Comparison of adhesion characteristics of common dairy sporeformers and their spores on unmodified and modified stainless steel contact surfaces

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ABSTRACT

The attachment of aerobic spore-forming bacteria and their spores to the surfaces of dairy processing equipment leads to biofilm formation. Although sporeformers may differ in the degree of attachment, various surface modifications are being studied in order 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 the thermotolerant sporeformer *Bacillus licheniformis* and the high-heat-resistant sporeformers *Geobacillus stearothermophilus* and *Bacillus sporothermodurans* on both native and modified stainless steel surfaces. We studied the effect of contact surface and cell surface properties (including surface energy, surface hydrophobicity, cell surface hydrophobicity, and zeta potential) on the adhesion tendency of both types of sporeformers and their spores. Attachment to native and modified (Ni-P-polytetrafluoroethylene, Ni-P-PTFE) stainless steel surfaces was determined by allowing interaction between the respective contact surface and vegetative cells or spores for 1 h at ambient temperature. The hydrophobicity of vegetative cells and spores of aerobic spore-forming bacteria was determined using the hexadecane assay, and zeta potential was determined using the Zeta sizer Nano series instrument (Malvern Panalytical, Malvern, UK). The results indicated a higher adhesion tendency of spores over vegetative cells for both thermotolerant and high-heat-resistant sporeformers. On comparing the sporeformers, *B. sporothermodurans* demonstrated the highest adhesion tendency followed by *G. stearothermophilus*; *B. licheniformis* exhibited minimal attachment on both surfaces. The tendency to adhere varied with cell surface properties, decreasing with lower cell surface hydrophobicity and higher cell surface charge. On the other hand, modifying contact

surface properties for higher surface hydrophobicity and lower surface energy decreased attachment.

Key words: aerobic sporeformer, spores, hydrophobicity, zeta potential, attachment

INTRODUCTION

High-heat-resistant sporeformers (HHRS) such as *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* and thermotolerant sporeformers (TTS) such as *Bacillus licheniformis* are common sporeformers encountered in the dairy industry and are largely associated with spoilage of milk and dairy products (Cheng et al., 2010). These aerobic sporeformers can be found in a variety of dairy products, including cheeses, milk powders, evaporated milk, and canned products, which demonstrates their ability to resist high temperature treatments such as pasteurization and ultra-high temperature processing (Scott et al., 2007). *Geobacillus stearothermophilus* and *B. sporothermodurans* are considered HHRS due to their ability to survive commercial (UHT) sterilization (Hill and Smythe, 2012). In contrast, *B. licheniformis*, although unable to survive UHT conditions, is capable of multiplying at both mesophilic and thermophilic temperatures and is thus regarded as a TTS (Burgess et al., 2010). These bacilli actively attach to stainless steel (SS) surfaces and result in the formation of biofilms (Burgess et al., 2010). The establishment of biofilms on the surface is generally described as a 2-stage process. Biofilm formation commences when microorganisms adhere to the surface by weak Van der Waals and electrostatic forces. This stage is reversible, as the bacteria can easily be detached from the surface (Hood and Zottola, 1995). Once bacteria produce exopolysaccharides and become embedded, irreversible attachment occurs. At this stage, biofilm is difficult to remove and requires strong shear force as well as higher concentrations of chemicals and detergents (Davey and O'Toole, 2000). Dairy processors pay close attention to these biofilms, as they have various detrimental effects once formed, including food spoilage and potential food-borne illness, resulting in huge economic losses. The bacteria

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detach from biofilms and enter the product stream, and thus have a high potential to contaminate milk and milk products (Flint et al., 1997; Jindal et al., 2018). Also, these biofilms provide resistance to heat transfer processes, and a biofilm that is about 0.05 mm deep can cut heat transfer by one-third (Russell, 1993). Metal surfaces are corroded due to the existence of biofilm and metabolic activity of the microorganisms present inside the biofilm, causing expensive structural damage to the surfaces (Bryers, 1987; Gupta and Anand, 2017). These biofilms can also result in blockages and decreased flow rates.

