), 559-565.

Goeres, D. M., Loetterle, L. R., Hamilton, M. A., Murga, R., Kirby, D. W., & Donlan, R. M. (2005). Statistical assessment of a laboratory method for growing biofilms. *Microbiology, 151*(3), 757.

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology, 2*(2), 95-108.

Harrison, J. J., Turner, R. J., & Ceri, H. (2005). Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*. *Environmental Microbiology, 7*(7), 981-994.

Hauert, R. (2003). A review of modified DLC coatings for biological applications. *Diamond and Related Materials, 12*(3-7), 583-589.

Helke, D., Somers, E., & Wong, A. (1993). Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel and buna-N in the presence of milk and individual milk components. *Journal of Food Protection, 56*(6), 479.

Hetrick, E., & Schoenfisch, M. (2006). Reducing implant-related infections: Active release strategies. *Chemical Society Reviews, 35*(9), 780-789.

Hibma, A. M., Jassim, S. A. A., & Griffiths, M. W. (1997). Infection and removal of L-forms of *Listeria monocytogenes* with bred bacteriophage. *International Journal of Food Microbiology, 34*(3), 197-207.

Holah, J., & Thorpe, R. (1990). Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Microbiology, 69*(4), 599-608.

Hood, S., & Zottola, E. (1995). Biofilms in food processing. *Food Control, 6*(1), 9-18.

Hood, S., & Zottola, E. (1997a). Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *International Journal of Food Microbiology, 37*(2-3), 145-153.

Hood, S., & Zottola, E. (1997b). Growth media and surface conditioning influence the adherence of *Pseudomonas fragi, Salmonella typhimurium, and Listeria*

monocytogenes cells to stainless steel. *Journal of Food Protection, 60*(9), 1034-1037.

Huang, C. F., Chiang, H. J., Lan, W. C., Chou, H. H., Ou, K. L., & Yu, C. H. (2011). Development of silver-containing austenite antibacterial stainless steels for biomedical applications Part I: microstructure characteristics, mechanical properties and antibacterial mechanisms. *Biofouling, 27*(5), 449-457.

Huang, C. T., Yu, F. P., McFeters, G. A., & Stewart, P. S. (1995). Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. *Applied and Environmental Microbiology, 61*(6), 2252-2256.

Hudson, J., Billington, C., Carey-Smith, G., & Greening, G. (2005). Bacteriophages as biocontrol agents in food. *Journal of Food Protection, 68*(2), 426-437.

Hughes, K., Sutherland, I., Clark, J., & Jones, M. (1998). Bacteriophage and associated polysaccharide depolymerases–Novel tools for study of bacterial biofilms. *Journal of Applied Microbiology, 85*(3), 583-590.

Hyde, F., Alberg, M., & Smith, K. (1997). Comparison of fluorinated polymers against stainless steel, glass and polypropylene in microbial biofilm adherence and removal. *Journal of Industrial Microbiology and Biotechnology, 19*(2), 142-149.

Ilg, Y., Bruckner, S., & Kreyenschmidt, J. (2011). Applicability of surfaces containing silver in domestic refrigerators. *International Journal of Consumer Studies, 35*(2), 221-227.

Ilg, Y., & Kreyenschmidt, J. (2011). Effects of food components on the antimicrobial activity of polypropylene surfaces containing silver ions (Ag^{+}). *International Journal of Food Science & Technology*. *46*(7), 1469-1476.

Izatt, R., Christensen, J., & Rytting, J. (1971). Sites and thermodynamic quantities associated with proton and metal ion interaction with ribonucleic acid, deoxyribonucleic acid, and their constituent bases, nucleosides, and and nucleotides. *Chemical Reviews, 71*(5), 439-481.

Jeong, D. K., & Frank, J. F. (1994). Growth of *Listeria monocytogenes* at 10C in biofilms with microorganisms isolated from meat and dairy processing environments. *Journal of Food Protection 57*(7), 576-586.

