Biofilm Formation by Common Dairy Sporeformers on Native and Modified Stainless Steel Surfaces

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BIOFILM FORMATION BY COMMON DAIRY SPOREFORMERS ON NATIVE AND MODIFIED STAINLESS STEEL SURFACES

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

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This thesis is dedicated to my parents for asking me to be who I am today, my little brother and my husband, Somil Gupta, for supporting me all the way!
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ABBREVIATIONS

SS: Stainless Steel
CIP: Cleaning-In-Place
PHE: Plate Heat Exchanger
EPS: Exo-polysaccharides
Ni-P-PTFE: Nickel Phosphorus Polytetrafluoroethylene
NFDM: Non Fat Dry Milk
PBS: Phosphate Buffered Saline
SEM: Scanning Electron Microscopy
CFU: Colony Forming Unit
RLU: Relative Light Units
HRS: Heat resistant spore formers
SEM: Scanning Electron Microscopy
BHI: Brain Heart Infusion
Ni-P-PTFE: Nickel phosphorous polytetrafluoroethylene
ATCC: American Type Culture Collection
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen
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ABSTRACT

BIOFILM FORMATION BY COMMON DAIRY SPOREFORMERS ON NATIVE AND MODIFIED STAINLESS STEEL SURFACES

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Aerobic sporeformers can be traced in a variety of dairy products such as milk powders, evaporated milk, and canned products, which demonstrates their capability of resisting high temperature treatments such as pasteurization and Ultra high temperatures. These bacilli also actively attach to the stainless steel surfaces, consequently resulting in the formation of biofilms. Product quality as well as its safety is undesirably affected by the growth of these sporeforming bacteria. Therefore, creating an ideal environment for the processing of dairy products is a critical challenge for the dairy industry. Hence, the objective of this research was to analyze various surface modifications of the conventional Stainless Steel (SS) and to study the extent of bacterial adhesion in order to develop a surface that is least vulnerable to bacterial attachment, thus reducing the formation of biofilms.

The first part of the study analyzed the adhesion tendency of aerobic spore-forming bacteria on native and modified Stainless Steel surface (AMC 18, Dursan, Ni-P-PTFE and Lectrofluor 641). Heat resistant aerobic spore-forming bacteria were specifically picked for this study as these can survive pasteurization, Ultra High Temperature (UHT) treatment and can also develop Heat Resistant Spores (HRS), which can potentially contaminate the dairy processing lines. The modified SS coupons were manufactured using spin coating and dip coating method. Biofilm development on native
and modifies SS coupons were compared for three common aerobic sporeformers namely *G. stearothermophilus, B. licheniformis* and *B. sporothermodurans*. Various surface properties including surface energy, surface hydrophobicity and surface roughness of the coupons were compared for their role on the adhesion tendency of the sporeformers. Bacterial attachment was observed to be directly proportional to the surface energy, whereas it was inversely proportional to the surface hydrophobicity. Biofilm development studies indicated that Ni-P-PTFE modified surface was least vulnerable to bacterial attachment whereas native SS surface was highly susceptible. Scanning Electron Microscopy showed the extent of bacterial attachment and biofilm formation.

The second part of the study compared native SS surface and Ni-P-PTFE modified surface plate heat exchangers (PHEs) for the extent of biofouling and shedding of biofilms. Milk was allowed to flow continuously for 17 hours through both the pasteurizers to mimic the conditions encountered in a dairy plant that have the potential to create a conducive environment for biofouling. 3M quick swabs and ATP swabs were employed for sampling from both the pasteurizers, for studying the biofilm formation and evaluating the efficacy of CIP, respectively. Milk samples were collected at the start of pasteurization run and at hourly intervals after the 10th hour of the operation from both the balance tanks (raw milk sample) and outlets (pasteurized milk sample) of both PHEs. It was observed that after the 15th hour, there was a sudden increase in the standard plate counts (SPC) of the native PHE. Consequently, the SPC of the native PHE turned out to be far higher, as compared to the modified PHE, as the experiment reached the 17th hour. Also, there was more biofilm formation in the regeneration section of native pasteurizer as compared to the modified pasteurizer.
The third part of the study compared the adhesion tendency of spores and vegetative cells on both native and Ni-P-PTFE modified SS surfaces of various spore-forming bacteria including *G. stearothermophilus*, *B. licheniformis* and *B. sporothermodurans*. The adhesion tendency of the sporeformers was observed to be also influenced by cell surface properties viz. cell surface hydrophobicity and cell surface charge (zeta potential). As per the results from the study, spores exhibited a far greater attachment tendency as compared to the vegetative cells of the same spore-forming bacteria. Amongst different sporeformers, *B. sporothermodurans* demonstrated greatest adhesion tendency followed by *G. stearothermophilus*, with *B. licheniformis* exhibiting least adhesion tendency. The tendency to adhere varied with the variations in cell surface properties as it decreased with lowering cell surface hydrophobicity and increasing cell surface charge.

All of the above studies provide useful information regarding the various factors (both contact surface and cell surface properties) that play a significant role in influencing the adhesion tendency and biofilm formation by the aerobic spore forming bacteria.
INTRODUCTION

Milk that emanates from udder of a healthy cow is sterile nevertheless contamination begins the moment it comes in contact with outside environment (Flint et al., 1997). This may be through the various spoilage and pathogenic microorganisms present in the environment and the vessels to which milk is being drawn. Nutritious components of the milk make it highly vulnerable to the microbial attack. It provides them with ideal environment vital for their growth. Consumption of raw milk poses high risks of illness associated with milk borne pathogens. Pasteurization of milk prior to consumption is one solution to this problem as it can inactivate most of the pathogenic and spoilage microorganisms (Flint et al., 1997). As the milk flows through the pasteurizer, the milk proteins denature resulting in fouling (Belmar-Beiny and Fryer, 1993). This accelerates bacterial adhesion on Plate Heat Exchanger (PHE) surface eventually leading to the development of biofilms (Barnes et al., 1999).

Fouling of PHEs during the long milk pasteurization runs is a serious concern in the dairy industry as the cleaning of Plate Heat Exchangers (PHEs) to remove the organic matter and bacteria attached to it incurs great costs (Chaudagne, 1991). Fouling occurs as a result of foulants being in constant contact with the surface of the PHEs and can ensue in various forms including scaling – precipitation of solids dissolved in the fluids, sedimentation of particulates, biological growth and various chemical reactions between fluid and the surface material such as in case of corrosion (Belmiloudi, 2011). Several factors including physical and chemical properties of the fluid, its velocity and temperature and surface properties of the PHEs play an important role in the extent of fouling (Belmiloudi, 2011). The flow of milk during pasteurization involves high
temperatures resulting in denaturation of proteins, which stick to the surface of the PHEs and make it highly vulnerable to attack by bacteria and other food borne pathogens. This combined with the precipitation of minerals leads to the fouling of the surface of PHEs consequently resulting in the formation of biofilms (Belmar-Beiny and Fryer, 1993). Many estimates are required to be taken to cover the risk of fouling and prevent production loss at the same time. For instance, the size of the PHE is overestimated by 30-50% to cover the loss of heat transfer that occur as a result of layers of foulants on its surface (Mitrovic, 2012).

Biofilms comprise of a higher concentration of bacteria implanted in extracellular polymeric substances (EPS), which are attached to, grow and multiply on the equipment surface (Carpentier and Cerf, 1993). It is beneficial for the bacteria to be a part of biofilms as it not only provides nutrients but also shields the bacteria from sanitizers, disinfectants, and other antimicrobial agents (Bower et al., 1996). The establishment of biofilms on the equipment surface takes place through various stages. The first step involves the conditioning of the surface with the attachment of organic compounds. The clean surface quickly adsorbs organic molecules resulting in preconditioning of the surface. The second step involves the attachment of bacteria, which occurs within 5-30 seconds. Initially, this attachment is reversible (Hood and Zottola, 1995), which is followed by irreversible attachment (Davey and O'toole, 2000). Bacteria adhere to the pre-conditioned surface through weak Vander Waal forces and hydrophobic interactions in case of reversible attachment and can be easily removed with the application of little force (Hood and Zottola, 1995).
Irreversible attachments result when bacteria attached to the surface start producing Extracellular polymeric substance (EPS) and get embedded in it (Davey and O'toole, 2000). This EPS matrix serves as a reservoir of nutrients and offers increased resistance for bacteria within the biofilms (Carpentier and Cerf, 1993). Following irreversible attachment, the growth and multiplication of bacteria results in micro colony formation, which is accompanied by production of EPS. This EPS matrix allows the expansion of micro colonies and results in the formation of multi layered biofilm structures (Stoodley et al., 1999). Once these multi layered biofilms structures are formed, there is an increased difficulty in eliminating bacterial cells and could potentially serve as a source of contamination to the products and equipment surface (Burgess et al., 2010).

Aerobic Spore forming bacteria are found to form biofilms on the surface of equipment in dairy processing conditions. They usually form biofilms in the regions of high temperature of 45-65°C (Flint et al., 1997), which includes regeneration section of the pasteurizers used for pasteurizing milk, preheaters and evaporators used for the concentration of milk. These aerobic sporeformers have the capability to form endospores, which undergo germination and multiply under favorable conditions (Andersson et al., 1995). Establishment of biofilms of aerobic sporeformers on the surface of processing equipment poses a great risk of contamination in the product stream due to their shedding and consequent multiplication (Flint et al., 2001).

This research explains the role of contact surface properties and bacterial cell surface properties in the process of biofilm formation by various aerobic sporeforming bacteria on plate heat exchangers (PHEs).
Stainless Steel (SS316) is the most common material used for the fabrication of PHEs. It has good corrosion and temperature resistance. Surface properties such as surface energy, hydrophobicity and surface roughness are of great importance influencing biofouling. Usually low surface energy is associated with little bacterial adhesion (Dexter, 1979). Some researchers also argue on the use of optimal value of surface energy for which bacterial adhesion is minimized (Baier, 2006). Studies conducted by Fletcher and Loeb, (1979) demonstrated that hydrophobic surface promotes lesser amount of deposit buildup of proteins, polysaccharides and bacterial adhesion. Although it seems practical that more bacteria should attach to the rough surface, it was found by many research studies that there was no correlation existing between surface roughness and bacterial attachment (Langeveld et al., 1972, Tide et al., 1999).

Apart from the substrate properties, characteristics of bacteria for instance cell surface hydrophobicity and zeta potential could also stimulate bacterial attachment on the surface. Bacteria generally exhibits a net negative charge (Dickson and Koohmaraie, 1989) and are hydrophobic but these characteristics vary in different microorganisms thereby affecting their affinity to attach to the surface. Spores are found to be more hydrophobic and adhere more on the surface due to the relative abundance of protein in the outer coats and exosporium as compared with the peptidoglycan on vegetative cell surfaces (Wiencek et al., 1990). Many research studies suggest that with the increase in hydrophobic character, the bacterial attachment would be enhanced (Rönner et al., 1990). However there are some other research studies, which contradicts the relations between intensified hydrophobic character of bacteria and its adhesion to the surface (Parkar et al., 2001).
Zeta potential is defined as the extent of electrostatic charge present on the cell walls of bacteria. It is also found to perform an important role influencing the adhesion properties of bacteria. Though as suggested by some researchers, adhesion tendency of bacteria should decrease with the enhancement in zeta potential (Rönner et al., 1990), other researchers claim that bacterial adhesion is independent of the zeta potential (Seale et al., 2008).

The project was initiated with the hypothesis that the modified surfaces would promote a lesser amount of deposit build up and adhesion of aerobic sporeformers viz. *Geobacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus sporothermodurans*. Also, based on some previous studies, which demonstrated a higher spore attachment it was hypothesized that there would be greater spore adhesion. The overall purpose of this study was to analyze the extent of biofouling on native as well as modified Stainless Steel (SS) surfaces. It was accomplished employing the following work plan:

- Screening of all the native and modified SS surface for their resistance to biofilms using High heat Resistant Sporeforming (HHRS) bacteria viz. *Geobacillus stearothermophilus* under static conditions.

- Examining the native and Ni-P-PTFE modified SS surface (which presented highest resistance to bacterial adhesion) using *Bacillus licheniformis*, *Bacillus sporothermodurans* and mixed species biofilm comprising of *Bacillus licheniformis* and *Geobacillus stearothermophilus*.

- Evaluation of native and Ni-P-PTFE modified SS surface on commercial scale for biofilm formation.
Comparison of the attachment of spores and vegetative cells of commonly encountered aerobic sporeformers such as *Geobacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus sporothermodurans* on the native and Ni-P-PTFE modified SS surfaces.

- Determination of cell surface properties namely cell surface hydrophobicity and zeta potential (expressed as surface charge).
- Investigation of any relationship existing between cell surface properties and bacterial adhesion on the surface.

**REFERENCES**


CHAPTER 1
LITERATURE REVIEW
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Processing of milk

Quality of dairy products continues to be a topic of intense debate in the dairy industry (Flint et al., 2000). Milk due to its high nutritional value is vulnerable to attack by a wide range of both gram negative and gram positive bacteria as it provides them with a highly nourishing environment to grow (Cherif-Antar et al., 2015). While milk is sterile when it is secreted out of cow’s udder, there are high possibilities of contamination occurring at various stages from milk transportation to processing and storage (Marchand et al., 2012). Contamination starts during the very first process of milking through the microbial flora present on the udder or in the milking machine. After milking, it is stored for around 48 to 72 hours at 7°C that restricts the growth of microorganisms (Flint et al., 1997). There is a severe risk of contamination from the transfer lines and storage containers during transportation and storage of milk at production site. Dairy manufacturers rely on the pasteurization process for defending the consumers from contaminations caused by spoilage and food borne pathogenic bacteria present as a result of contaminations (Visser and Jeurnink, 1997). This practice is used all around the globe to increase the shelf life and eradicate pathogens and heat sensitive spoilage organisms present in this highly perishable food product. To achieve this, the milk is normally heated to more than 72°C for not less than 15 seconds (Murphy et al., 1999) or 63°C for 30 minutes (Lau et al., 1991) and then cooled rapidly.
**Plate heat exchangers**

Plate Heat Exchangers (PHEs) (Figure 1) most common for market milk pasteurization purposes are commonly fabricated out of Stainless Steel (SS) surface. Tubular heat exchangers can be used when long running times are essential. Scraped-surface heat exchangers are used while dealing with viscous products. The size (dimensioning) of the heat exchangers can be determined after acknowledging the applicable parameters. The parameters for Plant PHE and R and D PHE used for the plant trials are shown in table 1.

**Service media**

The amount of required service media (steam, water and ice water) is calculated, as this considerably impacts the selection of valves used for regulating steam and ice water feed. Hot water is a highly used service media for heating milk, which is nearly 2 – 3°C higher than the optimum temperature required for the processing of the product. Similarly, ice-water is utilized for cooling milk after pasteurization. Steam is used to raise the temperature of water, which further heats the product to pasteurization temperature (Bylund, 2003).

**Temperature control**

A temperature controller acting on the steam-regulating valve maintains a constant pasteurization temperature. A sensor in the product line before the holding tube immediately detects any tendency for the product temperature to drop which opens the steam-regulating valve to supply more steam to the water. This results in increase in circulating water temperature.
Optimum milk flow and its appropriate level in the balance tank is maintained by making use of float-controlled inlet valve. The level of raw milk in the balance tank begins to drop as soon as its supply is interrupted. But it is necessary to maintain the level of milk in the pasteurizer or else the product will burn on to the plates. To accommodate this, the balance tank is fitted with a low-level electrode, which transmits a signal the moment level reaches the minimum point. This signal triggers the flow diversion valve that returns the product to the balance tank. The milk from the balance tank is delivered to the pasteurizer through the feed pump. The flow controller is responsible for maintaining the flow of milk through the pasteurizer. This provides optimum temperature throughout and a constant length of the holding time for achieving the requisite pasteurization effect.

The first section of the pasteurizer that is the pre-heating section receives the cold untreated milk. Here, the regenerative heating of untreated milk takes place with the pasteurized milk, which cools it down. Final heating takes place in the heating section at a temperature 2 – 3°C higher than the pasteurization temperature. The hot milk then passes through an external holding tube to achieve the required pasteurization effect. After passing through the tube, the pasteurized milk comes back to the regeneration section for cooling. Here again heat is transferred from the pasteurized milk to the incoming cold untreated milk. Flow of raw and pasteurized milk in the regenerative section of pasteurizer is shown in figure 2. Then, service media such as cold water, ice water, a glycol solution or some other refrigerant are employed to cool down the pasteurized milk to below 4°C. The temperature of the chilled milk is normally recorded, together with the pasteurization temperature.
As untreated milk passes through the PHE, there is denaturation of proteins resulting in fouling and accelerating the bacterial attachment on the surface of PHE. Hence, when raw milk is contaminated, there is increasing evidences of biofilms formed on the internal surface of plate heat exchanger. Thus, to get rid of these biofilms and to sustain a clean and safe environment, Cleaning in place (CIP) processes are employed. Bacteria that survive CIP and Pasteurization pose a great threat to the well being of consumers consuming pasteurized milk and other dairy products manufactured from pasteurized milk. Additionally, the bacteria that survive can potentially promote the formation of biofilm, which in turn protects the bacteria from high temperatures and chemical compounds. Thus the microbial colonization in food processing environment is a potential cause of contamination that can cause both food quality and food safety concerns. Therefore, good hygiene of plate heat exchangers is critical to ensure that it does not cross contaminate the product stream and the product is safe for consumption (Bylund, 2003).