The occurrence of aerobic bacteria in raw milk, especially those belonging to the genus *Bacillus*, is a matter of concern because of their ability to form endospores, which can resist high heat and remain dormant for long time (Andersson et al., 1995; Ryu and Beuchat, 2005). Sources for their entry into 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 raw milk at low concentrations, higher counts are often found in the final product (McGuiggan et al., 2002; Buehner et al., 2014, 2015). This 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 the dairy processing environment (Watterson et al., 2014). The physicochemical interactions between the surface and bacteria are responsible for the initial onset of biofilm formation (van Loosdrecht et al., 1989). However, limited information is available about the influence of the cell surface properties of bacteria on contact surface attachment and biofilm formation. Both contact surface properties, such as substrate hydrophobicity and surface energy, and cell surface properties, such as bacterial cell hydrophobicity and surface charge (expressed as zeta potential), facilitate attachment, which can lead to persistent biofilms. Because the process to eliminate these biofilms from the processing system is complex, a better approach would be to prevent the formation of biofilms (Hood and Zottola, 1997). One recent emphasis is to develop surface modifications that could help prevent or reduce biofilm formation on food contact surfaces. Incorporating silver or coating SS contact surfaces with Ni-P-polytetrafluoroethylene (Ni-P-PTFE) has been used in health care applications to reduce biofilm attachment and bacterial infections (Zhao and Liu, 2006; Chiang et al., 2010). Sol-Gel (a Thermolon-based surface modification of stainless steel; Porcelain Industries Inc., Dickson, TN) has also been used to reduce the establishment of biofilms (Liu

et al., 2017), and has been approved by the US Food and Drug Administration for use in fabricating food-processing equipment (FDA, 2018).

In our previous investigation, we demonstrated differences in biofilm formation on native and modified SS surfaces (Jindal et al., 2016). The objective of the present study was to investigate the effect of bacterial cell surface properties on the attachment behavior of HHRS and TTS and their spores commonly encountered in dairy industry.

MATERIALS AND METHODS

Source of Bacterial Cultures

Two HHRS, *Geobacillus stearothermophilus* ATCC 15952 and *Bacillus sporothermodurans* DSM 10599, and 1 TS, *Bacillus licheniformis* ATCC 6634, were examined for properties of bacterial cells that could influence their attachment to native and modified SS surfaces. The above bacteria were sourced from the American Type Culture Collection (ATCC, Manassas, VA), and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), respectively.

Preparation of Vegetative Cell Suspensions

The above sporeformers were grown individually in freshly prepared brain-heart infusion (BHI) broth (Oxoid/Thermo Scientific, Basingstoke, UK) by incubating at their optimum growth temperature (*G. stearothermophilus*, ATCC 15952, 50°C; *B. licheniformis*, ATCC 6634, 30°C; *B. sporothermodurans*, DSM 10599, 30°C) as recommended by the supplier, and were maintained for future use in cryogenic vials as described by Perry (1995). Overnight cultures were then centrifuged at $4,500 \times g$ for 30 min. The resulting pellets were subsequently diluted in PBS at pH 7.4, and maintained in 1.8-mL cryogenic vials containing sterile beads and glycerol (Cryobank, Copan Diagnostic Inc., Murrieta, CA). 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). Before use, the pellets were suspended in PBS, and final suspensions adjusted to a concentration of 1×10^7 cfu/mL.

Preparation of Endospores

Spore stocks of *G. stearothermophilus*, *B. licheniformis*, and *B. sporothermodurans* were prepared by the method of Novak et al. (2005). One milliliter of each of the actively growing cultures was separately spread-plated on BHI agar plates and incubated at the

optimum growth temperature for 10 d to obtain spores. Spore staining was performed intermittently during the incubation period to check the extent of sporulation. Spores were harvested after approximately 90% sporulation was attained by washing off the agar surface with 10 mL of sterile distilled water. After soaking for 2 min, the wet agar surface was scraped using sterile spreaders and the spore suspension was collected in 50-mL sterile centrifuge tubes. The tubes containing spore suspensions were centrifuged at $4,500 \times g$ for 30 min. The spore pellet thus obtained was washed twice in 20 mL of sterile distilled water followed by centrifugation at $4,500 \times g$ for 30 min. The washed pellets were then suspended in 10 mL of sterile distilled water, heated at 80°C for 12 min to inactivate the remaining vegetative cells, cooled, and stored at -20°C.