Jeong, Y., Jeon, Y., Seong, H., & Lim, S. (2009). Electrochemical characteristics of 316l stainless steel processed with a thermally treated Ni-and Cr-rich surface coating. *Metals and Materials International, 15*(1), 37-42.

Jones, C., Adams, M., Zhdan, P., & Chamberlain, A. (1999). The role of surface physicochemical properties in determining the distribution of the autochthonous microflora in mineral water bottles. *Journal of Applied Microbiology, 86*(6), 917-927.

Kalishwaralal, K., BarathManiKanth, S., Pandian, S. R. K., Deepak, V., & Gurunathan, S. (2010). Silver nanoparticles impede the biofilm formation by *Pseudomonas*

aeruginosa and *Staphylococcus epidermidis*. *Colloids and Surfaces B: Biointerfaces, 79*(2), 340-344.

Kampf, G., Dietze, B., Große-Siestrup, C., Wendt, C., & Martiny, H. (1998). Microbicidal activity of a new silver-containing polymer, SPI-ARGENT II. *Antimicrobial Agents and Chemotherapy, 42*(9), 2440-2442.

Kampmann, Y., De Clerck, E., Kohn, S., Patchala, D., Langerock, R., & Kreyenschmidt, J. (2008). Study on the antimicrobial effect of silver containing inner liners in refrigerators. *Journal of Applied Microbiology, 104*(6), 1808-1814.

Knight, G., Nicol, R., & McMeekin, T. (2004). Temperature step changes: A novel approach to control biofilms of *Streptococcus thermophilus* in a pilot plant-scale cheese-milk pasteurisation plant. *International Journal of Food Microbiology, 93*(3), 305-318.

Kumar, C., & Anand, S. (1998). Significance of microbial biofilms in food industry: A review. *International Journal of Food Microbiology, 42*(1-2), 9-27.

Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy, 45*(4), 999-1007.

Lindsay, D., Brözel, V., Mostert, J., & Von Holy, A. (2002). Differential efficacy of a chlorine dioxide containing sanitizer against single species and binary biofilms of a dairy associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. *Journal of Applied Microbiology, 92*(2), 352-361.

Lindsay, D., & Von Holy, A. (1999). Different responses of planktonic and attached *Bacillus subtilis* and *Pseudomonas fluorescens* to sanitizer treatment. *Journal of Food Protection 62*(4), 368-379.

Liu, C., Zhao, Q., Liu, Y., Wang, S., & Abel, E. (2008). Reduction of bacterial adhesion on modified DLC coatings. *Colloids and Surfaces B: Biointerfaces, 61*(2), 182-187.

Meyer, B. (2003). Approaches to prevention, removal and killing of biofilms. *International Biodeterioration & Biodegradation, 51*(4), 249-253.

Mittelman, M. (1998). Structure and functional characteristics of bacterial biofilms in fluid processing operations. *Journal of Dairy Science, 81*(10), 2760-2764.

Mohammed Fayaz, A., Balaji, K., Girilal, M., Kalaichelvan, P., & Venkatesan, R. (2009). Mycobased synthesis of silver nanoparticles and their incorporation into sodium alginate films for vegetable and fruit preservation. *Journal of Agricultural and Food Chemistry, 57*(14), 6246-6252.

Monteiro, D. R., Gorup, L. F., Takamiya, A. S., Ruvollo-Filho, A. C., Camargo, E. R. d., & Barbosa, D. B. (2009). The growing importance of materials that prevent microbial adhesion: Antimicrobial effect of medical devices containing silver. [doi: 10.1016/j.ijantimicag.2009.01.017]. *International Journal of Antimicrobial Agents, 34*(2), 103-110.

Moretro, T., & Langsrud, S. (2011). Effects of materials containing antimicrobial compounds on food hygiene. *Journal of Food Protection, 74*(7), 1200-1211.