**Fouling in plate heat exchanger**

Fouling of PHEs is a common event in the process of pasteurization of raw milk. These PHEs are therefore required to be cleaned regularly to remove the organic matter and bacteria attached to its surface, which incurs great costs to the dairy industry. In France, the fouling in the dairy industry resulted in an expenditure of around 1000 million French francs in 1991 (Chaudagne, 1991). Moreover, mere cleaning is not sufficient to remove all the bacterial attachments on the surface of PHEs. The proteins denature as a result of heating of milk during pasteurization and are adsorbed by the PHEs, which consequently
accelerates bacterial adhesion to their surface. The aggregation of these proteins along with mineral deposits (mainly calcium) leads to the fouling of the surface of PHEs (Belmar-Beiny and Fryer, 1993). Based on the experiments conducted by Burton, white voluminous precipitate (type-A) is developed on the surface of the PHE between 80°C and 105°C. β-lactoglobulin (β-LG) is found as the major protein in this deposit whereas calcium and phosphate compose the mineral part of the precipitate. Generally, type-A deposit is found to be composed of 30-50% minerals, 50-60% protein, and 4-8% fat. Type-B deposits are developed on the surface of PHE at high temperatures surpassing 100°C. Such deposits, as stated by Burton, has a brittle, granular structure with grey color and are composed of 70-80% minerals, 15-20% proteins and 4-8% fat. These deposits are harder as compared to the type-A deposits and consequently more challenging to clean leaving behind a larger volume of organic matter and bacterial attachments. The amount of deposit is inversely proportional to the temperature, which is the precipitated amount diminishes with the increasing the temperature further. Major proteins that compose type-B deposits are αs1 casein (27%) and β-casein (50%). Bouman et al., (1982) also analyzed the fouling of PHEs and found that fouling took place on the raw milk side instead of the pasteurized one. The composition of the deposit upon analyzing in the regeneration compartment of the raw milk side with a temperature reaching to 57°C after 12 hours of processing was found to be 30mg/plate for Phosphorous, 51mg/plate for Calcium and 52mg/plate for protein. The composition changed significantly in the heating section where a temperature of 70°C is maintained and was found to be (in mg/plate) 36, 95 and 133 for P, Ca and protein respectively. From the above statistics, it can clearly be inferred that calcium phosphate to protein weight ratio in the deposit is nearly a factor 30 greater
than that in milk. The composition of the deposit is entirely transformed on preheating milk at 80-85°C for 5-10 min. The mineral component dominates the composition of the deposit as a consequence of pre denaturation of the whey proteins.

**Establishment of Biofilms**

Biofilms consists of viable and nonviable microorganisms implanted in extracellular polymeric substances (EPS) sticking to equipment surface (Carpentier and Cerf, 1993). EPS normally contain proteins, sugars, lipids and other polymeric substances hydrated by water (Costerton et al., 1981). Nutrients are concentrated in the EPS, which gives protection to microorganisms in the biofilm by insulating from heat, sanitizers and disinfectants (Carpentier and Cerf, 1993). The resistance of microorganisms to harsh environment help the survival and growth of food borne pathogens in food processing industry (Bower et al., 1996). These mass of cells further becomes large enough to accelerate the deposition of food and minerals on the food contact surfaces resulting in the formation of biofilms (Bakke et al., 1984). These food and mineral deposits help to provide protection to microorganisms in the biofilms (Hood and Zottola, 1995). Biofilms can be formed from single or multiple species of bacteria. These microorganisms may form a single layer or multiple structures and generally exist in the form of aggregates or clusters (Bryers, 1987).

The development of biofilm is carried out through various stages. Initial onset of biofilm formation takes place when microorganisms attach to the food contact surface. This attachment is reversible. Bacteria are loosely attached to the food contact surface at this stage with van der Waals & electrostatic forces and can be easily removed with
application of a little force (Hood and Zottola, 1995). Once bacteria are embedded into EPS produced by them, this results in irreversible attachment (Davey and O'toole, 2000). For removing the irreversible attachment strong shear forces is required along with application of chemicals and detergents (Bower et al., 1996). Therefore, Selection of effective chemicals combined with proper cleaning protocol (such as correct concentration of chemicals, temperature, flow rate and contact time) is very important for efficient cleaning of food processing equipment to prevent the formation of matured biofilms.

Biofilm formation is influenced by a lot of factors, however the major is composition, topography, chemistry of the surface and flow of product (Mittelman, 1998). Poorly or un-cleaned surfaces accelerate the soil accumulation, which promote the biofilm formation in the presence of water. Once the biofilms are formed on equipment surfaces, it can also affect the chemical and physical state of equipment. These biofilms lead to resistance in heat transfer (Sandu and Singh, 1991) and it is found that even 0.05mm deep biofilm can decrease the heat transfer by one third (Russell, 1993). In few cases metal surfaces gets corroded (Bryers, 1987) due to the presence of biofilm and metabolic activity of the microorganisms present inside the biofilm cause heavy damage to surface. These biofilms can also result in blockages and decreased flow rates. This results in major costs for repair and replacement of equipment. There is no clear substantiation that biofilms with pathogens spread the food born disease because detection and detailing of biofilms has not been incorporated so far in the research of food born disease.
Surface properties such as surface energy, hydrophobicity and surface roughness also effects the bacterial attachment and biofilms. Several studies were conducted to link surface energy, hydrophobicity and surface roughness to mineral deposits and bacterial cell adhesion, however no theory was found to be consistent. Research conducted by (Liu et al., 1997) suggests that the deposition will be higher for the increasing values of surface energy. Studies were also conducted such as those by Tsibouklis et al., (2000) who argued for the use of low surface energy to inhibit bacterial adhesion whereas Zhao, (2004) presented the existence of an optimum value of surface energy (30 N/M) for which bacterial adhesion could be minimized. There are also various disagreements regarding the influence of surface roughness on biofilm formation. Researchers like Masurovsky and Jordan, (1958), Hoffman, (1983) and Pedersen, (1990) report that bacterial adhesion increases with the increasing surface roughness. Whereas other researchers like Langeveld et al., (1972) and Vanhaecke et al., (1990) believe that there is no correlation between surface irregularities and the ability of bacteria to attach to the surface. Several researchers have also reported that the surface having least hydrophobicity will be the one exhibiting greatest bacterial attachment (Fletcher and Loeb, 1979, Pringle and Fletcher, 1983).

**Contribution of aerobic sporeformers as biofilm constitutive micro flora**

Aerobic sporeforming bacteria are characterized by their ability to form endospores, which can resist harsh environmental conditions such as high temperatures, pressures and could stay in dormant state for centuries (Andersson et al., 1995, Ryu and Beuchat, 2005). The presence of these aerobic spore-forming bacteria especially those
belonging to the genus *Bacillus* is a matter of great concern because of their ubiquitous nature that makes it impossible to avert their entry in food ingredients (Baril et al., 2012). They contaminate the untreated milk both in vegetative form and in the form of endospores. The sources for their entry in raw milk are present throughout the dairy chain including water, air, soil, and equipment. Hydrophobic property and high heat resistance allow them to attach to the dairy processing equipment and survive CIP, thus leading to the formation of biofilms (Simmonds et al., 2003). Presence of these aerobic spore formers is responsible for premature food spoilage and food poisoning leading to huge economic losses. Majorly, the food poisoning cases are related to the presence of these sporeformers that germinate, multiply, and release hazardous level to toxins during storage. Sporeformers such as *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus amyloliquefaciens* are associated with the production of toxin resulting in food poisoning (Salkinoja-Salonen et al., 1999, Mikkola et al., 2004). The production of spoilage enzymes such as proteases, lipases, and lecithinases by these bacteria can result in off flavors and structural defects in dairy products. The proteolytic activity results in bitter off flavors while rancid off flavors are due to lipolytic activity. These aerobic spore-forming bacteria also interfere in the production of cheese by reducing nitrate and production of gas during fermentative growth (Meer et al., 1991, in't Veld, 1996, Heyndrickx and Scheldeman, 2002). Although the dairy sector has studied a lot about the diversity of these spore-forming bacteria, information about fast and simple diagnostic tools to detect these sporeformers is still lacking resulting in the entry of these sporeformers in food ingredients.
**Geobacillus stearothermophilus, Bacillus licheniformis and Bacillus sporothermodurans** are the test isolates used in the current study. **Geobacillus stearothermophilus** and **Bacillus licheniformis** exhibit a cordial growth and were found on the surface of preheaters and evaporators.

**Geobacillus stearothermophilus**

*Geobacillus* species was previously classified within genus *Bacillus* and hence *Geobacillus stearothermophilus* was earlier known as *Bacillus stearothermophilus*. These are thermophilic, rod shaped, aerobic spore forming bacteria with optimum growth temperature ranging between 55°-65°C (Nazina et al., 2001). These bacilli actively attach to stainless steel surface consequently resulting in the formation of biofilms (Burgess et al., 2010). It can cause long-term persistent contamination of dairy processing lines. These are considered as a potential contaminant in Dairy manufacturing industries and act as a biological indicator for sterility validation, hence is of great interest to the dairy industries (Cheng et al., 2010, Guizelini et al., 2012). These can be traced in variety of dairy products such as milk powders, evaporated milk and canned products suggesting its capability of surviving high temperature treatments such as pasteurization (Scott et al., 2007). Presence of this bacterium in higher concentration in the finished dairy products as compared to raw milk suggests the extensive multiplication of this bacterium during the manufacturing process. Spores of *Geobacillus stearothermophilus* can resist canned food heat treatment and is responsible for flat-sour spoilage of low acid canned food (Ito, 1981). This bacterium has also been spotted in various raw and processed foods such as dehydrated vegetables and fruit preparations. In dairy industry, this dormant thermophile
is mostly associated with raw milk, milk powder, evaporated milk and many other dairy products. Doubling time of 25 minutes was reported for this bacterium (Flint et al., 2001).

**Bacillus licheniformis**

These are gram positive, rod shaped, aerobic spore forming bacteria with the optimum growth temperature of 30°C. It is capable of growing at both mesophilic and thermophilic temperatures (Hill and Smythe, 2012). *B. licheniformis* possess irregular shaped colonies which are opaque with a white tint. It was also reported by (Chen et al., 2004) that *B. licheniformis* produce both extracellular and intracellular proteinases and lipases. *Bacillus licheniformis* shows significant proteolytic and lipolytic activity but there could be strain dependent variation. Greater than 70% of the strains show both this type of behaviors. It is also found to ferment lactose (De Jonghe et al., 2010). *B. licheniformis* can produce toxic components that can result in food poisoning (Lücking et al., 2013). Review conducted by (Burton, 2012) suggests that the thermophilic spore level in raw milk tends to be low with the dominance of *Bacillus licheniformis*. 28 milk powder samples from 18 different countries analyzed by (Rückert et al., 2004) suggested that *A. flavithermus, B. licheniformis, G. stearothermophilus* were the dominant organisms. It has been reported that *B. licheniformis* sporadically contaminate the UHT milk by production of highly heat resistant spores.

**Bacillus sporothermodurans**

This bacterium first discovered by Pettersson et al., (1996) is characterized as gram positive, aerobic high heat resistant sporeformer with a optimum growth temperature of 30°C. It survives the commercial sterilization and ultra high temperature processing of milk, as it has been isolated from UHT milk of several countries. Countries
like USA, Mexico and various parts of Europe have experienced non sterility due to *Bacillus sporothermodurans* (Klijn et al., 1997). Endospores of *Bacillus sporothermodurans* are capable of surviving UHT treatment, which subsequently germinate back resulting in a non-sterile product. These high heat resistant spores were first detected in UHT treated milk in southern Europe in 1985. Although this bacterium can grow to an extent of $10^5$/ml during storage for 5 days yet cause no noticeable changes in milk and is nonpathogenic (Bertil et al., 1996). Growth of *Bacillus sporothermodurans* can result in spoilage in the form of slight change in color of milk, destabilization of casein micelles and off flavor.

**Factors affecting microbial attachment**

There are numerous factors that influence the nature of biofilms and attachment of microorganisms to dairy processing equipment. This includes physical & chemical properties of the cell surface, equipment surface and composition of fluid.

**Free Surface Energy**

Surface energy is the extra energy required to detach the bacteria that is in contact with the fouled surface. With the reduction in surface energy, the binding energy between the surface and bacteria attached to it will also be lowered and further will lead to an increase in the detachment tendency of the attached bacteria. The surfaces that have high surface energy (high wettability) are more hydrophilic. Therefore, more microbial attachment takes place on the surfaces with higher wettability (Boulange-Petermann et al., 1993). Stainless steel and Glass are two examples of hydrophilic surfaces that have much higher microbial attachment than Teflon and Rubber which are hydrophobic in nature.
Blackman and Frank, 1996). Boulange Petermann et al., (1997) used Streptococcus thermophilus, a well-recognized strain of heat exchanger plates in dairy industry to develop a relationship between the metallic surface wettability and bacterial adhesion. According to his results, the total surface energy has no role to play with the wettability of metallic surfaces rather it is driven by the equilibrium between the polar components and the dispersive components of the total surface energy. The experiments conducted by Baier, (2006) suggested the existence of an optimum value of free surface energy for which bacterial attachment to the surface of metals is minimized. Investigations by McGuire and Swartzel, (1989) also showed similar results when he was trying to figure out the influence of solid surface energy on macro-molecular protein adsorption from milk.

Cell surface charge

Bacterial cell wall has negative charge but its magnitude varies in different strains. Researchers have used methods like electrophoretic mobility and electrostatic interaction chromatography to measure the net negative charge of the cell wall. Dickson and Koohmaraitae, (1989) found that there is more bacterial attachment with the increase in negative charge. On the other hand Gilbert et al., (1991) used electrophoretic mobility and found that with the increase in negative charge in the cell wall of bacteria, there will be less bacterial attachment to glass by E. coli while inverse relationship exist for S. epidermidis. Research conducted by Parker et al., (2001) conclude that there is no correlation found between the surface charge and attachment of vegetative cells. So there is no strong basis yet to conclude the relationship of bacterial attachment to the negative charge in the bacterial cell wall.
Construction/Finish of food contact surfaces

There is a significant impact of properties of food contact surface on the establishment of biofilms on its surface (Frank and Chmielewski, 1997). There is more deposit of soil on eroded or rough surfaces and hence are more difficult to clean than novel surfaces (Boulangé-Petermann et al., 1997). This results in survival of bacteria, which can regrow and produce a biofilm. Holah and Thorpe (1990) has reported that there will be higher sticking of food residues on rough surface which cause proliferation of bacteria on the equipment surfaces and provides protection from cleaning and sanitation. Conversely many other researchers suggest that there is hardly any correlation between surface roughness and bacterial adhesion (Langeveld et al., 1972, Tide et al., 1999). Hilbert et al., (2003) suggests that although the microbial attachment has little to do with the surface roughness, there is a significant improvement in corrosion resistance by smoothing the surface. Several other studies demonstrated similar results proposing the increase in corrosion resistance with various surface treatments such as mechanical polishing, electro polishing or pickling (Hong and Nagumo, 1997, Zuo et al., 2002).

Surface pre-condition

Clean surfaces quickly adsorb the organic layer in the aqueous solution and get charged with organic molecules. The adsorption of organic layer occurs within seconds on the surface of equipment. As milk flows through the PHE, there is a denaturation of protein as a result of heating of milk, which sticks to its surface. According to Rosmaninho and Melo (2006) whey proteins present in milk especially B lactoglobulin denature and aggregate upon heating which is responsible for fouling on the surface of PHE. Also, Calcium and phosphate ions precipitate upon heating, which deposits minerals on the
surface of PHE and hence further contributes to fouling. This process is termed as pre-conditioning and is considered an essential prerequisite for the establishment of biofilms. Bacterial attachment is accelerated once the organic layer is formed on the equipment surfaces because of the presence of charged ions (Barnes et al., 1999). However, the experiments conducted by Parker et al., (2001) demonstrated the decrease in ability of spores and vegetative cells to adhere to a surface pre-conditioning with 1 and 10% of skim milk protein. Helke et al., (1993) also found that when a clean surface is conditioned with milk and milk protein, there was less attachment by S. typhimurium and L. monocytogenes. Speers et al., (1984) reported similar results. This indicates that conditioning film might not be crucial in the attachment of bacteria and biofilm formation.