Influence of Bacterial Cell Surface Properties on Adhesion Tendency

Bacterial cell surface hydrophobicity was determined using microbial adhesion to hydrocarbon (MATH), as suggested by Rosenberg et al. (1980). Overnight grown cultures of *G. stearothermophilus*, *B. sporothermodurans*, and *B. licheniformis* were centrifuged at $4,500 \times g$ for 30 min and then the pelleted cells were suspended in sterile distilled water to an optical density at 600 nm (OD_{600}) of 1.2 to 1.6. Three milliliters of this suspension was added to 3.0 mL of hexadecane, followed by vigorous mixing on a vortex mixer at room temperature for 60 s and incubating at 30°C for 10 min. After 10 min of incubation, the suspension was agitated on a vortex mixer for 2 min and allowed to stand for 20 min at ambient temperature. The absorbance of aqueous layer was measured at 600 nm using a visible spectrophotometer (Spectronic 200, version 2.06; Thermo Fisher Scientific, Waltham, MA) and cell surface hydrophobicity (expressed as percent transfer to hexadecane layer) was calculated using the formula

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

Similar studies were conducted with the endospores of *G. stearothermophilus*, *B. sporothermodurans*, and *B. licheniformis*.

Zeta potential was measured using a Malvern Nano ZS Zetasizer (Malvern Panalytical, Malvern, UK) at 25°C. Overnight cultures of *G. stearothermophilus*, *B. sporothermodurans*, and *B. licheniformis* were centrifuged at $4,500 \times g$ for 30 min at 4°C and then the pel-

leted cells were suspended in sterile distilled water to an OD_{600} of 1.2 to 1.6. A spectrophotometer (Spectronic 200, version 2.06) was used to measure the OD_{600} of the culture at various dilutions. Samples were prepared for analysis by suspending 1 mL of the suspension in 9 mL of phosphate buffer at pH 7.4. A similar procedure was carried out to determine the zeta potential of spores of TS and HHRS (Denyer et al., 1993).

Bacterial Attachment to Native and Modified SS Coupons

We evaluated the attachment of vegetative cells and spores of HHRS and TS to 2 SS-based surfaces. Corrugated stainless steel (SS316) plates (AGC Heat Transfer, Portland, OR; used as the native SS surface) were cut into coupons (25.4 mm $\times$ 25.4 mm $\times$ 0.54 mm thick) to mimic the surface of a plate heat exchanger. The Ni-P-PTFE coating technique was used to modify the corrugated SS surface and the coated coupons were 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 7.62 μm of nickel was coated by electroless deposition onto cleaned, Wood's struck 316SS, followed by co-deposition of PTFE particles (~ 0.0000002 m diameter) in a second electroless nickel deposition step.

Before initiating the experiment, native and modified SS coupons were washed first with deionized water and then with 70% alcohol, rinsed with deionized water, and finally sterilized by autoclaving at 121°C for 15 min.

Three trials, with 2 coupons each, were conducted for each experiment. The attachment study was performed according to the method described by Parkar et al. (2001) with slight modifications involving an increase in the incubation time to ensure adequate establishment of bacteria on both the surfaces. Clean and sterile coupons of both native and modified SS were incubated in petri dishes with washed vegetative cells or spores of *G. stearothermophilus*, *B. sporothermodurans*, and *B. licheniformis* at 1×10^6 cfu/mL in sterile distilled water (Parkar et al., 2001) and placed in a shaking incubator (150 rpm) for 1 h at ambient temperature. Sterile distilled water was used for incubation to prevent ionic germination of spores. Zero-hour counts were taken immediately after inoculation.