Mulligan, A., Wilson, M., & Knowles, J. (2003). Effect of increasing silver content in phosphate based glasses on biofilms of *Streptococcus sanguis*. *Journal of Biomedical Materials Research Part A, 67*(2), 401-412.

Palmer, J., Flint, S., & Brooks, J. (2007). Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology and Biotechnology, 34*(9), 577-588.

Palmer, J., Flint, S., Schmid, J., & Brooks, J. (2010). The role of surface charge and hydrophobicity in the attachment of *Anoxybacillus flavithermus* isolated from milk powder. *Journal of Industrial Microbiology and Biotechnology*, 1-9.

Parkar, S., Flint, S., & Brooks, J. (2004). Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *Journal of Applied Microbiology, 96*(1), 110-116.

Parkar, S., Flint, S., Palmer, J., & Brooks, J. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of Applied Microbiology, 90*(6), 901-908.

Percival, S. L., Hill, K. E., Malic, S., Thomas, D. W., & Williams, D. W. (2011). Antimicrobial tolerance and the significance of persister cells in recalcitrant chronic wound biofilms. *Wound Repair and Regeneration, 19*(1), 1-9.

Poulsen, L. V. (1999). Microbial Biofilm in Food Processing. *Food Science and Technology, 32*(6), 321-326.

Quarini, J. (2002). Ice-pigging to reduce and remove fouling and to achieve clean-in-place. *Applied Thermal Engineering, 22*(7), 747-753.

Rautray, T. R., Narayanan, R., Kwon, T. Y., & Kim, K. H. (2010). Surface modification of titanium and titanium alloys by ion implantation. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 93*(2), 581-591.

Rosmaninho, R., Santos, O., Nylander, T., Paulsson, M., Beuf, M., Benezech, T., Yiantsios, S., Andritsos, N., Karabelas, A., Rizzo, G., Müller-Steinhagen, H., & Rizzo, G. (2007). Modified stainless steel surfaces targeted to reduce fouling-Evaluation of fouling by milk components. *Journal of Food Engineering, 80*(4), 1176-1187.

Seale, R., Bremer, P., Flint, S., & McQuillan, A. (2010). Characterization of spore surfaces from a *Geobacillus* sp. isolate by pH dependence of surface charge and infrared spectra. *Journal of Applied Microbiology, 109*(4), 1339-1348.

Sharma, M., & Anand, S. (2002). Biofilms evaluation as an essential component of HACCP for food/dairy processing industry- A case. *Food Control, 13*(6-7), 469-477.

Sillankorva, S., Oliveira, R., Vieira, M. J., Sutherland, I., & Azeredo, J. (2004). Bacteriophage S1 infection of *Pseudomonas fluorescens* planktonic cells versus biofilms. *Biofouling, 20*(3), 133-138.

Silvestry-Rodriguez, N., Bright, K. R., Slack, D. C., Uhlmann, D. R., & Gerba, C. P. (2008). Silver as a residual disinfectant to prevent biofilm formation in water distribution systems. *Applied and Environmental Microbiology, 74*(5), 1639-1641.

Simões, M., Simões, L., & Vieira, M. (2010). A review of current and emergent biofilm control strategies. *Food Science and Technology*.

Sinde, E., & Carballo, J. (2000). Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: The influence of free energy and the effect of commercial sanitizers. *Food Microbiology, 17*(4), 439-447.

Speers, J., & Gilmour, A. (1985). The influence of milk and milk components on the attachment of bacteria to farm dairy equipment surfaces. *Journal of Applied Microbiology, 59*(4), 325-332.

Spoering, A. L., & Lewis, K. (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *Journal of Bacteriology, 183*(23), 6746-6751.

Sutherland, I. (2001). The biofilm matrix-an immobilized but dynamic microbial environment. *Trends in Microbiology, 9*(5), 222-227.

Sutherland, I., Hughes, K., Skillman, L., & Tait, K. (2004). The interaction of phage and biofilms. *FEMS microbiology letters, 232*(1), 1-6.