**Sticking ability**

Different bacterial cells need varied contact time for attachment to food contact surfaces. Microorganisms that have a good sticking ability (example L. monocytogenes) need lesser contact time for attachment (Lundén et al., 2000). Sticking ability of microorganisms is decided by the Physical & chemical properties of their cell surface. Growth rate, growing media and condition of the media has a direct influence over the physical and chemical properties of cell surface. Mortality of bacterial cells decides active or passive sticking to the food contact surface. In case of active adhesion, the properties of microbial cells (flagella, protein & surface charge) decide the sticking to food contact surface. Flagellum helps bacteria for moving to attachment site. Surface charge has an impact on the cell chemistry, cell proteins & polysaccharides which influence the adhesion properties (Davey and O'toole, 2000). Bacterial cells with negative surface
charge are generally hydrophobic in character. More the hydrophobicity of bacteria, more will be the capability of bacteria to attach to the surface and hence lesser will be the sticking time. Hydrophobicity change with growth phase, which decreases with increase in growth rate (Boulangé-Petermann et al., 1997). Physical properties of the fluid directly impact the passive sticking of bacteria (specific gravity, flow rate etc). As compared to vegetative cells, the sticking ability of spores is much more because of hydrophobic nature of their hairy surfaces (Bower et al., 1996).

**Role of Extracellular Polymeric Substances (EPS)**

Extracellular Polymeric Substances are composed of proteins, sugars, lipids and other polymeric substances hydrated by water (Costerton et al., 1981). It has been recorded that the Extracellular polysaccharides are involved in both fresh water bacterial attachment (Jones et al., 1969) and marine bacterial attachment (Floodgate, 1972). However, some studies suggest the role of EPS in the initial phase or reversible phase of attachment (Corpe, 1970) whereas others believe its role at later stages during irreversible attachment (Zobell, 1943, Marshall et al., 1971). Experiments directed by Brown et al., (1977) proposed that the excessive EPS production can even resist the attachment even though small amount of it is required initially. Research conducted by Allison and Sutherland, (1987) proved that the EPS is mainly involved in the formation of micro colonies and development of microbial biofilms and is not directly involved with the bacterial attachment as the non- polysaccharide producing mutant attached to the glass slide in equal number as the polysaccharide producing bacteria.
Equipment design

Equipment design is also very important to prevent the deposition of food residues and allow its easy cleaning, and in turn prevent the development of biofilms. Material of construction for equipment must be selected, which is compatible for food application and CIP regime needed. Material of construction of equipment should be such that it provides resistance to the attachment of bacteria so that there is less formation of biofilm. Correct layout of equipment connected with well-designed CIP system minimizes the cleaning problems and prevents the formation of biofilms (GISE, 1991). Dead ends, gaskets, valves, and joints are the areas that are more to biofilms (Wong, 1998). Dead ends should be avoided to ensure sufficient exposure of CIP chemical in all parts of the equipment and food processing line. Cleaning of biofilm becomes difficult at dead ends or near the gaskets.

Biofilm removal and control strategies

In ideal conditions, it is better to prevent the formation of biofilm then controlling or removing it. However, at present there is no such technique available that can prevent or control the biofilm formation. Important factors that play key role in the formation of biofilm are equipment design, availability of nutrients, aqueous environment and temperature control. However, in the food industry, all these factors are most often difficult to control. Biofilms will form in the aqueous environment even with presence of little nutrients. Sticking of extracellular polymeric substances on the surface makes the biofilms further more difficult to clean. Therefore, most effective control to avoid or remove biofilms is effective cleaning (Simões et al., 2010). To prevent the biofilm
formation, surface should be cleaned and sanitized regularly so that there is no firm attachment of bacteria to the surface. Quality of the final product will greatly depend on the efficiency of cleaning operations.

**Cleaning and sanitization**

Proper cleaning regimes should be selected to ensure the removal food residues effectively from the surfaces because the presence of food particles accelerates the microbial growth (Simões et al., 2010). Standard cleaning protocol involves flushing with water to remove the loosely adhered residues, reused water (containing traces of chemicals) is also used for the removal of loose residues, followed by circulation of chemicals, flushing and finally sanitation. Correct concentration of chemicals, temperature, flow rate and time of circulation are the important factors for ensuring effective cleaning. These parameters are worked out on the basis of type of food product, temperature of processing lines and length of production etc. Most common chemical used for cleaning in the food industry are alkalis for dissolving the fats and proteins (Forsythe and Hayes, 1998). Wetting agents may be used with specific alkalis to lower the surface tension and better penetration of chemical. Occasionally acids are used for dissolving the mineral residues. Extended cleaning with alkali is necessary to remove biofilm (Simões et al., 2010). Sanitation after cleaning is important to remove left over microorganisms from the equipment surfaces. Proper cleaning of surfaces is important for effective sanitation because the organic matter act as protection from sanitizer and reduces the activity of sanitizers. Most common sanitizers used in food industry are Chlorine, Hydrogen peroxide and Per acetic acid. Table 2 shows the protocol followed for CIP of plate heat exchanger at South Dakota State University dairy plant.
Use of modified surface

It is well known that SS316 is widely used for fabrication of plate heat exchangers and have a very good corrosion resistance. However, they are very much prone to bacterial adhesion and formation of biofilms, as they do not exhibit any bacterial inhibiting properties and hence completely lack defense against microbial growth. Hence it is of high relevance to modify the SS surface and to use antimicrobial coatings to have less deposition, bacterial adhesion and establishment of biofilms. Researchers have used different methods to modify the surface such as use of different coatings and alloying modifications.

Silver addition to SS316. Silver has been widely used in the past for bacterial inhibition (Davies and Etris, 1997). As silver is having the ability to reduce risk of infection, it is also considered as a potential surface for hospitals and health care applications where high hygiene is required (Bragg and Rainnie, 1974, Schierholz et al., 1998). Research conducted by Chiang et al., (2010) proved that adding silver to SS increases the bacterial inhibiting properties and could be used in place of traditional SS to reduce the incidence of bacterial contamination. Also, when the silver content in SS was increased to 0.09 wt%, SS exhibited an exceptional bacterial inhibiting effect similar to that as pure silver. However, addition of silver in SS results in slightly decreased corrosion resistance and mechanical strength.

Nickel– phosphorus – polytetrafluoroethylene (Ni-P-PTFE) Coating. Ni –P-PTFE coatings are fabricated out of autocatalytic plating process. Ni-P-PTFE Coating promoted lesser amount of fouling. It also has a very high cleaning efficiency as the
initial layer of organic material did not adhere as strongly as on the native SS surface. Greater percentage of homogeneously distributed PTFE particles incorporated in Ni-P matrix is effective in reducing fouling. Till now, the greatest amount of PTFE particle that could be achieved on the outer surface is 20%. The effect of modifying SS surface on bacterial attachment and biofilm formation was studied by Rosmaninho et al., (2007) and found that Ni-P-PTFE surface promoted a lesser amount of deposit build up and was simplest to clean. Also it was reported by (Zhao, 2004) that these coatings hindered the bacterial adhesion by 82-97%.

**Non-stick coating.** Non-stick coating prepared from polymeric materials with low surface energy has the potential to inhibit the early bacterial adhesion and biofilm formation. Research conducted by Tsibouklis et al., (2000) proves that good quality thermally formed films free from cracks are resistant to bacterial attack when exposed to the cultures of *Bacillus megaterium, staphylococcus aureus,* sulphate-reducing bacteria, and mixed marine *pseudomonas* for up to 48 hours under static growth conditions. A flexible liner backbone of the constituent polymer is recommended onto which pendent side chains exhibiting low intermolecular interactions are attached (Kobayashi and Owen, 1995).

**Silver zeolite coating.** Research conducted by Griffith et al., (2015) demonstrated the use of silver zeolite coating on SS as an effective antimicrobial food contact surface that is capable of inhibiting the food borne pathogenic biofilm formation. Silver zeolite also exhibits anti corrosive properties. This coating consists of a porous crystalline alumino silicate base and silver over the metallic zeolite. This coating inhibits the microbial growth by releasing silver ions which enters the cell and leads to cellular
damage (Matsumura et al., 2003). This coating proved as an effective antimicrobial coating against the biofilms of *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Cowan et al., 2003). Research conducted by Chen et al., (2009) indicate that increased hydrophobic character and electrostatic interactions of the coated surface was responsible for reduced microbial adhesion. Antibacterial activity in membrane processing is boosted by the application of silver zeolite to polyvinyl fluoride (Liao et al., 2011).

**Regulatory aspects.** Food processing equipment is the surface, which comes in direct contact with the food product. Therefore, It must be fabricated out of a material that does not allow the migration of deleterious substance, impart color, odor or taste to the food. If any modification is done in fabrication of equipment, it should be such that it ensures the hygienic design criteria. It should be safe, corrosion resistant, and durable to withstand repeated washings and processing. It should be easy to clean and resist any sort of pitting, distortion, and chipping. It should be nonporous, nonabsorbent, nonreactive, and nontoxic. It should be smooth and free of cracks and crevices. Stainless steel surface modified using titanium, platinum or gold is highly desirable for food processing equipment however their usage is limited due to their high cost. Equipment fabricated out of copper is generally used in brewing industries. Leaching of copper could result when used for processing high acid products. Aluminum coated with polytetrafluoroethylene could also be used as a food contact material in food processing equipment. Materials such as ceramics are used in membrane filtration applications (Schmidt and Erickson, 2009). According to 21CFR 175 subpart C, food grade coatings could comprise of aluminum stearate, aluminum lauryl sulfate, borax, Disodium hydrogen phosphate,
Formaldehyde, Glyceryl monostearate, Methyl cellulose Mineral oil, Paraffin wax, Potassium hydroxide, Potassium persulfate, Tallow, Tetrasodium pyrophosphate, and Titanium dioxide.

**Biofilm detectors and Mechatronic surface sensors**

Biofilm detectors have been developed to analyze or observe the attachment of bacteria on the surface thus it helps in early detection of biofilm and controlling the biofilm before it is converted to an immature biofilm (Philip-Chandy et al., 2000).

Similar to Biofilm detectors, Mechatronic surface sensors have also been developed by Pereira et al., (2008) to observe the attachment of bacteria on the surface in initial stages of biofilm formation. In addition to the biofilm detection, it is also able to detect the presence of any cleaning solution hence Mechatronic surface sensors are capable of sensing both biological and chemical cleanliness of the surface. If there is detection of biofilms at early stage of its formation, then there is a potential to prevent the formation of irreversible biofilms.

**Use of Green Chemicals**

Green chemicals are those chemicals that are enzyme based and are also known as bio-cleaners. These chemicals can be used to effectively to degrade the biofilms using the mixture of enzymes. Augustin et al., (2004) reported that green chemicals can be used as an effective enzymatic cleaning products that can be used to degrade the biofilms formed by those microorganisms found in Dairy industry. However the problem with the use of these green chemicals in Dairy industry is that the enzymatic action particularly that of proteolytic enzymes is reduced in the presence of milk (Oulahal-Lagsir et al., 2003).

Wettability of biofilms can be increased by the combination of surfactants and proteolytic
enzymes. With the increase in wettability of biofilm, cleaning efficiency can be increased (Parkar et al., 2004). It is difficult to identify the enzyme that can be used against all types of biofilm because each enzyme has its own specific mode of action. These green chemicals containing different enzymes can be very efficient in cleaning process but their use is limited because of the high price enzymes and less availability as compared to chemicals that are used commercially (Simões et al., 2010).

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Table 1. Parameters designed for Plant PHE and R and D PHE used for the plant trials

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Plant PHE</th>
<th>R and D PHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity, lb./hr.</td>
<td>6000 lb./hr.</td>
<td>4000 lb./hr.</td>
</tr>
<tr>
<td>Temperature programme, F</td>
<td>40°F -178°F -42°F</td>
<td>40°F -178°F -40°F</td>
</tr>
<tr>
<td>Regeneration efficiency</td>
<td>90%</td>
<td>90%</td>
</tr>
<tr>
<td>Heating medium temperature</td>
<td>180°F</td>
<td>180°F</td>
</tr>
<tr>
<td>Coolant temperature</td>
<td>28°F</td>
<td>28°F</td>
</tr>
</tbody>
</table>
Table 2. CIP protocol for Plate Heat Exchanger (PHE) followed at South Dakota State University dairy plant.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Time (minutes)</th>
<th>Temperature (°C)</th>
<th>Temperature (°F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre rinse</td>
<td>4</td>
<td>100</td>
<td>37.8</td>
</tr>
<tr>
<td>Caustic wash</td>
<td>45</td>
<td>175</td>
<td>79.4</td>
</tr>
<tr>
<td>Rinse</td>
<td>8</td>
<td>100</td>
<td>37.8</td>
</tr>
<tr>
<td>Acid wash</td>
<td>20</td>
<td>140</td>
<td>60</td>
</tr>
<tr>
<td>Final rinse</td>
<td>15</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>
**Figure 1.** Flow of milk and service media in milk pasteurizer.

Orange represents raw chilled milk from balance tank, green- Regenerated milk, purple- separated milk, black- homogenized milk, red- heated milk, dotted black– hot water, blue line - chilled pasteurized milk, dotted blue - chilled water, dotted red – steam, dotted purple - diverted milk
**Figure 2.** Flow of raw and pasteurized milk in the regenerative section of pasteurizer.

Pasteurized milk is demonstrated by purple line and orange line shows raw milk.

1. Pasteurized milk into regeneration section
2. Pasteurized cold milk into cooling section
3. Raw milk into regeneration section
4. Raw hold milk to separator
   - ● Closed port
   - ○ Open port
CHAPTER 2
EVALUATION OF MODIFIED STAINLESS STEEL SURFACES TARGETED TO REDUCE BIOFILM FORMATION BY COMMON MILK SPOREFORMERS
SHIVALI JINDAL

ABSTRACT

Development of bacterial biofilms on stainless steel surfaces poses a great threat to the quality of milk and other dairy products as the biofilm embedded bacteria can survive thermal processing. Established biofilms also offer cleaning challenges as they are resistant to most of the regular cleaning protocols. Sporeforming thermotolerant organisms entrapped within biofilm matrix can also form heat resistant spores, and may result in a long-term persistent contamination. The main objective of this study was to evaluate the efficacy of different non-fouling coatings (AMC 18, Dursan, Ni-P-PTFE, Solgel and Lectrofluor 641) on Stainless Steel (SS) plate heat exchanger surfaces, to resist the formation of bacterial biofilms. It was hypothesized that modified SS surfaces would promote a lesser amount of deposit build up and bacterial adhesion as compared to the native SS surface. Vegetative cells of aerobic sporeformers; *Geobacillus stearothermophilus* (ATCC 15952), *Bacillus licheniformis* (ATCC 6634), and *Bacillus sporothermodurans* (DSM 10599) were used to study biofilm development on the modified and native SS surfaces. The adherence of these organisms though influenced by surface energy and hydrophobicity, exhibited no apparent relation with surface roughness. The Ni-P-PTFE coating exhibited the least bacterial attachment and milk solid deposition, hence, was most resistant to biofilm formation. Scanning electron microscopy, which was used to visualize the extent of biofilm formation on modified and
native SS surfaces, also revealed lower bacterial attachment on the Ni-P-PTFE as compared to the native SS surface. This study thus provides evidence on the reduced biofilm formation on the modified SS surfaces.

**Keywords**: Nickel– phosphorus – polytetrafluoroethylene (Ni-P-PTFE), Biofilms, Sporeformers, Coatings

**INTRODUCTION**

Heat resistant spore formers in milk present a significant challenge to product quality and safety, due to their ability to survive thermal processing treatments. These bacteria are known to colonize in large numbers during the long processing runs and develop cleaning resistant biofilms (Lücking et al., 2013), enabling cross contamination of finished products. The plate heat exchangers (PHEs) that are commonly used for pasteurizing milk are fabricated out of stainless steel SS316. As milk flows through the Plate Heat Exchanger (PHE), the proteins undergo thermal denaturation and foul on its surface, accelerating bacterial attachment. According to Rosmaninho and Melo (2006), even whey proteins present in milk, especially β-lactoglobulin, denature and aggregate upon heating, which is responsible for fouling on the surface of PHE. Also, calcium phosphate formed as a result of precipitation of calcium and phosphate ions, deposits minerals on the surface of PHE, and hence further contributes to fouling. It was reported by Hinton et al., (2002) that more bacterial activity was observed on the fouled surface, to a level of $10^5$ cfu/cm$^2$, as compared to the unfouled surface. Food residues further protect microorganisms within biofilms, which makes them more resistant to regular cleaning protocols. Once the biofilms are formed on equipment surfaces, they also lead to
resistance in heat transfer, and it was reported that even 0.05mm deep biofilm can
decrease the heat transfer by one third (Sandu and Singh, 1991). It has further been
reported that metal surfaces even get corroded (Bryers, 1987) due to the presence of
biofilms, and the metabolic activity of the microorganisms present inside the biofilms.
This results in major costs for repair and replacement of equipment. There is thus a
significant opportunity to improve the safety, quality, and operating efficiency of dairy
processing operations by reducing fouling and biofilm formation on PHEs.