Enumeration of Adherent Bacterial Cells and Spores

At the end of 1 h of incubation, immersed coupons were removed from petri dishes using sterile tweezers

and washed with sterile distilled water to remove loosely adhered cells or spores. Then, the attached bacterial cells or spores were swabbed from the surface of the coupon using sterile 3M Quick Swabs (3M, St. Paul, MN) and spread plated to obtain counts of adherent bacterial cells or spores. The swab tube was vortexed to release all cells from the swab tip and then the swab tip was twisted against the wall of the swab tube to facilitate recovery of all bacterial cells or spores. The contents in the tube were mixed and appropriate serial dilutions were made with sterile PBS at pH 7.4. Aerobic plate counts were performed on BHI agar plates (Khanal et al., 2014) using the spread plate technique (Downes and Ito, 2001). Colonies that appeared on the agar plates were counted after 24 h of incubation at the optimum growth temperature of the bacteria.

Scanning Electron Microscopy to Observe Attachment

Images were obtained using scanning electron microscopy (Hitachi S-3400N, Hitachi America Ltd., Tarrytown, NY) and used to analyze the surface of native and modified SS surfaces before and after attachment of vegetative cells and spores of *G. stearothermophilus*, which served as a representative sporeformer. The air-drying method was used for 12 h to obtain a partially dehydrated biofilm for electron microscopy with minimum structural damage (Hassan et al., 2010). This was followed by sputter coating with a 10-nm-thick layer of 99% gold to make the sample more conductive for microscopy. The scanning electron microscope was exposed to 10 kV accelerating voltage to observe biofilms from a distance of 10 mm from the coupon.

Statistical Analysis

All experiments related to the adhesion of vegetative cells and spores on native and modified SS surface were performed 3 times with 2 coupons in each experiment. The bacterial counts were calculated as mean values and standard errors. Means were compared using the Tukey multiple comparison test using SAS software (version 9.3, SAS Institute Inc., Cary, NC) with least significance difference at $P < 0.05$.

Twelve scans (6 independent samples examined in duplicate) were performed to analyze the cell surface properties of the vegetative cells and spores of the aerobic sporeformers. These cell surface properties were correlated with different aerobic sporeformers using Microsoft Excel for Mac (2011, Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

Pasteurization is a commonly used thermal process to inactivate pathogenic and spoilage-causing organisms. However, sporeformers readily form spores when subjected to harsh environmental conditions, and the spores can resist high temperature and pressure (Andersson et al., 1995; Heyndrickx, 2011). Many of the thermotolerant spore-forming vegetative cells and their spores attach to contact surfaces and later develop into biofilms. This study was conducted to analyze and compare the attachment behavior of vegetative cells and spores of common dairy sporeformers to both native and modified SS contact surfaces. High-heat-resistant sporeformers such as *G. stearothermophilus* and *B. sporothermodurans* and TTS such as *B. licheniformis* are the commonly encountered aerobic sporeformers (Lücking et al., 2013), and they are associated with the spoilage of milk and dairy products. Hence, these bacteria were studied for their cell surface properties, including cell surface hydrophobicity and zeta potential, and their tendency to attach to native and Ni-P-PTFE-modified SS surfaces.

Our previous study, based on the surface properties of native and Ni-P-PTFE-modified SS surfaces, revealed that the modified surface had lower surface energy (15.96 ± 1.21 mN/m) than the native SS surface (42.94 ± 0.67 mN/m). In contrast, the native SS surface had lower surface hydrophobicity than the modified SS surface (Jindal et al., 2016). In the current study, we analyzed the cell surface properties of various sporeformers and their spores.

Influence of Cell Surface Hydrophobicity on Bacterial Adhesion

Cell surface properties play a key role in the attachment of sporeformers or their spores to the surface of processing equipment. The attachment of vegetative cells and spores is highly influenced by cell surface proteins (Parkar et al., 2001).