Tang, X., Flint, S., Bennett, R., & Brooks, J. (2010). The efficacy of different cleaners and sanitisers in cleaning biofilms on UF membranes used in the dairy industry. *Journal of Membrane Science, 352*(1-2), 71-75.

Teh, K. H., Flint, S., Palmer, J., Lindsay, D., Andrewes, P., & Bremer, P. (2011). Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. *International Dairy Journal. 21*(10), 742-747.

Teixeira, P., Lopes, Z., Azeredo, J., Oliveira, R., & Vieira, M. J. (2005). Physico-chemical surface characterization of a bacterial population isolated from a milking machine. *Food Microbiology, 22*(2-3), 247-251.

U.S. Food and Drug Administration. 2012. Inventory of effective food contact substance (FCS) notifications. Database search: silver. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?filter=silver&sortColumn=&rpt=fcsListing. Accessed 14 March, 2012.

Valappil, S. P., Pickup, D. M., Carroll, D. L., Hope, C. K., Pratten, J., Newport, R. J., Smith, M.E., Wilson, M., & Knowles, J. C. (2007). Effect of silver content on the structure and antibacterial activity of silver-doped phosphate-based glasses. *Antimicrobial Agents and Chemotherapy, 51*(12), 4453-4461.

Van Loosdrecht, M., Lyklema, J., Norde, W., & Zehnder, A. (1990). Influence of interfaces on microbial activity. *Microbiology and Molecular Biology Reviews, 54*(1), 75.

Vilain, S., Pretorius, J. M., Theron, J., & Brözel, V. S. (2009). DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. *Applied and Environmental Microbiology, 75*(9), 2861-2868.

Wang, G., & Zreiqat, H. (2010). Functional Coatings or Films for Hard-Tissue Applications. *Materials, 3*(7), 3994-4050.

Wassall, M., Santin, M., Isalberti, C., Cannas, M., & Denyer, S. (1997). Adhesion of bacteria to stainless steel and silver-coated orthopedic external fixation pins. *Journal of Biomedical Materials Research Part A, 36*(3), 325-330.

Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., & Mattick, J. S. (2002). Extracellular DNA required for bacterial biofilm formation. *Science, 295*(5559), 1487.

Wimpenny, J., Manz, W., & Szewzyk, U. (2000). Heterogeneity in biofilms. *FEMS Microbiology Reviews, 24*(5), 661-671.

Wirtanen, G., Husmark, U., & Mattila-Sandholm, T. (1996). Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems. *Journal of Food Protection, 59*(7), 727-733.

Wong, L., & Amy, C. (1998). Biofilms in food processing environments. *Journal of Dairy Science, 81*(10), 2765-2770.

Zhao, Q. (2004). Effect of surface free energy of graded Ni-P-PTFE coatings on bacterial adhesion. *Surface & Coatings Technology, 185*(2-3), 199-204. doi: 10.1016/j.surfcoat.2003.12.009

Zhao, Q., Liu, Y., & Wang, C. (2005 a). Development and evaluation of electroless Ag-PTFE composite coatings with anti-microbial and anti-corrosion properties. *Applied Surface Science, 252*(5), 1620-1627. doi: 10.1016/j.apsusc.2005.02.098

Zhao, Q., Liu, Y., Wang, C., Wang, S., & Müller-Steinhagen, H. (2005 b). Effect of surface free energy on the adhesion of biofouling and crystalline fouling. *Chemical Engineering Science, 60*(17), 4858-4865.

Zhao, Q., Liu, Y., Wang, C., Wang, S., Peng, N., & Jeynes, C. (2008). Reduction of bacterial adhesion on ion-implanted stainless steel surfaces. *Medical Engineering & Physics, 30*(3), 341-349.