The likelihood of biofouling by proteins, mineral deposits, and thermoduric
bacteria depends on the surface properties of PHEs, such as surface energy, surface
roughness, and hydrophobicity. It was reported by Liu et al., (1997) that fouling was
higher for surfaces having greater surface energy. Studies were also conducted to link
surface energy to cell adhesion, however, none of the theories provide conclusive
evidence. Tsibouklis et al., (2000) supported the use of low surface energy to inhibit
bacterial adhesion, whereas Zhao, (2004) presented a case for an optimum value of
surface energy (30 N/M) for which bacterial adhesion could be minimized. Similarly,
there are conflicting reports regarding the influence of surface roughness on bacterial
adhesion and biofilm formation on the surface of PHEs. Some researchers reported that
bacterial adhesion increases with the increasing surface roughness (Masurovsky and
believe that there is no correlation between surface irregularities and the ability of
bacteria to attach (Langeveld et al., 1972, Vanhaecke et al., 1990). This may be due to
differences in degree of physico-chemical parameters of the surface studied, the bacterial
species tested, the bulk fluid phase under study, and the method used to detect bacterial adhesion (Flint et al., 2000).

Sol Gel modified surface tested in this study has been regarded as environmentally friendly, durable and approved by FDA for its use as a food contact surface (FDA CFR 21, 175.300). We have also previously reported on an electroless nickel coating process in which fluoropolymer particles (PTFE) are codeposited in a nickel phosphorus coating on 316SS, resulting in a coating that is resistant to both protein and mineral fouling during dairy processing, bacterial adhesion, and biofilm formation by Bacillus cereus (Barish and Goddard, 2013). Our results were promising, yet the coatings were prepared on a laboratory scale, limiting the commercial translatability of the technology. An opportunity remains for demonstrating the efficacy of commercially scalable techniques in surface modification of SS316.

The main focus of the current research was to evaluate the four commercially available surface modifications of SS316, so as to select the coated surface that is most resistant to adhesion and biofilm formation by thermoduric sporeformers. The four surface modifications, listed in Table 1, were also evaluated for differences in their surface energy, surface roughness, and hydrophobicity. Scanning Electron Microscopy (SEM) technique was used to image the native and modified surfaces for visually comparing the biofilm formation.

MATERIALS AND METHODS

Source of native and modified SS coupons

Actual corrugated SS heat exchanger plates were cut into SS316 (0.0254 m x
0.0254 m; 0.0005 m thick) coupons to mimic the surface of PHEs. These were donated by AGC Heat Transfer (Portland, OR), and used as SS surface control or was modified using the four commercial coating technologies as listed in Table 1. Each coating was applied on 12 corrugated and 12 flat SS coupons using spin coating or dip coating method. Corrugated coupons were used for microbiological analysis and the flat coupons were used for surface characterization. For coating purposes the AMC 18 was supplied by AMCX (Tyrone, PA) and is commercially available. The Dursan is composed of carboxy silicon material, inter-diffused with the SS substrate, resulting in a 0.0000004 – 0.0000016 m coating and was supplied by SilcoTek Corporation, Bellefonte, PA. The Lectrofluor 641 is a fluoro polymer-based coating and was supplied by General Lectrofluor 641 Corporation, Linden, NJ. The Ni-P-PTFE coatings were prepared by a previously reported method (Barish and Goddard, 2013) in which approximately 0.00000762 m of nickel is coated by electroless deposition onto cleaned, Wood’s struck SS316, followed by codeposition of PTFE particles (~0.0000002 m diameter) in a second electroless nickel deposition step.

Native SS 316 and Sol gel modified SS flat coupons were also examined for their resistance to biofilm formation. Sol gel modified coupons were fabricated out of an inorganic ceramic polymer and were procured from Porcelain industries (Dickson, TN). Native SS 316 flat coupon were supplied by stainless supply (Monroe, NC).

**Surface Characterization of native and modified SS316**

Examination of surface characteristics for native and modified SS surface such as Dynamic contact angle, Surface energy and Surface roughness were conducted in the lab of Dr. Julie Goddard at University of Massachusetts. Following are the protocols used for
the analysis (Jindal et al., 2016). This information is reproduced here for discussion purpose in context of our study.

**Dynamic Contact Angle.** Kruss DSA 100 Drop Shape Analyzer (Hamburg, Germany) was used to measure the contact angle of HPLC water on the native and modified SS surface (Schmidt et al., 2004). Contact angle fitting method from the Drop Shape Analysis software version 1.91.0.2 was used for the measurement. A steady temperature of 25±1°C was maintained while taking all the measurements. While taking the measurements, single drop of water (5µl) flowing at 25µl/min was credited on the SS surface. A total of nine scans were performed (three measurements on each of three independent samples) for every treatment.

**Surface Energy.** Surface energy was quantified with the help of a Neumann model by using the advancing values of four test liquids with known surface tension: HPLC water (72.8 mN/m), glycerol (64 mN/m), formamide (58 mN/m), and ethylene glycol (48 mN/m). The relationship between the advancing value and the surface tension of four liquids was fitted with Neumann model. The critical surface tension of the test surface was calculated when \( \cos \theta = 1 \). The Neumann model was shown as below:

\[
\cos \theta = -1 + 2 \sqrt{\gamma_{SV}} e^{-\beta(\gamma_{LV} - \gamma_{SW})}
\]

where \( \theta \) is the contact angle (degree), \( \gamma_{LV} \) is the surface energy of the liquid (mN/m), \( \gamma_{SV} \) is the surface energy of the solid (mN/m), and \( \beta \) is the constant (m^2/mN^2), respectively.

**Surface Roughness.** Roughness of different modified surfaces was determined using optical profilometry that was carried out on Zeta-20 optical profilometer (Barish
and Goddard, 2014). A 20x camera lens (WD=3.10mm) was used with a total scanning area of 1173 X 880 µm². 3D Zdot™ software (Zeta Instruments, San Jose, CA) was employed to examine the images. Native and modified SS coupons were compared for their surface roughness. The results were reported as an average ± standard error for a total of nine scans (three analyses on each of three independently prepared coupons).

**Bacterial biofilm formation on native and modified SS coupons**

**Sourcing and maintaining bacterial isolates.** Biofilm formation related studies were performed using three different aerobic spore forming bacteria namely *Geobacillus stearothermophilus* (ATCC 15952), *Bacillus licheniformis* (ATCC 6634), and *Bacillus sporothermodurans* (DSM 10599). Reference strains of these aerobic sporeforming bacteria were sourced from the American Type Culture Collection (ATCC, Manassas, Virginia) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Germany. The bacterial strains were grown in freshly prepared Brain Heart Infusion (BHI) Broth (Oxoid, Thermo Scientific, UK) by incubating at their optimum growth temperatures (Table 2) as recommended by ATCC and DSM, and were preserved for future use in cryogenic vials (Perry, 1995).

Each activated culture was centrifuged at 4500 x g for 30 minutes at 4°C using Avanti JE centrifuge (Beckman Coulter, Inc.). The pellets thus obtained were subsequently suspended in Phosphate Buffer Saline (PBS) at pH 7.4 and maintained in a 1.8 mL cryogenic vials (Copan diagnostic Inc., Murrieta, CA) that contained sterile beads and glycerol. The vials were stored in NuAire ultralow deep freezer (NuAire Inc. Plymouth, MN) at -80°C until further use (Khanal et al., 2014).

**Preparation of vegetative cell suspensions.** Vegetative cells of the respective
organisms were prepared by transferring the culture from cryo-vials in to BHI broth and incubating at their optimum growth temperature in shaking incubator (at 150 rpm). After overnight incubation, vegetative cells were harvested by centrifugation at 4500 x g for 10 minutes at 4°C. The pellets obtained were washed in Phosphate Buffer Saline (PBS), and adjusted to a concentration of log 7.0 cfu mL\(^{-1}\), which were then used for spiking the growth medium for biofilm formation studies on coated coupons.

**Development of biofilms on native and modified SS coupon.** Three trials, with two coupons each, were conducted for each experiment. Before initiating the experiment, native and four types of modified SS coupons were washed with deionized water, followed by washing with 70% alcohol, and rinsing the coupons with de-ionized water, which were then sterilized by autoclaving at 121°C for 15 minutes. Two coupons of each type were separately submerged in petri dishes containing 25 mL each of 11% total solids sterile reconstituted Non Fat Dry Milk (NFDM). The NFDM was chosen as a growth medium as the fat interacts with the hydrophilic surface of SS surface (Barnes et al., 1999). Overnight grown culture of *G. stearothermophilus* was spiked into reconstituted NFDM in the petri dish to a level of around log 6.0 cfu mL\(^{-1}\). These petri dishes (having milk inoculated with the organism, and SS316 native and modified coupons) were incubated at 50°C (being the optimum growth temperature as recommended by ATCC and also the temperature encountered in regeneration section of PHE) with the purpose of forming biofilms on the surface of coupons. Zero hour counts of milk were taken right after inoculation, to ascertain the actual initial counts of culture being inoculated in to growth medium (sterile reconstituted NFDM). The growth medium was replaced every 15 hours, without causing any disturbance to the biofilms already formed on the coupons.
This was done to provide fresh nutrients to the bacteria and also to remove toxic substances and acids being generated in the growth medium as a result of bacterial metabolism. This process was continued for 72 hours. As the preliminary experiments to evaluate the formation of *G. stearothermophilus* biofilms indicated that the Ni-P-PTFE modified SS316 was the most resistant to biofilms, therefore, the Ni-P-PTFE coupons were further evaluated for biofilm development using the other two cultures; *B. licheniformis* (ATCC 6634), and *B. sporothermodurans* (DSM 10599) using the same protocol as described above. Although *B. licheniformis* had a supplier recommendation of 30°C, it showed comparable growth at 50°C.

**Development of mixed species biofilm on native and modified SS coupons.** The biofilm formation was also studied on native SS surface and modified SS surface (Ni-P-PTFE) using a mixed culture of *B. licheniformis* and *G. stearothermophilus* following the same protocol as described above.

**Establishment of biofilm on native and Sol gel modified flat SS coupons.** Aerobic sporeformer viz. *B. licheniformis* was employed for the development of biofilms on native and Sol gel modified SS coupon at 50°C using the same protocol as described above.

**Enumeration of viable cells embedded within biofilm matrix.** At the end of 72 hours incubation, the coupons immersed in bacterial growth medium were removed from the petri dish using sterile tweezers, and rinsed with phosphate buffer to remove non-adherent and loosely adhered cells. This was followed by swabbing the targeted area (6.25 cm²) using sterile 3M Quick swabs (3M center, St. Paul, MN). The swab tube was vortexed for 10 seconds to release all cells from the swab tip. Swab tip was pressed and
twisted against the wall of swab tube to facilitate the recovery of all bacterial cells. The contents in the tube were then mixed and serially diluted in sterile phosphate buffer saline (PBS) solution at pH 7.4. Aerobic plate counts were performed on BHI agar plates (Khanal et al., 2014) using spread plate technique (Downes and Ito, 2001). The plates were incubated for 24 to 48 hours at 50°C. The Colony Forming Units (CFUs) were calculated and the counts were reported as log cfu/cm² (Wehr and Frank, 2004)

Microscopic visualization of bacterial biofilms. The electron micrographs, for studying the microstructure of matured biofilms formed on different surfaces, were obtained using a Scanning Electron Microscope (SEM) (Hitachi S-3400N, Hitachi America Ltd, Tarrytown, NY), located in the Daktronics Engineering Hall, South Dakota State University. Air drying method (Hassan et al., 2010) with slight modifications involving a reduction in air drying time was used to fix the biofilm on the coupon surface with minimum structural damage, and to obtain a partially dehydrated biofilm for electron microscopy. Samples were air dried for 12 hours after moderate rinsing with phosphate buffer (pH 7.0) at room temperature under laminar airflow, followed by sputter coating with a 10-nm thick layer of deposition of 99% gold. Biofilms were then observed under The SEM at 10kV accelerating voltage by maintaining a distance of 10mm from the coupon.

Statistical Analysis

Trials were repeated thrice with two replicates in each experiment. The bacterial counts were calculated for mean values and standard error. Means were compared using Tukey multiple comparison test using SAS 9.3 software (SAS Institute Inc. Cary, NC) with least significance difference at p < 0.05. The surface properties such as surface
energy, surface roughness, and hydrophobicity were correlated with the various stainless steel modifications using Microsoft excel.

**RESULTS AND DISCUSSION**

As explained on Page 53, the surface properties analyzed in the lab of Dr. Julie Goddard at University of Massachusetts (under a collaborative project, jointly published, Jindal et al., 2016). The results of that study are being used here for completeness of discussion purposes (It includes figure 1 to 3).

**Effects of surface properties on formation of bacterial biofilms**

Biofilm formation on the food contact surface involves the events of milk solid deposition, bacterial adhesion and colonization, and finally the biofilm formation. A deep insight into the surface properties of various coated surfaces and their relationship with the extent of bacterial adhesion is essential in order to minimize the biofilm formation. The surface properties of various modified surfaces were investigated in terms of surface energy, surface roughness, and hydrophobicity.

**Surface energy of coated surfaces.** Surface energy is defined as the extent of attractive or repulsive force that a material surface exerts on another surface. In the context of this experiment, it is the extra energy required to detach the bacteria that are in contact with the fouled surface. With the reduction in surface energy, the binding energy between the surface and bacteria attached to it could be lowered and further could lead to an increase in the detachment tendency of the attached bacteria. Consequently, the biofilm formation can thus be minimized (Dexter, 1979). Experimental results obtained in our study also supports the above study, suggesting a decrease in the extent of bacterial
attachment with the descent of surface energy (Figure 1) of native and modified SS surfaces. Native SS surface with the highest value of surface energy (42.94 ±0.67 mN/m) recorded maximum bacterial attachment (log 5.11 ±0.03) to its surface. On the other hand, Ni-P-PTFE and Lectrofluor 641 with lowest values of surface energies (15.96 ±1.21 mN/m and 14.34 mN/m respectively) recorded least bacterial adhesion (log 3.15 ±0.04 and 3.42 ±0.04 respectively) to their surface (Table 3). Similarly, the other two modified SS surfaces (AMC 18, Dursan) with lower values of surface energies than native stainless steel also showed less bacterial adhesion to their surfaces. With the lowering values of surface energies for different modified SS surfaces (AMC 18, Dursan), the extent of bacterial attachment to the respective surfaces also decreased. Some studies have previously been conducted by other researchers to investigate the effect of surface energy on the extent of bacterial adhesion on the surface. According to a study conducted by Baier, 2006, there exist an optimum value (20-30 mN/m) of surface energy for which bacterial adhesion is minimum. The bacterial adhesion would thus be greater for surface energies more or less than the optimum value. The possible reason proposed by them for this typical attachment behavior is the influence of bacterial properties (cell surface hydrophobicity and zeta potential), which alter their attachment to stainless steel surface and resulting in minimal attachment at optimal value of surface energy. Research study conducted by Ronner et al., (1990) states that greater the absolute charge on the cell wall of bacteria, less will be its tendency to adhere to the surface.

**Surface roughness of coated surfaces.** Surface roughness is the deviation of the vector perpendicular to the real surface from ideal direction. If the angle of deviations is large, the surface is said to be rough or else if the deviations are trivial, the surface is said
to be smooth. In general, it is expected and seems obvious that more bacteria should attach on a rough surface as a result of bacterial entrapment (Hoffman, 1983, Pedersen, 1990). However, other studies suggest that there may not be a correlation between the surface irregularities and bacterial adhesion (Langeveld et al., 1972, Mafu et al., 1990, Tide et al., 1999).

Zeta-20 optical profilometer was used to obtain optical profilometry images which relates to surface roughness of native and modified SS surface and which is further qualified from the Sa values of different SS surfaces (Figure 3). According to the results of the experiments conducted, Lectrofluor 641 with the greatest surface roughness (2.64 μm) and Ni-P-PTFE with the least surface roughness (1.06 μm) shared almost similar extent of bacterial adhesion (Table 3). This suggests that there may not be a clear relationship between surface texture and the extent of bacterial adhesion. The complexity of the surface and microbial interactions thus makes it impossible to predict biofilm formation based on any single factor (e.g. roughness, surface energy, and bacterial species) alone.