In our study, cell surface hydrophobicity values were greater for spores than for vegetative cells of the same sporeformer for both HHRS and TTS (Figure 1). This could be explained by the relative abundance of protein in the outer coat and exosporium (the outer layer that provides chemical and enzymatic resistance to spores) 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 was much more hydrophobic than the spore

Figure 1. Cell surface hydrophobicity (%) of vegetative cells and spores of the aerobic sporeformers *Geobacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus sporothermodurans*. Values are an average of 12 measurements $\pm$ SE. Values with different lowercase letters (a–f) are significantly different at $P < 0.05$.

coat. Spores possessing a layer of exosporium exhibited a hydrophobic character (one of the most relevant factors influencing bacterial attachment and biofilm formation, which determines the contact between the bacterial cell wall and the substrate) greater than 70% compared with those lacking this layer (hydrophobic character $<30\%$).

Additionally, when the hydrophobicity of different sporeformers was compared, vegetative cells of *B. sporothermodurans* were significantly ($P < 0.05$) more hydrophobic than those of *G. stearothermophilus* (28.48%), with *B. licheniformis* demonstrating the lowest hydrophobicity (3.09%; Figure 1). Spores of the species demonstrated a similar trend. Higher cell surface hydrophobicity would lead to an enhanced tendency for the bacteria to attach and hence greater biofilm formation. This can be seen in the results shown in Table 1, where attachment was less for vegetative cells (lower cell surface hydrophobicity) than for spores (higher cell surface hydrophobicity) of the same sporeformer. Rönner et al. (1990) demonstrated that the attachment tendency of bacteria increases with increasing cell surface hydrophobicity. Another important observation from our study was that the hydrophobicity of

B. sporothermodurans and *G. stearothermophilus* varied significantly: vegetative cells and spores of *B. sporothermodurans* had greater hydrophobicity than those of *G. stearothermophilus*. It is also important to note that any preconditioning of the surface, due to the presence of a foulant, for example, would result in differences in attachment behavior compared with clean surfaces (Jindal et al., 2016).

Table 1. Attachment of vegetative cells and spores (mean $\pm$ SE) on native stainless steel surface

Strain	Count ($\log_{10}$ cfu/cm ²)
Vegetative cells	
<i>Geobacillus stearothermophilus</i>	2.94 $\pm$ 0.03 ^c
<i>Bacillus sporothermodurans</i>	3.30 $\pm$ 0.00 ^b
<i>Bacillus licheniformis</i>	ND ¹
Spores	
<i>G. stearothermophilus</i>	3.17 $\pm$ 0.06 ^b
<i>B. sporothermodurans</i>	4.07 $\pm$ 0.04 ^a
<i>B. licheniformis</i>	2.92 $\pm$ 0.05 ^c

^{a–c}Values with different lowercase superscript letters are significantly different at $P < 0.05$.

¹Not detected.

Figure 2. Zeta potential (mV) of vegetative cells and spores of the aerobic sporeformers *Geobacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus sporothermodurans*. Values are an average of 12 measurements $\pm$ SE. Values with different lowercase letters (a–d) are significantly different at $P < 0.05$.

Effect of Zeta Potential on Bacterial Adhesion

Another cell surface property—zeta potential—plays a significant role in determining the adhesion properties of the sporeformers. Zeta potential is the electrical potential at the particle-slipping plane (shear surface) relative to the bulk dispersion medium. Although the magnitude may vary from strain to strain, the net charge on the bacterial cell wall is always negative (Dickson and Koohmaraie, 1989). Our results demonstrated that the magnitude of absolute charge on the cell walls was higher for vegetative cells than for spores of the same sporeformer. However, we did not detect significant differences between the zeta potential of vegetative cells and spores of *G. stearothermophilus* and *B. licheniformis* (Figure 2).