APPENDIX

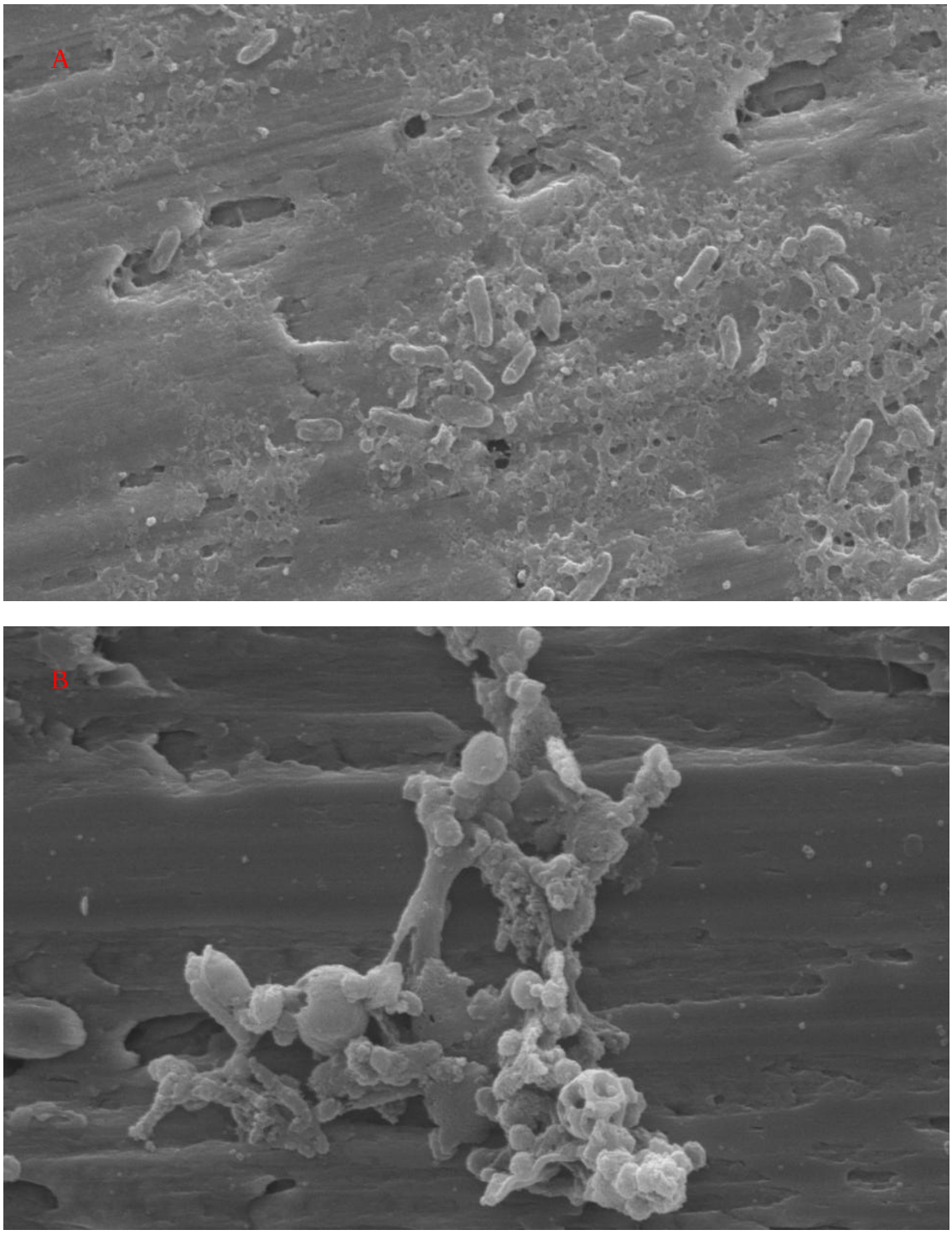

Figure 1: *P. fluorescens* (A) and *S. thermophilus* (B) cells trapped in the conditioning film.