Hydrophobicity of coated surfaces. Dynamic contact angle can be used to quantify hydrophobicity or wettability of a surface. If the contact angle is less than 90°, the solid surface is considered to be hydrophilic resulting in the spreading of liquid on the surface of solid. On the other hand, if the contact angle is greater than 90°, the solid surface is said to be hydrophobic (Bhushan and Jung, 2007).

According to the results achieved for the dynamic contact angle and bacterial adhesion, it is found that native SS being the least hydrophobic (Figure 4) was the one exhibiting the highest bacterial adhesion. Lectrofluor 641 and Ni-P-PTFE being the most
hydrophobic (Figure 4) demonstrated the least bacterial attachment to their surface (Table 3). Consequently, it can be inferred that with the increase in hydrophobicity of the SS surfaces, there is a decline in the extent of bacterial adhesion. Some previous researchers have also reported similar results with hydrophobic surfaces (Fletcher and Loeb, 1979, Pringle and Fletcher, 1983).

In summation, both surface energy and hydrophobicity were found to have a significant role in the bacterial adhesion. Surface energy was observed to be directly related to the adhesion tendency of bacteria, reflecting that the bacterial tendency to attach to the contact surface increases with the surface energy. On the contrary, hydrophobic character of the coated surface exhibited an inverse relationship with the bacterial adhesion. Surface roughness showed almost no role in influencing the tendency of bacteria to attach to various coated surfaces.

**Comparison of biofilm development by different sporeformers**

Development of biofilms on the surface of native and modified SS coupons by common thermoduric sporeformers was studied at 50°C to obtain a biofouling behavior at a temperature similar to that encountered in the regeneration section of PHEs. Moreover, this was also the optimum growth temperature for *G. stearothermophilus* (ATCC 15952). Both, *B. licheniformis* (ATCC 6634) and *B. sporothermodurans* (DSM 10599) strains used in this study also showed good growth at 50°C.

A superior attachment and biofilm forming ability of *G. stearothermophilus* (ATCC 15952) has been reported previously. Doyle et al., (2015) stated that *G. stearothermophilus* (ATCC 15952) caused long term persistent contamination of dairy processing lines as they formed biofilm on SS surface of processing equipment.
Therefore, in our study, all the coatings were first screened for their resistance to bacterial adhesion and biofilm formation using *G. stearothermophilus* under static condition.

The initial counts of *G. stearothermophilus*, after its inoculation in the growth medium, were around log 6.0 cfu/mL. The biofilm embedded cells of *G. stearothermophilus* (ATCC 15952) were quantified by swabbing an area of 6.25 cm² after 72 hours of biofilm formation on native and modified SS coupon surfaces, used in the study. Native SS surface attracted the most bacteria. The bacterial counts after the incubation period of 72 hours for native SS coupons were around log 5.1 cfu/cm², which were significantly higher as compared to the modified surfaces (Table 3). A possible reason could be the lower conditioning of modified surfaces due to reduced amount of milk solids sticking on its surface as compared to the native SS surface. It was previously reported by (Barnes et al., 1999) that the pre-conditioning of the surface with organic molecules accelerated the bacterial attachment. It can thus be inferred that as the bacterial adhesion is also influenced by the conditioning of the surface, it could be minimized by modifying the surface such that the milk solid deposition is minimum. The effect of modifying SS surface on fouling was studied by (Rosmaninho et al., 2007), who reported that a Ni-P-PTFE surface promoted a lesser amount of deposit build up and was easiest to clean. In another previous study (Zhao, 2004) surface coatings were reported to hinder the bacterial adhesion by 82-97%.

A comparison of the number of adhered cells of *G. stearothermophilus* to different modified and native SS surfaces after 72 hours of incubation revealed that Ni-P-PTFE was most resistant to bacterial attachment, whereas native SS surface recorded a higher bacterial adhesion (Table 3). Similar results were obtained with *B. licheniformis*
(Table 4). It was interesting to note that while the biofilm formation trend for *B. sporothermodurans* was also similar for the two surfaces, overall it showed lower biofilm formation on both native and modified surfaces as compared to the other two sporeformers. One of the probable reason for this could be the slower multiplication of *B. sporothermodurans* (Klijn et al., 1997). Murphy et al., (1999) also reported that *G. stearothermophilus* and *B. licheniformis* exhibited good growth in preheaters and evaporators. In order to evaluate the biofilm formation by mixed species, the Ni-P-PTFE and native SS coupons were examined for their resistance to a mixed species biofilm comprising of the above two aerobic sporeformers. Ni-P-PTFE exhibited significantly less bacterial attachment as compared to the native SS surface, even for the mixed species biofilms (Table 4).

Native and Sol gel modified flat SS coupons when examined for their resistance to biofilm formation using *B. licheniformis* clearly illustrates the presence of more bacterial attachment on native SS surface as compared to modified SS surface (Table 5).

It was also observed that the viable counts in spent growth medium (reconstituted nonfat dried milk) at the time of changeovers were always around 7 logs, which were comparatively higher to the biofilm embedded bacterial counts. Flint et al., (2001) has reported that bacteria entrapped in the biofilm by *G. stearothermophilus* may also shed in the milk passing over them.

*Biofilm visualization using Scanning Electron Microscope.* Scanning electron microscope was used to examine the microstructures of native and modified SS surfaces, before and after the biofilm formation. While examining the SEM micrographs of various modified and native SS surfaces, it is evident that the surfaces were very different.
visually Figure (5A, 5B, 5C, 5D, 5E). No particles were seen on Lectrofluor 641, AMC-18, and Dursan whereas large number of homogeneously distributed PTFE particles could be seen on Ni-P-PTFE modified surface. Also, it was observed that the surface of Lectrofluor 641 was extremely rough as compared to the other coatings, which is also evident from the observed results. The micrographs obtained for native as well as modified SS surface after the biofilm formation clearly illustrates that Ni-P-PTFE and Lectrofluor 641 resisted the bacterial attachment and biofilm formation to a greater extent as compared to other surfaces. These two surfaces exhibited less bacterial attachment on its surface. On the other hand, the native SS surface demonstrated the greatest microbial adhesion and biofilm formation Figure (6A, 6B, 6C, 6D, 6E).

CONCLUSIONS

The main objective of this study was to evaluate the influence of surface modifications on surface characteristics and biofilm formation by thermoduric sporeformers commonly encountered during dairy processing. Of the several coatings tested, Ni-P-PTFE showed the highest resistance to attachment of bacteria and milk solid deposition, consequently resulting in minimal biofilm formation. The study suggests that SS316 modified with Ni-P-PTFE blend can improve the bacterial inhibiting properties resulting in lesser biofilm formation. Therefore, Ni-P-PTFE (most resistant to bacterial biofilm formation) can effectively serve in reducing the bacterial attachment and cross contamination of dairy products during processing.

Surface properties of SS316 also influenced the extent of bacterial adhesion. Less hydrophobic SS surface attracted more bacteria than more hydrophobic surfaces. Ni-P-
PTFE being the most hydrophobic, demonstrated the least bacterial attachment, clearly indicating that bacterial adhesion decreases with increments in surface hydrophobicity. Bacterial adhesion was also found to decrease with the reduction in surface energy. Native SS surface with the highest value for surface energy also showed maximum bacterial attachment and biofilm formation. In this study, biofilm formation by the tested sporeformers could not be related to the roughness of the surface. The findings from this research provide evidence for the potential of using modified SS surfaces in the dairy and food industry to reduce the biofilm formation, thereby resulting in lower cross contamination and enhanced microbial quality of end products. The reduced biofilm formation would also help in a greater cost effectiveness because of more efficient cleaning operations, saving time, labor, and money.

ACKNOWLEDGEMENTS

This work was financially supported by Dairy Management Inc. (Rosemont, IL) and administered by the Dairy Research Institute. Authors also acknowledge the support of the Agricultural Experimentation Station, SDSU in conducting this study, and the Electrical Engineering Department, SDSU for carrying out SEM work. AGC heat transfer (Portland, OR) is also acknowledged for donating the SS coupons. Disclaimer: The results and views expressed in this study by no means promote any one coating over other or for any commercial use. The available coatings were analyzed for their resistance to develop biofilms and bacterial adhesion studies under lab conditions for an academic purpose only.
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   forming bacilli in milk during the manufacture of low heat powders. Int J Dairy


### Table 1. Identification of native SS surface and four commercial coating technologies evaluated in this work.

<table>
<thead>
<tr>
<th>Coating Abbreviation</th>
<th>Description</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lectrofluor 641</td>
<td>Fluoro polymer-based coating on SS</td>
<td>General Magnaplate Corporation, Linden, NJ</td>
</tr>
<tr>
<td>AMC-18</td>
<td>Anti-stiction coating available commercially</td>
<td>Advanced Materials Components Express, Lemont, PA</td>
</tr>
<tr>
<td>Ni-P-PTFE</td>
<td>Electroless deposition of nickel followed by co-deposition of PTFE particles</td>
<td>Avtec Finishing Systems, New Hope MN</td>
</tr>
<tr>
<td>Dursan</td>
<td>Composed of carboxy silicon material inter-diffused with SS</td>
<td>SilcoTek Corporation, Bellefonte, PA</td>
</tr>
<tr>
<td>Native SS316</td>
<td>SS containing molybdenum imparting anticorrosive properties</td>
<td>AGC Heat Transfer, Portland, OR</td>
</tr>
</tbody>
</table>
Table 2. Test isolates, their source and Growth temperature.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
<th>Optimum growth temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Geobacillus stearothermophilus</em></td>
<td>ATCC® 15952™</td>
<td>50°C</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>ATCC® 6634™</td>
<td>30°C</td>
</tr>
<tr>
<td><em>Bacillus sporothermodurans</em></td>
<td>DSM 10599</td>
<td>30°C</td>
</tr>
</tbody>
</table>
Table 3. Viable counts in biofilms of *G. stearothermophilus* formed on native and modified SS surface

<table>
<thead>
<tr>
<th>Types of Coupons</th>
<th>SS 316</th>
<th>AMC 18</th>
<th>Lectrofluor 641</th>
<th>Dursan</th>
<th>Ni-P-PTFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average counts</td>
<td><em>(Log$_{10}$ CFU/cm$^2$)</em></td>
<td>5.11±0.03$^a$</td>
<td>3.89±0.04$^c$</td>
<td>3.42±0.04$^d$</td>
<td>4.40±0.02$^b$</td>
</tr>
</tbody>
</table>

*Mean ± SE

Values with different lowercase superscript letters (a-e) within a row are significantly different at p value <0.05.
Table 4. Viable counts in biofilms of *G. stearothermophilus*, *B. licheniformis*, *B. sporothermodurans* and mixed species (*G. stearothermophilus* and *B. licheniformis*) formed on modified (Ni-P-PTFE) and native SS coupons.

<table>
<thead>
<tr>
<th></th>
<th><em>G. stearothermophilus</em></th>
<th><em>B. licheniformis</em></th>
<th><em>B. sporothermodurans</em></th>
<th>Multi species</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS316</td>
<td>5.10±0.03<em>aA</em></td>
<td>5.13±0.02<em>aA</em></td>
<td>3.09±0.02<em>bA</em></td>
<td>5.09±0.02<em>aA</em></td>
</tr>
<tr>
<td>Ni-P-PTFE</td>
<td>3.15±0.04<em>abB</em></td>
<td>3.11±0.04<em>abB</em></td>
<td>1.88±0.06<em>bbB</em></td>
<td>3.09±0.02<em>abB</em></td>
</tr>
</tbody>
</table>

*Mean ± SE

Values with difference lowercase superscript letters (a-b) within a row are significantly different at p value <0.05.

Values with different uppercase superscript letters (A-B) within a column are significantly different at p value <0.05.
Table 5. Viable counts in biofilms of *B. licheniformis* formed on native SS flat coupon and Sol gel modified SS flat coupon.

<table>
<thead>
<tr>
<th></th>
<th>Average counts *(Log$_{10}$ CFU/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native SS 316</td>
<td>4.35 ± 0.07$^a$</td>
</tr>
<tr>
<td>Sol gel modified surface</td>
<td>3.38 ± 0.10$^b$</td>
</tr>
</tbody>
</table>

*Mean ± SE

Values with difference superscript letters (a-b) within a column are significantly different at p value <0.05.
Figure 1. Surface energy of native and modified 316 stainless steel coupons. Values are an average of (3 independent samples) ± standard error.
Figure 2. Surface roughness (Sa) of native and modified 316 stainless steel coupons. Values are an average of 9 measurements (from 3 distinct regions on 3 independent samples) ± standard error.
**Figure 3.** Advancing and receding dynamic contact angle of native and modified 316 stainless steel coupons. Values are an average of 9 scans (from 3 distinct regions on 3 independent samples) ± standard error.
Figure 4A. Scanning electron micrograph of native SS coupon at 1000X magnification before biofilm formation.
**Figure 4B.** Scanning electron micrograph of modified coupon (AMC 18) at 1000X magnification before biofilm formation.
Figure 4C. Scanning electron micrograph of modified coupon (Dursan) at 1000X magnification before biofilm formation.
Figure 4D. Scanning electron micrograph of modified coupon (Lectrofluor 641) at 1000X magnification before biofilm formation.
Figure 4E. Scanning electron micrograph of modified coupon (Ni-P-PTFE) at 1000X magnification before biofilm formation.
Figure 5A. Scanning electron micrograph of native SS coupon at 5000X magnification after biofilm formation showing attachment of *G. stearothermophilus* and absorption of thick layer of foulants on the surface. Black arrows show location of rods of *G. stearothermophilus* in the biofilm formed over native SS coupon.
Figure 5B. Scanning electron micrograph of modified SS coupon (AMC 18) at 5000X magnification after biofilm formation showing attachment of *G. stearothermophilus* and absorption of foulants on the surface. Black arrows show location of rods of *G. stearothermophilus* in the biofilm formed over native SS coupon.
Figure 5C. Scanning electron micrograph of modified SS coupon (Dursan) at 5000X magnification after biofilm formation showing attachment of *G. stearothermophilus* and absorption of foulants on the surface. Black arrows show location of rods of *G. stearothermophilus* in the biofilm formed over native SS coupon.
**Figure 5D.** Scanning electron micrograph of modified SS coupon (Lectrofluor 641) at 5000X magnification after biofilm formation showing attachment of *G. stearothermophilus* and absorption of foulants on the surface. Black arrows show location of rods of *G. stearothermophilus* in the biofilm formed over native SS coupon.
Figure 5E. Scanning electron micrograph of modified SS coupon (Ni-P-PTFE) at 5000X magnification after biofilm formation showing attachment of *G. stearothermophilus* and absorption of foulants on the surface. Black arrows show location of rods of *G. stearothermophilus* in the biofilm formed over native SS coupon.
CHAPTER 3

A COMPARISON OF BIOFILM DEVELOPMENT ON STAINLESS STEEL AND MODIFIED SURFACE (NI-P-PTEF) PLATE HEAT EXCHANGERS DURING A 17H MILK PASTEURIZATION RUN.

SHIVALI JINDAL

ABSTRACT

Flow of milk through a Plate Heat Exchanger (PHE) results in denaturation of proteins, resulting in fouling. This also accelerates bacterial adhesion on PHE surface, eventually leading to the development of biofilms. During prolonged processing, these biofilms result in shedding of bacteria that cross contaminate the milk being processed, thereby limiting the duration of production runs. Altering the surface properties of PHEs such as surface energy and hydrophobicity could be an effective approach to reduce biofouling. This study was conducted to compare the extent of biofouling on native stainless steel (SS), and modified surface (Ni-P-PTEF) PHE during pasteurization of raw milk for an uninterrupted processing run of 17 hours. For microbial studies, raw and pasteurized milk samples were aseptically collected from inlets and outlets of both PHEs at various time intervals to examine shedding of bacteria in the milk. At the end of the run, 3M quick swabs and ATP swabs were used for sampling plates from different sections of the pasteurizers (regeneration, heating, and cooling) for biofilm screening and to estimate the efficiency of Cleaning In Place (CIP), respectively. The data were analyzed for analysis of variance and means were compared. Modified PHE experienced lower mesophilic and thermophilic attachment and biofilm formation (average log 1.0 and 0.99 cfu/cm², respectively) in the regenerative section of the pasteurizer as compared
to the SS PHE (average 1.49 and 1.47 logs). Similarly, higher Relative Light Units (RLUs) were observed for SS PHE, as compared to the modified PHE, illustrating the presence of more organic matter on the surface of SS PHE at the end of the run. In addition, at the 17th hour, milk collected from the outlet of SS PHE had significantly higher plate counts of 5.44 cfu/cm², as compared to pasteurized milk collected from the modified PHE (4.12 log cfu/cm²). This provided further evidence in favor of the modified PHE in achieving improved microbial quality of pasteurized milk in long process runs. Moreover, since cleaning of SS PHE involves acid treatment step, while only alkali treatment step is sufficient for the modified surface PHE, use of the latter is both cost and time effective, making it a more ideal surface for thermal processing of milk and other fluid dairy products.

**Key words:** Biofilms, Stainless Steel, Plate heat exchanger, pasteurization, cleaning

**INTRODUCTION**

Milk is considered an ideal medium for growth of many different pathogenic and spoilage causing bacteria as it contains essential nutritional components for their growth (Degeest and De Vuyst, 1999, Cherif-Antar et al., 2015). Thermal processes such as pasteurization of milk are practiced to not only reduce spoilage bacteria but also pathogens to provide protection from illness (Visser and Jeurnink, 1997). Plate heat exchangers (PHEs) are used for pasteurizing milk and other products in the dairy industry, which are fabricated out of Stainless Steel (SS-316) surface (Shah et al., 1988). As milk flows through PHEs, milk proteins denature as a result of heating of milk, and stick to the equipment surface (Rosmaninho and Melo, 2006). In an earlier study,
Bouman et al., (1982) observed that fouling of PHEs was greater in the regeneration section of the pasteurizer. The composition of the deposit with a temperature reaching to 57°C after 12 hours of processing was found to be 30mg/plate for Phosphorous, 51mg/plate for Calcium, and 52mg/plate for protein. However, the composition changed significantly in the heating section where a temperature of 70°C was maintained and was found to be (in mg/plate) 36, 95 and 133 for Phosphorous, Calcium and protein, respectively. Further, the fouling due to protein and mineral deposits on the surface of PHEs during pasteurization also accelerates bacterial adhesion to the surfaces, leading to biofilm development. Thermophiles were reported by Hinton et al., (2002) at a level of $10^5$ cfu/cm$^2$ on fouled surfaces, whereas no bacterial activity was detected on the clean surface. An important source of microbial cross contamination in the dairy industry is the formation of microbial biofilm during milk storage and processing, due to the adherence of bacterial contaminants on stainless steel surfaces (Flint et al., 1997). Developments of biofilms in milk processing environments leads to increased opportunity for microbes to cross contaminate the processed dairy products. Moreover, spoilage and pathogenic bacteria entrapped within biofilms are protected from sanitizers due to multispecies cooperation and the presence of extracellular polymeric substances, which result in their survival (Watnick and Kolter, 2000). Rapid fouling of plate heat exchangers is thus undesirable for both economic and technical reasons. According to a study conducted by Van Asselt et al., (2005), 80% of the total production cost is attributed to fouling and cleaning in dairy processing industries. Further, the production run times are limited due to both shedding of bacteria in the product leading to microbiological concerns, and loss of heat transfer characteristics of PHEs (Sandu and Singh, 1991). It has also been
reported that metal surfaces can be corroded (Bryers, 1987) due to metabolic activity of the micro-organisms present inside the biofilm.

The initial establishment of biofilm highly depends on the properties of the surface material such as surface energy and hydrophobicity (Flint et al., 1997). Many researchers reported that there will be decreased bacterial adhesion with the lowering of surface energy and enhanced surface hydrophobicity (Fletcher and Loeb, 1979, Pringle and Fletcher, 1983, Tsibouklis et al., 2000). Hence, it is of high relevance to modify the SS surface and to use antimicrobial coatings to have less deposition, bacterial adhesion, and establishment of biofilms. One of our recent studies (Jindal et al., 2016) using modified coupons, revealed that SS316 surface modified with Ni-P-PTFE blend supports lower bacterial adherence, consequently resulting in reduced biofilm formation. Results from this study were promising, yet all the experiments were conducted on coupons at a laboratory scale in a static environment. As a follow up, the purpose of this study was to investigate the extent of biofouling on native (SS) and modified (Ni-P-PTFE) PHEs under dynamic conditions for an extended run time of 17 hours.

**MATERIALS AND METHODS**

Two separate pasteurizers used for this study included a Native (SS) and a Ni-P-PTFE modified surface plates PHE AGC Heat Transfer, Portland, OR. Milk was allowed to flow continuously through both the pasteurizers for 17 hours in order to simulate the conditions encountered in a typical dairy plant. Prior to the pasteurization step, the incoming raw milk was split into two balance tanks. At the end of pasteurization, both PHEs were flushed with water and were dismantled for sampling. Plates were re-
assembled in both the PHEs for Cleaning-In-Place (CIP) followed by dismantling again to check the efficiency of CIP. Two stage CIP treatment was followed, which involved the use of sodium hydroxide and an acid wash of nitric acid for native PHE. On the other hand, the Ni-P-PTFE modified surface PHE, being sensitive to the acid treatment, was treated with only alkali during the CIP cycle, as recommended by the manufacturer.

**Sampling**

Milk samples were collected at the start of the run, and at hourly intervals from 11th hour onwards from both the balance tanks (raw milk sample) and outlets (pasteurized milk sample) of both pasteurizers. Raw and pasteurized milk samples were maintained below 4°C from collection to completion of analysis. Appropriate serial dilutions were made aseptically and plated on the Plate Count Agar (PCA) followed by incubation at 32°C for 48h. The Colony Forming Units (CFUs) were calculated and the counts were reported as log cfu/cm² (Wehr and Frank, 2004).

**Enumeration of viable cell count adhered in biofilm matrix**

3M Quick swabs were used to recover biofilms from the regeneration section at the end of the run, when PHEs were flushed with water. Standard protocols were followed to enumerate mesophiles and thermophiles embedded in the 17h old biofilms. In order to validate the surface cleanliness of each section of both PHEs, Charm’s ATP swabs were used. The Bioluminescence Luminometer (Charm Sciences Inc., Lawrence, MA) was employed to measure the Relative Light Units (RLU). The Matrix Assisted Laser Desorption/ Ionization- Time of Flight (MALDI-TOF) was used to identify the selected isolates from raw milk, pasteurized milk, and the surface of both the PHEs. It is an extremely reliable method for identification of bacteria and examines ribosomal
proteins based on mass spectrophotometry. The counts were analyzed using analysis of variance and means were compared.

**RESULTS AND DISCUSSION**

*Viable counts in raw and pasteurized milk*

The results obtained in this study are presented in Figures 1-3. It was observed that the standard plate counts started to increase in the pasteurized milk samples after the 11th hour. The SPC count at the 11th hour for the pasteurized milk of native PHE (log 2.54 cfu/cm²) is higher as compared to the modified surface PHE (nil), however the rate of their growth is steeper for the modified PHE as compared to the steady count for the former. This is fairly justified since the bacterial counts for the pasteurized milk obtained from native PHE started to increase (after 10th hour) before the pasteurized milk obtained from modified PHE (counts increased after 11th hour). Hence, by the 11th hour, the pasteurized milk obtained from native PHE had already increased whereas the pasteurized milk obtained from modified PHE just started to increase resulting in its steeper slope after the 11th hour. But, as we go past 15th hour, native PHE experiences a sudden increase in the SPC thus raising its count (log 5.44 cfu/cm²) far more than that for modified PHE (log 4.12 cfu/cm²) as we reach the 17th hour. Similar results were reported in a study conducted by Lehmann et al., (1992) in which the bacterial count increased slightly (7 X 10³/ml to 2 X 10⁴/ml) in the pasteurized milk over the initial 10 hours of operation and more rapidly (2 X 10⁶/ml) over the remaining period of 21 hour of operating time. The probable reason for the increasing counts in pasteurized milk could be the shedding of bacteria entrapped in biofilm into the product stream. Flint et al.,
(2001) also reported that the bacteria entrapped in biofilms of *Geobacillus stearothermophilus* could shed into the milk passing over them.

**Viable counts in biofilm**

By quantifying the cells adhering to the regeneration section of both the PHEs, it was found that regeneration section of native PHE attracted more bacteria (log 1.48 cfu/cm$^2$) as compared to the modified PHE (log 0.995 cfu/cm$^2$). This is likely due to less conditioning of modified surface due to the reduced amount of milk solids sticking on its surface as compared to the native SS surface. It was reported by Rosmaninho et al., (2007) that Ni-P-PTFE coated surface promoted lesser amount of deposit build up and Zhao, (2004) stated that these coatings hindered bacterial adhesion by 82-97%. On isolating bacteria from the regeneration section of both the PHEs, mesophiles traced in native (log 1.49 cfu/cm$^2$) and modified (log 1.0 cfu/cm$^2$) PHEs were in comparable amounts to the thermophiles traced in both native (log 1.47 cfu/cm$^2$) and modified (log 0.99 cfu/cm$^2$) PHEs. This indicates that the mesophiles isolated from the regeneration section of pasteurizer can potentially survive as well as multiply in the high temperatures (up to 70°C). Research study conducted by Sharma and Anand, (2002) also demonstrated the active multiplication of both mesophiles (6 isolates in pre-pasteurized line to 29 isolates in post- pasteurized line) and thermophiles (1 isolates in pre-pasteurized line to 6 isolates in post- pasteurized line) during the commercial pasteurization of milk, indicating the ability of mesophiles and thermophiles to survive and multiply in the pasteurizer.

**Identification of bacterial isolates**

The species identification studies carried out using MALDI-TOF revealed similar bacteria in the biofilms developed in the regeneration section of both the PHEs as
compared to isolated from the raw milk samples. A total of 21 different isolates were obtained from the raw milk samples and swabbed solutions. Using MALDI-TOF, isolates isolated from the balance tank were identified as *Candida krusel, Pseudomonas azotoformans, Staphylococcus aureus, Escherichia coli, Bacillus licheniformis, Pseudomonas rhodesiae, Pseudomonas chlororaphis, Pseudomonas putida, Acinetobacter sp., Bacillus Altitudinis, Pseudomonas chlororaphis, Lactococcus gravieae, Bacillus cereus, Streptomyces, Staphylococcus warneri, and Bacillus sonorensis. Streptomyces, Staphylococcus warneri, Bacillus cereus and Bacillus licheniformis, Bacillus sonorensis, Brevibacillus para brevis, Kocura rhizophila, Streptococcus salivarium ssp, Bacillus pumilus* were isolated from the surface of regeneration section of both the PHEs. After 10th hour it was found that there was a gradual bacterial build up in the product stream and the isolates isolated from pasteurized milk were identified as *Bacillus pumilus, Pseudomonas putida, Staphylococcus aureus, Bacillus licheniformis, and Bacillus cereus.*

**Estimation of Relative Light Units**

The relative cleaning efficiency was monitored by measuring relative light units (RLU) using a Bioluminescence Luminometer (Charm Sciences Inc., Lawrence, MA) after cleaning the surfaces of both native and modified surface PHEs at the end of the 17h run. These RLUs were observed to be around 10 times higher for native PHE as compared to the modified one. This clearly indicates the presence of more organic matter on the surface of native PHE after CIP making it highly vulnerable to attack by bacteria. As ATP swabs detect the total ATP present on the surface and do not differentiate between the ATP from microbial cells and the ATP from organic matter so direct
comparison of RLUs and Colony Forming Units (CFUs) to biofilms is not feasible. However, the overall fouling was clearly established to be higher for the native PHE as compared to the modified one.

Further to this, in the native pasteurizer, the highest RLU counts were observed in the regeneration section whereas the least RLU counts were observed in the cooling section after pasteurization. On the other hand, in the modified pasteurizer, the maximum RLU counts were observed in the heating section whereas the least RLU counts were observed in the cooling section after pasteurization. An important observation from the RLU counts obtained indicates the presence of smallest amount of organic matter after cleaning (as indicated by low RLU counts) in the cooling section of both the native and modified plate heat exchangers. This is apparent from the fact that the heating and regeneration sections of the pasteurizers involve the denaturation of protein (as opposed to the cooling section) that stick to their surface and pose a critical challenge to get cleaned.

CONCLUSION

This study provides a proof of concept supporting the superiority of modified surface (Ni-P-PTFE) plate heat exchanger in terms of lower bacterial adhesion in comparison to the native PHE. The lower fouling would thus be helpful in conducting longer production runs without any cleaning interruptions. Moreover, since cleaning of native PHE involve an acid treatment step as compared to only alkali treatment step required for modified surface PHE, use of the latter is both cost and time effective making it more ideal surface as compared to the former.
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REFERENCES


Figure 1. Standard Plate Count (SPC) in pasteurized milk from native and modified PHE.
**Figure 2.** Biofilm counts in the regeneration section of native and modified PHE.
Figure 3. RLUs on native and modified SS surface after cleaning.
CHAPTER 4

COMPARISON OF THE ADHESION CHARACTERISTICS OF COMMON DAIRY SPOREFORMERS AND THEIR SPORES

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ABSTRACT

The initial attachment of aerobic spore forming bacteria to the surfaces of dairy processing equipment leads to biofilm formation and biofouling. Although sporeformers may vary in attachment, various surface modifications are being studied to develop a surface that is least vulnerable to attachment. This study was conducted to compare the extent of adhesion of spores and vegetative cells of common dairy sporeformers such as *Geobacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus sporothermodurans*, on both native and modified stainless steel surfaces. Influence of various contact surface and cell surface properties including surface energy, surface hydrophobicity, cell surface hydrophobicity and zeta potential on the adhesion tendency of bacteria was analyzed to establish their relationship. The ability of the vegetative cells and spores of different aerobic sporeformer to attach to native, and modified (Ni-P-PTFE) stainless steel surfaces was determined by allowing the interaction between the contact surface, and spores or vegetative cells for an hour at ambient temperature. Hexadecane assay was employed to determine the hydrophobicity of vegetative cells and spores of aerobic sporeforming bacteria, while the surface charge (expressed as zeta potential) was determined using Zeta sizer Nano series instrument. The results clearly indicated higher adhesion tendency of spores over vegetative cells of aerobic sporeforming bacteria. On comparing the sporeformers, *Bacillus sporothermodurans*
demonstrated greatest adhesion tendency followed by *Geobacillus stearothermophilus* and *Bacillus licheniformis*, respectively. The tendency to adhere varied with the variations in cell surface properties as it decreased with lower cell surface hydrophobicity and higher cell surface charge. On the other hand, modifying the contact surface properties caused the attachment tendency to decrease with the lowering surface energy and increasing surface hydrophobicity.

**Keywords**: Aerobic sporeformers, hydrophobicity, zeta potential

**INTRODUCTION**

Heat resistant aerobic spore forming bacilli such as *Bacillus sporothermodurans*, *Bacillus licheniformis*, and *Geobacillus stearothermophilus* are some of the common contaminants in dairy industry and are largely associated with spoilage of milk and milk products (Cheng et al., 2010). These aerobic sporeformers can be found in a variety of dairy products such as cheeses, milk powders, evaporated milk, and canned products, which demonstrates their capability of resisting high temperature treatments such as pasteurization and Ultra high temperatures (Scott et al., 2007). These bacilli actively attach to the stainless steel surfaces, consequently resulting in the formation of biofilms (Burgess et al., 2010). The establishment of biofilms on the surface is generally described as a two-stage process. The initial commencement of biofilm formation takes place when microorganisms adhere to the surface by weak Vander Waals and electrostatic forces. This attachment is reversible, as the bacteria can easily be detached from the surface (Hood and Zottola, 1995). Once the bacteria produce exopolysaccharides and gets embedded, it results in an irreversible attachment. These irreversible attachments are
difficult to remove and require strong shear force beside the use of increased concentration of chemicals and detergents (Davey and O’toole, 2000). Dairy processors impart high attention to these biofilms as once formed they have various detrimental effects including food spoilage and potential food borne illness resulting in huge economic losses. The bacteria detach from biofilms and enter the product stream, and thus have a high potential to contaminate milk and milk products (Flint et al., 1997). Also, these biofilms provide resistance to the heat transfer processes, and just about 0.05mm deep biofilm can cut down the heat transfer by one third (Russell, 1993). Metal surfaces get corroded (Bryers, 1987) due to the existence of biofilm and metabolic activity of the microorganisms present inside the biofilm, causing expensive structural damage to the surfaces. These biofilms can also result in blockages and decreased flow rates.

The occurrence of aerobic bacteria in raw milk, especially those belonging to genus bacillus, is a matter of concern because of their ability to form endospores, which can resist high heat treatments, and stay in dormant state for long (Andersson et al., 1995, Ryu and Beuchat, 2005). The sources for their entry in raw milk are present throughout the dairy chain including water, air, soil, and equipment (Wirtanen et al., 1996). Although, spores of these bacteria are reported to be present in low concentration in raw milk, higher counts are often obtained in the final product (McGuiggan et al., 2002). This clearly illustrates that the presence of biofilms of aerobic sporeformers on the surface of processing equipment can potentially contaminate the product stream by shedding bacteria into it (Flint et al., 2001). Vegetative cells and spores of sporeformers have been reported to exhibit a strong attachment in dairy processing environment (Watterson et al.,
However, very limited information is available about the influence of their cell surface properties on the contact surface attachment and biofilm formation. The physiochemical interactions between the surface and bacteria are responsible for the initial onset of biofilm formation (van Loosdrecht et al., 1989).

Both contact surface properties such as substrate hydrophobicity & surface energy, and the cell surface properties such as bacterial cell hydrophobicity & surface charge, expressed as zeta potential, facilitate the attachment, which can lead to everlasting biofilms. Since, the process to eliminate these biofilms from the system is very complex (Hood and Zottola, 1997), a better approach would be to prevent the formation of biofilms. One of the recent emphases is to develop surface modifications that can help prevent or reduce biofilm formation on food contact surfaces. Incorporation of silver or coating with Ni-P-PTFE for stainless steel contact surfaces have been employed in health care applications to reduce the attachment of bacterial infections (Zhao and Liu, 2006, Chiang et al., 2010). Sol-Gel surface modification of stainless steel has also been tried with a potential to reduce the establishment of biofilms, and has been approved by FDA for its use to fabricate food-processing equipment (FDA CFR 21, 175,300). In our previous investigation (Jindal et al., 2016), we demonstrated differences in biofilm formation on native and modified stainless steel coupons. In continuation to that, the objective of present study was to investigate the effect of cell surface properties on the attachment behavior of different aerobic spore forming bacteria and their spores that are commonly encountered in dairy industry.
MATERIALS AND METHODS

Source of bacterial cultures

Three different aerobic spore forming bacteria namely *Geobacillus stearothermophilus* (ATCC 15952), *Bacillus sporothermodurans* (DSM 10599), and *Bacillus licheniformis* (ATCC 6634) were used to examine the properties of bacterial cell that could impact their attachment to native and modified stainless steel (SS) surfaces. *Geobacillus stearothermophilus* and *Bacillus sporothermodurans* are considered to be high heat resistant sporeformers (HHRS) due to their ability to survive commercial sterilization (Ultra High Temperature) (Hill and Smythe, 2012). On the other hand, *Bacillus licheniformis* is inept in surviving through Ultra High Temperature (UHT) and capable of multiplying at both mesophilic and thermophilic temperatures, thus regarded as Thermo-tolerant sporeformer (Burgess et al., 2010). The above bacteria were sourced from the American type culture collection (ATCC, Manassas, Virginia), and Deutsche Sammlung von Mikroorganisem und Zellkulturen (DSM, Germany), respectively.

Preparation of vegetative cells suspension

The reference strains of the above sporeformers were grown in freshly prepared Brain Heart Infusion (BHI) Broth (Oxoid, Thermo Scientific, UK) by incubating at their optimum growth temperature as recommended by the supplier, and were maintained for future utilization in cryogenic vials as proposed by Perry, (1995). As per the process, overnight grown cultures were centrifuged at 4500 x g for the duration of 30 minutes. The pellets obtained were subsequently diluted in Phosphate Buffer Saline (PBS) at pH 7.4 and maintained in 1.8 mL cryogenic vials (CRYOBANK - Copan diagnostic Inc., Murrieta, CA) that contained sterile beads and glycerol. The vials were stored in a deep
freezer (NuAire ultralow freezer, NuAire Inc. Plymouth, MN) at -80°C for future experiments (Khanal et al., 2014). Prior to use, the pellets were suspended in Phosphate Buffer Saline (PBS), and the final suspension was adjusted to a concentration of $1 \times 10^7$ cfu mL$^{-1}$.

**Preparation of Endospores**

Spore stocks of *Geobacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus sporothermodurans* were prepared by the method proposed by Novak et al., (2005). 1.0 mL of each of the actively growing culture of above aerobic sporeformer was separately spread plated on BHI agar plate and incubated at their optimum growth temperature for 10 days. Spore staining were performed during the incubation period to check the extent of sporulation. Spores were harvested, after approximately 90% of sporulation was attained, by flooding 10ml of sterile distilled water on agar surface. After soaking for 2 minutes, surface was scraped using sterile spreaders and spore suspension was collected in 50 ml sterile centrifuge tubes. The tube containing spore suspension was centrifuged at 4500 x g for 30 minutes. Spore Pellet was washed two times in 20 ml of sterile distilled water followed by centrifugation at 4500 x g for 30 minutes. These washed pellets were then suspended in 10ml of sterile distilled water, heated at 80°C for 12 minutes to kill all the remaining vegetative cells, cooled and stored at – 20°C.

**Influence of bacterial cell surface properties on their adhesion tendency**

*Estimation of degree of surface hydrophobicity of bacterial cells.* Bacterial cell surface hydrophobicity was determined using Microbial Adhesion to Hydrocarbon (MATH) as suggested by Rosenberg et al., (1980). Overnight grown cultures of *Geobacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus sporothermodurans*
were centrifuged at 4500g for 15 minutes followed by suspending the pelleted cells in sterile distilled water to an O.D. of 1.2 to 1.6. 3.0 mL of this suspension was added to 3.0 mL of hexadecane, followed by vigorously mixing on a vortex mixer at room temperature for 60 seconds and incubating at 30°C for 10 minutes. After 10 minutes of incubation, it was agitated on a vortex mixer for 2 minutes at ambient temperature and allowed to stand for 20 minutes at ambient temperature. The absorbance of aqueous layer was measured at 600 nm using visible spectrophotometer (Spectronic 200, version 2.06) and cell surface hydrophobicity (expressed as percent transfer to hexadecane layer) was calculated using the formula:

\[
\text{% Hydrophobicity} = \frac{(\text{OD}_{600} \text{ before treatment with hexadecane} - \text{OD}_{600} \text{ after treatment with hexadecane}) \times 100}{\text{OD}_{600} \text{ before treatment with hexadecane}}
\]

**Determination of zeta potential of bacterial cells.** The cell surface charge (expressed as zeta potential) was measured using Malvern Zetasizer (Nano ZS, Worcestershire, UK). Overnight grown cultures of *Geobacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus sporothermodurans* were centrifuged at 4500g for 15 minutes at 4°C followed by suspending the pelleted cells in sterile distilled water to an O.D. of 1.2 to 1.6. Spectrophotometer (Spectronic 200, version 2.06) was employed to examine the optical density of the culture at various dilutions at 600nm. Samples were prepared for analysis by suspending 1ml of above suspension to 9 ml of phosphate buffer. Similar procedure was carried out for the determination of zeta potential of spores of above aerobic sporeforming bacteria (Denyer et al., 1993)

**Bacterial attachment studies to native and modified stainless steel coupons**
Native (SS) and Ni-P-PTFE modified SS coupons. This study covers the attachment of vegetative cells and spores of three different heat resistant aerobic spore formers on two stainless steel based surfaces. Corrugated stainless steel (SS316) plates were cut into (1 in x 1 in; 0.019in thick) coupons to mimic the plate heat exchanger surface. These were donated by AGC Heat Transfer (Portland, OR), and used as native SS surface. The Ni-P-PTFE coating technique was used for the modification of the modified SS surface examined in this work and was sourced from Avtec Finishing Systems (New Hope, MN). The Ni-P-PTFE coatings were prepared by a previously reported method (Barish and Goddard, 2013) in which approximately 0.0003” of nickel was coated by electroless deposition onto cleaned, Wood’s striked 316SS, followed by codeposition of PTFE particles (~200 nm diameter) in a second electroless nickel deposition step (0.0003”).

Before initiating the experiment, native (SS) and modified corrugated SS coupons were washed with deionized water, followed by washing with 70% alcohol, and re-rinsing the coupons with de-ionized water, which were then sterilized by autoclaving at 121°C for 15 minutes.

Attachment of vegetative cells or spores to native and modified SS surface.

Three trials, with two coupons each, were conducted for each experiment. Attachment study was performed according to the method proposed by Parker et al., (2001) with slight modifications involving an increase in the incubation time to further the establishment of bacteria on both the surfaces. Clean, sterile native and modified SS coupons were incubated with washed cells or spores, as the case may be, of Geobacillus stearothermophilus, Bacillus licheniformis, and Bacillus sporothermodurans separately at
a level of $1 \times 10^6$ cfu ml$^{-1}$ in sterile distilled water (Parker et al., 2001) and placed in shaking incubator (150 rpm) for 1 hour at ambient temperature. Sterile distilled water was used for the experiment to prevent the ionic germination. Zero hour counts were taken right after inoculation to know the initial count of culture being inoculated in sterile distilled water. *Bacillus licheniformis* was also studied for its attachment to native and modified SS surface after 2 and 4 hours of incubation.

**Enumeration of bacterial cells or spores adhered to the surface.** At the end of one hour of incubation, immersed coupons were removed from petridish using sterile tweezers followed by washing with sterile distilled water to remove loosely adhered cells or spores, as the case may be. Following this, the attached bacterial cells or spores were swabbed from the surface of the coupon using sterile 3M quick swabs (3M, MN, USA) and spread plated to get the count for bacterial cells or spores adhered to the surface. The swab tube was vortexed to release all cells from the swab tip followed by twisting the swab tip against the wall of swab tube to facilitate the recovery of all bacterial cells or spores. The contents in the tube were then mixed and appropriate serial dilutions were made with sterile phosphate buffer saline (PBS) solution at pH 7.4. Aerobic plate counts were performed on BHI agar plates (Khanal et al., 2014) using spread plate technique (Downes and Ito, 2001). Colonies that appeared on the agar plates were counted after 24 hours of incubation at the optimum growth temperature of bacteria.

**Scanning Electron Microscopy**

Scanning Electron Micrographs obtained from scanning electron microscopy (Hitachi S-3400N, Hitachi America, Ltd., Tarrytown, NY) were employed to analyze the surface of native and modified SS surface before and after attachment of vegetative cells and spores.
of *Geobacillus stearothermophilus*. Air drying method was employed for 12 hours in order to obtain a partially dehydrated biofilm for electron microscopy with minimum structural damage (Hassan et al., 2010). This was followed by sputter coating with 10nm thick layer of 99% gold to make the sample more conductive for microscopy. The SEM was exposed to 10kv accelerating voltage to observe biofilms from a distance of 10mm from the coupon.

**Statistical analysis**

Studies relating to the adhesion of spores and vegetative cells on native and modified SS surface were performed three times with two coupons in each experiment. The bacterial counts were calculated for mean values and standard error. Means were compared using Tukey multiple comparison test using SAS 9.3 software (SAS Institute Inc. Cary, NC) with least significance difference at p < 0.05.

Twelve scans (6 independent samples examined in duplicate) were performed for the analysis of cell surface properties of the vegetative cells and spores of all the aerobic sporeformers examined in this work. These cell surface properties were correlated to different aerobic sporeformers using Microsoft® excel® for Mac, 2011.

**RESULTS AND DISCUSSION**

Pasteurization and Ultra High Temperature are two commonly used thermal processes to destroy the spoilage causing and pathogenic organisms. However, sporeformers when subjected to harsh environmental conditions readily form spores, which can resist high temperature and pressure (Andersson et al., 1995, Heyndrickx, 2011). Keeping this in regard, this study was conducted to analyze and compare the attachment behavior of
spores and vegetative cells of common dairy sporeformers to native and modified stainless steel contact surfaces.

High Heat Resistant Sporeformers including *Geobacillus stearothermophilus* and *Bacillus sporothermodurans*, as well as Thermo-tolerant sporeformer such as *Bacillus licheniformis* are the most commonly encountered aerobic sporeformers (Lücking et al., 2013) and are also associated with the spoilage of milk and milk products. Hence, these bacteria were researched in this study for their cell surface properties including cell surface hydrophobicity & cell surface charge, and their attachment tendency on native and Ni-P-PTFE modified stainless steel surface.

Findings from our previous study based on the surface properties of native and Ni-P-PTFE modified SS surfaces concluded that the modified surface demonstrated lower surface energy as compared to the native SS surface. On the contrary, surface hydrophobicity was lower for native SS surface as compared to the modified SS surface (Jindal et al., 2016). The cell surface properties for the various sporeformers analyzed in this study are discussed in the following sections.

*Influence of cell surface hydrophobicity on development of bacterial biofilms*

Cell surface properties have a key role in the attachment of sporeformers or their spores to the surface of processing equipment. The attachment of vegetative cells as well as spores is highly influenced by the cell surface proteins (Parkar et al., 2001). The cell surface hydrophobicity for spores was found to be greater than that of vegetative cells (Figure 1). This fact can be explained on the basis of relative abundance of protein in the outer coats and exosporium compared with the peptidoglycan on vegetative cell surfaces (Wiencek et al., 1990). Another study conducted by Koshikawa et al., (1989) on
the hydrophobicity of spores revealed that the protein and lipid content of the exosporium possessed far higher hydrophobicity as compared to the spore-coat. It was found that the spores possessing a layer of exosporium exhibited a hydrophobic character greater than 70% as compared to the ones lacking this layer (hydrophobic character less than 30%). The results from the trials conducted to determine cell surface hydrophobicity of vegetative cells and spores using hexadecane assay also demonstrated greater hydrophobicity for spores as compared to vegetative cells for all the aerobic sporeformers (Figure 1).

The hydrophobicity of HHRS viz., *Geobacillus stearothermophilus* & *Bacillus sporothermodurans*, and thermo-tolerant sporeformer, *Bacillus licheniformis* was determined using hexadecane assay in our study. The vegetative cells as well as spores of *Bacillus sporothermodurans* exhibited significantly higher hydrophobicity as compared to those of *Geobacillus stearothermophilus*, with *Bacillus licheniformis* demonstrating least hydrophobicity (Figure 1). Hence, the overall hydrophobicity of High Heat Resistant Sporeformers (HHRS) was observed to be greater as compared to that of Thermo-tolerant Sporeformers. Higher cell surface hydrophobicity would lead to an enhanced tendency for the bacteria to attach and hence greater biofilm formation.

Ronner et al., (1990) demonstrated that with the increment in cell surface hydrophobicity, the attachment tendency of bacteria increases. Another important observation that could be drawn from the result of the experiment is that the hydrophobicity of sporeformers varies significantly between the two HHRS sporeformers tested, since *Bacillus sporothermodurans* exhibited greater hydrophobicity as compared to *Geobacillus stearothermophilus*. In view of this, it is not possible to categories the biofilm forming
capabilities of HHRS and Thermo-Tolerant species.

**Effects of cell surface charge on development of bacterial biofilms**

Another cell surface property i.e. cell surface charge, evaluated in terms of zeta potential, also played a significant role in determining the adhesion properties of the sporeformers. Zeta potential is the amount of repulsive/attractive electrostatic charge present on the surface of cell walls of bacteria. Although the magnitude may vary from strain to strain, the net charge on the bacterial cell wall is always negative (Dickson and Koohmarae, 1989). The results analyzed from the trials conducted to determine zeta potential of vegetative cells and spores using Zetasizer Nano series instrument demonstrated that the magnitude of absolute charge on the cell walls was higher for vegetative cells in comparison to spores for all the aerobic sporeformers tested under this study (Figure 2). This demonstrates greater zeta potential (cell surface charge) for vegetative cells as compared to spores.

Comparing the HHRS and Thermo-tolerant sporeformers analyzed in the experiment, it was found that *Bacillus licheniformis* presented significantly higher zeta-potential as compared to the other two sporeformers, thus indicating an overall greater absolute cell surface charge on Thermo-tolerant sporeformers as compared to the HHRS. *B. licheniformis* delivered minimum bacterial attachment indicating that there exist an inverse relation between cell surface charge and bacterial attachment. Ronner et al., (1990) demonstrated an inverse relation between the attachment tendency of bacteria and cell surface charge i.e. the attachment tendency of bacteria decreases with the increment in the absolute cell surface charge. Similar to the cell surface hydrophobicity, differences in zeta potential were also encountered among the different High Heat Resistant
Sporeformers with *Geobacillus stearothermophilus* demonstrating higher zeta potential than *Bacillus sporothermodurans*. In view of this, different characteristics like cell surface hydrophobicity and cell surface charge can influence the attachment behavior in different manner. More studies in this area can help us learn in depth their influence on attachment behavior.

**Comparison of attachment of aerobic sporeformers and spores**

**Attachment of Spores and Vegetative cells.** Results presented in Table 1 indicate a greater adhesion tendency of the spores as compared to the vegetative cells of the sporeformers tested, on both the native and Ni-P-PTFE modified stainless steel surfaces. Research studies conducted by previous researchers also demonstrated a greater spore attachment as compared to vegetative cells on stainless steel surface (Parkar et al., 2001, Peng et al., 2001). These spores, under optimized conditions could germinate back to the vegetative cells, which then could multiply, initiating the formation of biofilms (Aouadhi et al., 2012). Hence, keeping in regard the higher cell surface hydrophobicity and lower zeta potential (cell surface charge) of spores as compared to the vegetative cells, it could well be concluded that cell surface hydrophobicity demonstrates a direct relation to the attachment tendency of bacteria that is the attachment tendency of bacteria increases with the increments in cell surface hydrophobicity. Similar observations were reported from the studies of other researchers (Rönner et al., 1990). Conversely, Parkar et al., (2001) reported hardly any relationship between bacterial adhesion and hydrophobic character of the cells. On the contrary, cell surface charge exhibits an inverse relation to the attachment tendency of bacteria. Similar results were reported by Ronner et al., (1990). However, according to Seale et al., (2008), zeta potential does not influence the bacterial
adhesion in any way.

**Influence of surface properties.** The attachment tendency of vegetative cells seem to lower when analyzed on modified SS surface as compared to the native SS surface. This can be related to the observation from our previous study (Jindal et al., 2016) that attachment of bacteria decreases with the decrements in surface energy and increments in surface hydrophobicity. Thus, Ni-P-PTFE modified SS surface with lower surface energy and higher hydrophobicity considerably lowers the attachment of vegetative cells on its surface. However, spores demonstrated similar attachment over both native as well as modified SS surface.

**Attachment of Vegetative cells of HHRS and Thermo-Tolerant Sporeformers.**

The study revealed greater attachment of vegetative cells of *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* as compared to *Bacillus licheniformis* on both native as well as modified SS surface. Hence, HHRS demonstrated more attachment than Thermo-Tolerant sporeformers on either kind of surface. Variations could also be observed among different High Heat Resistant Sporeformers with vegetative cells of *Bacillus sporothermodurans* exhibiting greater attachment than vegetative cells of *Geobacillus stearothermophilus*. This again can be related to cell surface properties of different sporeformers. Vegetative cells of *Bacillus sporothermodurans* with greatest cell surface hydrophobicity and least cell surface charge exhibited greatest adhesion to either surface whereas vegetative cells of *Bacillus licheniformis* with least cell surface hydrophobicity and highest cell surface charge demonstrated least adhesion tendency. Once again, it confirms the above established fact that cell surface hydrophobicity is directly proportional to the attachment tendency whereas cell surface charge is inversely
proportional to the same.

**Attachment tendency of B. licheniformis over extended incubation.**

Owing to the negligible attachment of vegetative cells of *Bacillus licheniformis* on native SS surface after one hour of incubation, its attachment was also analyzed on both native and modified surface over an extended incubation period of 2 and 4 hours. Results, as presented in Table 2, show the attachment of vegetative cells of *Bacillus licheniformis* on native SS surface over 2 and over 4-hour incubation periods, as log 1.0 cfu/cm² and log1.31 cfu/cm², respectively. This is still lower than the attachment shown by *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* after 1 hour of incubation. However, the attachment on the modified SS surface was still found to be negligible after 2 and 4 hours of incubation period.

**Attachment of vegetative cells in milk vs water.** This study employed water as the medium to study the attachment tendency of the High Heat Resistant and Thermo-Tolerant sporeformers as opposed to milk that was employed as the medium for previous study (Jindal et al., 2016). In the current study, vegetative cells of High Heat Resistant Sporeformers (*Bacillus sporothermodurans* followed by *Geobacillus stearothermophilus*) exhibited greater attachment on SS surface, while the Thermo-Tolerant *Bacillus licheniformis* demonstrated the least attachment on SS surface. However, the previous study that employed milk as the primary test medium, the results indicated greater attachment of Thermo-Tolerant *Bacillus licheniformis* as compared to High Heat Resistant *Bacillus sporothermodurans*. This anomaly can be explained on the basis of the different mediums utilized for each of the studies. The milk medium employed in the previous study leads to the preconditioning of surface due to milk
proteins present in milk, which ultimately accelerate the bacterial attachment on the SS surface resulting in their exponential growth (Barnes et al., 1999). Thus, milk provides an ideal medium for the multiplication of sporeformers. Since *Bacillus sporothermodurans* demonstrates longer generation time leading to the slower multiplication among the three bacteria analyzed, its concentration in the milk medium is less, thus exhibiting lower attachment when milk is employed as the medium. However, water (as a test medium) does not provide any proteins or other nutrients that could lead to preconditioning of surface. This leaves no incentive for any bacteria to multiply and grow in this medium. Hence, the multiplication tendency plays no role in the attachment tendency of bacteria when water is employed as the test medium. This is evident from the greater attachment of High Heat Resistant *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* as compared to the Thermo-Tolerant *Bacillus licheniformis* in water as the test medium.

**Influence of surface properties.** Modifications in the surface properties also play a significant role in influencing the adhesion tendency of the vegetative cells of both HHRS and Thermo-Tolerant sporeformers. Hence, Ni-P-PTFE modified SS surface with lower surface energy (directly proportional to adhesion tendency) and higher hydrophobicity (inversely proportional to adhesion tendency) exhibits less attachment of vegetative cells of both HHRS including *Bacillus sporothermodurans* & *Geobacillus stearothermophilus* and Thermo-Tolerant sporeformers such as *Bacillus licheniformis* as compared to the native SS surface. However, the overall observations among different sporeformers still remain the same with vegetative cells of *Bacillus sporothermodurans* demonstrating the highest attachment tendency followed by *Geobacillus stearothermophilus* and *Bacillus licheniformis*. 
Attachment of Spores of HHRS and Thermo-Tolerant Sporeformers. The general pattern of the attachment of spores as compared for the HHRS such as Bacillus sporothermodurans & Geobacillus stearothermophilus, and Thermo-Tolerant sporeformers such as Bacillus licheniformis showed a similar trend to the vegetative cells of these sporeformers (Table 1). As the spores of Bacillus sporothermodurans and Geobacillus stearothermophilus demonstrated significantly greater attachment as compared to the spores of Bacillus licheniformis thus it can be interpreted that spores of HHRS show great attachment tendency over spores of Thermo-Tolerant sporeformers. Similar to the case in the attachment of vegetative cells, differences in attachment were observed among HHRS spores. Bacillus sporothermodurans spores exhibited greater attachment than spores of Geobacillus stearothermophilus (Table 1).

Influence of surface properties. Unlike the variation in the attachment tendency of vegetative cells with the variations in surface properties such as surface energy and surface hydrophobicity, the adhesion tendency among spores remains relatively unaffected by the differences observed in the surface properties. This is evident from the observation presented in Table 1 that the extent of spore attachment for all the three sporeformers remained comparable for both the native and modified SS surface.

Scanning Electron Microscopy
Scanning Electron Microscopy was employed to visualize the extent of attachment of the vegetative cells and spores taking Geobacillus stearothermophilus as a model organism, on both native as well as modified SS surface. The observations and conclusions drawn in the above sections were thus validated by the electron micrographs that demonstrated greater attachment of spores as compared to vegetative cells for both native and modified
SS surface. The electron micrographs also demonstrated a greater attachment of vegetative cells of *Geobacillus stearothermophilus* on native SS surface as compared to modified SS surface, while spore attachment was similar on both native (SS) and modified surfaces (Figure 3A, 3B, 4A, 4B, 5A, 5B).

**CONCLUSIONS**

The main objective of this study was to analyze the influence of various bacterial cell surface properties including cell surface hydrophobicity, and cell surface charge (zeta potential) on the attachment of vegetative cells and spores of common dairy sporeformers including High Heat Resistant Sporeformers such as *Geobacillus stearothermophilus* and *Bacillus sporothermodurans*, and Thermo-tolerant sporeformers such as *Bacillus licheniformis*. In the US dairy industry, there have been several instances where *Bacillus licheniformis* has been very frequently reported as a predominant species in milk products (Hill and Smythe, 2012, Lücking et al., 2013, Buehner et al., 2015). As this sporeformer showed lower attachment, hence, it should be easier to clean from the contact surfaces. Further, the use of higher heat processing treatments, such as UHT that are capable of inactivating the thermo tolerant species, could be helpful in keeping their numbers low in the final product (Hill and Smythe, 2012). On the other hand, as the high heat resistant species (HHRS) tested in this study (*Bacillus sporothermodurans* and *Geobacillus stearothermophilus*) showed greater attachment to the surfaces examined, surface modifications coupled with higher heat treatments would be of great help in reducing their occurrences in dairy products (Warth, 1978, Tabit and Buys, 2010, Jindal et al., 2016).
The above results also provide useful information about the cell surface properties of vegetative cells and spores of HHRS and Thermo-Tolerant sporeformers. This information coupled with our previously reported data on the contact surface properties can be helpful to minimize the development of biofilms of aerobic sporeformers commonly encountered in the dairy processing industry. All these findings would prove useful in the dairy processing environment to ensure food quality and safety. This would also save time and resources that otherwise are required in cleaning and sanitation purposes. However, as the modified SS surface still exhibited some spore attachment, more research needs to be carried out in developing novel surfaces that will have negligible spore attachment. This will ensure a comprehensive and credible food safety measure.

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REFERENCES


### Table 1. Attachment of vegetative cells and spores on native (control) and modified (Ni-P-PTFE) SS surface

<table>
<thead>
<tr>
<th>Strains</th>
<th>Average counts *(Log\textsubscript{10} CFU/cm\textsuperscript{2})</th>
<th>Native</th>
<th>Ni-P-PTFE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vegetative cells</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. stearothermophilus</em></td>
<td>2.94 ± 0.03\textsuperscript{aA}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>3.3 ± 0.01\textsuperscript{ahA}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Spores</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. stearothermophilus</em></td>
<td>3.19 ± 0.06\textsuperscript{cA}</td>
<td>3.05 ± 0.07\textsuperscript{aB}</td>
<td></td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>2.92 ± 0.05\textsuperscript{chA}</td>
<td>2.53 ± 0.05\textsuperscript{bB}</td>
<td></td>
</tr>
<tr>
<td><em>B. sporothermodurans</em></td>
<td>4.07 ± 0.03\textsuperscript{dA}</td>
<td>4.03 ± 0.05\textsuperscript{cB}</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE

ND stands for Not Detected

Values with different lowercase superscript letters (a-d) within a column are significantly different at p value <0.05.

Values with different uppercase superscript letters (A-B) within a row are significantly different at p value <0.05.
**Table 2.** Attachment of vegetative cells of *Bacillus licheniformis* on native (control) and modified (Ni-P-PTFE) SS surface over 4 hours of incubation

<table>
<thead>
<tr>
<th></th>
<th>1 hour</th>
<th>2 hour</th>
<th>4 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native SS surface</td>
<td>ND</td>
<td>1 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.31 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ni-P-PTFE modified SS surface</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Mean ± SE

ND stands for Not Detected

Values with different lowercase superscript letters (a-b) within a row are significantly different at p value <0.05.
**Figure 1.** Cell surface hydrophobicity of vegetative cells and spores of different aerobic sporeformers.

Values are an average of (12 measurements) ± standard error.
Figure 2. Cell surface charge (Zeta potential) of vegetative cells and spores of different aerobic sporeformers.

Values are an average of (12 measurements) ± standard error.
Figure 3A. Scanning electron micrograph of native SS coupon at 1000X magnification.
Figure 3B. Scanning electron micrograph of modified SS coupon (Ni-P-PTFE) at 1000X magnification.
Figure 4A. Scanning electron micrograph of native SS coupon at 5000X magnification after attachment of vegetative cells of *Geobacillus stearothermophilus*.

Black arrows indicate the location of rods of *Geobacillus stearothermophilus* on native SS coupon.
**Figure 4B.** Scanning electron micrograph of modified SS coupon (Ni-P-PTFE) at 5000X magnification after attachment of vegetative cells of *Geobacillus stearothermophilus.*
Figure 5A. Scanning electron micrograph of native SS coupon at 5000X magnification after attachment of spores of *Geobacillus stearothermophilus*.

Black arrows indicate the location of spores of *Geobacillus stearothermophilus* on native SS coupon.
Figure 5B. Scanning electron micrograph of modified SS coupon (Ni-P-PTFE) at 5000X magnification after attachment of spores of *Geobacillus stearothermophilus*.

Black arrows indicate the location of spores of *Geobacillus stearothermophilus* on Ni-P-PTFE modified SS coupon.
SUMMARY AND CONCLUSION

The objective of this study was to evaluate the ability of various modified Stainless Steel surfaces to reduce bacterial adhesion and biofilms of commonly encountered aerobic sporeformers in dairy industry such as *Geobacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus sporothermodurans*.

The first phase of the study dealt with attachment tendency of various aerobic spore-forming bacteria on native as well as modified Stainless Steel surfaces viz. AMC 18, Dursan, Ni-P-PTFE, Sol gel and Lectrofluor 641. Surface properties such as surface energy and surface hydrophobicity of various modified and native SS surface were found to influence the extent of formation of biofilms. Bacterial adhesion was found to increase with the increment in surface hydrophobicity and decrements in surface energy. However, surface roughness hardly played any role in altering the adhesion tendency of bacteria. Both Lindgren with least surface roughness and magnaplate exhibiting highest surface roughness showed less bacterial attachment to their surface. Most hydrophobic Ni-P-PTFE modified surface exhibited least surface energy demonstrating least bacterial attachment, clearly illustrating the decrease in bacterial adhesion with the increment in surface hydrophobicity and descent in surface energy. Lindgren was trailed by Magnaplate, AMC-18, Dursan with native Stainless Steel surface being highly vulnerable to the attack by aerobic spore forming bacteria. Thus, it can be inferred from the above results that bacterial attachment preventing properties are substantially enhanced through various modifications of native SS surface especially when modified with Ni-P-PTFE blend. Similar pattern of bacterial adhesion was observed when native and modified SS surface were examined for *Bacillus licheniformis*, *Bacillus sporothermodurans* and multi
species comprising of \textit{Geobacillus stearothermophilus} and \textit{Bacillus licheniformis}. However, evaluations with \textit{Bacillus sporothermodurans} demonstrated a significant drop in the adhered bacteria. One of the probable reasons for this could be poor growth of this bacterium as compared to the other spore forming bacteria.

The experiments were conducted in static environment in the first phase of the study. Therefore, the second phase focused on examining the behavior of Ni-P-PTFE modified SS surface and native SS surface under dynamic conditions. Both Ni-P-PTFE modified and native SS surface PHEs were compared for the extent of biofouling and shedding of bacteria when examined over a 17 hour-long pasteurization run. It was observed that the SPC of the pasteurized milk started to increase after the 11\textsuperscript{th} hour and there was a sudden increase in the SPC of pasteurized milk of native PHE after 15\textsuperscript{th} hour thus raising its counts far higher as compared to the modified PHE. Due to the rapidly increasing SPC of the pasteurized milk of native PHE after 15\textsuperscript{th} hour, use of modified PHE is recommended for conducting long run plant trials.

Increasing bacterial load in the raw milk leads to the more biofilm formation as is evident from results that the same bacteria as the one isolated from raw milk was also traced from pasteurized milk and regeneration section of both the pasteurizers. On isolating bacteria from regeneration section of both the pasteurizers, both mesophiles and thermophiles were found in comparable numbers. More RLUs were also observed for native PHE as compared to modified PHE after CIP, clearly indicating the presence of more organic matter on the surface of native PHE thus making it highly vulnerable to the bacterial attachment and biofilm formation.
The third phase of the study focused on examining the influence of various surface properties and cell surface properties including surface energy, surface hydrophobicity, cell surface hydrophobicity and cell surface charge (zeta potential) on the adhesion tendency of several aerobic spore-forming bacteria including *Geobacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus sporothermodurans*. Spores and vegetative cells of these aerobic spore-forming bacteria were also distinctively compared for their adhesion tendency. Results from this study suggest that spores exhibited a far greater adhesion tendency as compared to the vegetative cells to both the native and Ni-P-PTFE modified Stainless Steel surface. Among the individual sporeformers, *Bacillus licheniformis* demonstrated minimum adhesion to both native and modified SS surface followed by *Geobacillus stearothermophilus* with *Bacillus sporothermodurans* exhibiting greatest attachment tendency. As *B. licheniformis* demonstrated lower attachment, hence, it should be easier to clean from the contact surfaces. Further, the use of higher heat processing treatments, such as UHT that are capable of knocking down these thermo tolerant species, could be helpful in keeping their numbers low in the final product. On the other hand, surface modifications coupled with higher heat treatments would be of great help in reducing the occurrences of HHRS in dairy products. Analyzing the surface properties, it could be concluded that bacterial tendency to attach is directly proportional to the surface energy whereas inversely proportional to the surface hydrophobicity. However, cell surface hydrophobicity shares a direct relation with the bacterial adhesion whereas cell surface charge is inversely proportional to the attachment by bacteria.

The information collected from this study strongly indicates the use of Ni-P-PTFE modified surface for fabricating the dairy processing equipment as it result in lower
bacterial attachment and hence biofilm formation. This would result in lower cross contamination and enhanced microbial quality of end products. This will also save time and resources that otherwise are required to be utilized in cleaning and for other sanitation purposes. However, as the Ni-P-PTFE modified SS surface was found to exhibit greater spore attachment, more research needs to be carried out in developing novel surfaces that will have negligible spore attachment along with vegetative cells. A thorough understanding of the specific characteristics of various bacteria and surfaces is essential to devise a material that is the most resistant to bacterial attachment. This will help ensure a comprehensive and credible food quality and safety measure.