On comparing the sporeformers analyzed in this experiment, we observed that *B. licheniformis* had significantly higher zeta potential than the other 2 sporeformers, indicating an overall greater absolute cell surface charge on *B. licheniformis* than on *G. stearothermophilus* and *B. sporothermodurans*. Further, vegetative cells and spores of *B. licheniformis* showed minimum bacterial attachment, indicating an inverse relationship between zeta potential and bacterial attachment when coupons were suspended in distilled

water and bacteria interacted with a clean contact surface. Rönner et al. (1990) demonstrated an inverse relation between the attachment tendency of bacteria and cell surface charge; that is, attachment of bacteria decreased with increases in absolute cell surface charge. Similar to cell surface hydrophobicity, differences in zeta potential were encountered among the HHRS, with *G. stearothermophilus* demonstrating higher zeta potential than *B. sporothermodurans*.

Comparison of Attachment of Sporeformers and Spores

Results presented in Table 1 and Table 2 indicate a greater attachment tendency of spores compared with vegetative cells on both the native and modified SS surfaces. Previous researchers demonstrated greater attachment of spores than vegetative cells on stainless steel surfaces (Parkar et al., 2001; Peng et al., 2001). Under optimal conditions, these spores could germinate back to vegetative cells, which could then multiply, initiating the formation of biofilms (Aouadhi et al., 2012). Hence, in view of the higher cell surface hydrophobicity of spores, we conclude that cell surface hydrophobicity demonstrates a direct relationship with attachment tendency. Spores of *B. sporothermodurans* (which had

the highest cell surface hydrophobicity) exhibited maximum attachment on both native and modified SS surfaces. Similar observations have been reported by other researchers (Rönnert et al., 1990). However, according to Seale et al. (2008), zeta potential does not influence bacterial adhesion in any manner. The general pattern of attachment of spores showed a trend similar to that of the corresponding vegetative cells of these sporeformers (Table 1).

The attachment tendency of vegetative cells was negligible on the modified SS surface. This also relates well to the observation from our previous study (Jindal et al., 2016) that attachment of bacteria decreases with lower surface energy and higher surface hydrophobicity of the contact surface. Thus, the Ni-P-PTFE-modified SS surface with lower surface energy and higher hydrophobicity considerably reduced the attachment of vegetative cells to its surface. However, spores demonstrated greater attachment to both native and modified SS surfaces when tested on clean surfaces without any pre-conditioning foulant layer.

The study revealed greater attachment of vegetative cells of *B. sporothermodurans* and *G. stearothermophilus* compared with those of *B. licheniformis* on native SS surface. Hence, the HHRS demonstrated greater attachment than TTS on either surface. Variations were observed among the 2 HHRS, with vegetative cells of *B. sporothermodurans* exhibiting greater attachment than those of *G. stearothermophilus*. This could be related to cell surface properties of different sporeformers. Vegetative cells of *B. sporothermodurans* (the highest cell surface hydrophobicity and lowest cell surface charge) exhibited maximum adhesion to both surfaces, whereas vegetative cells of *B. licheniformis* (the lowest cell surface hydrophobicity and highest cell surface charge) demonstrated least adhesion. Thus, cell surface hydrophobicity is directly related to attachment tendency, whereas cell surface charge is inversely related.

Table 2. Attachment of vegetative cells and spores (mean $\pm$ SE) on the Ni-P-polytetrafluoroethylene-modified stainless steel surface

Strain	Count ($\log_{10}$ cfu/cm ²)
Vegetative cells	
<i>Geobacillus stearothermophilus</i>	ND ¹
<i>Bacillus sporothermodurans</i>	ND
<i>Bacillus licheniformis</i>	ND
Spores	
<i>G. stearothermophilus</i>	3.19 ± 0.07^b
<i>B. sporothermodurans</i>	4.03 ± 0.05^a
<i>B. licheniformis</i>	2.54 ± 0.04^c

^{a-c}Values with different lowercase superscript letters are significantly different at $P < 0.05$.

¹Not detected.

Observations Under the Scanning Electron Microscope

Using *G. stearothermophilus* as a representative organism, scanning electron microscopy was used to visualize the extent of attachment of the vegetative cells and spores on both native and modified SS surfaces. The electron microscopy validated our other results, demonstrating greater attachment of spores than vegetative cells to both native and modified SS surfaces. The electron micrographs also demonstrated greater attachment of vegetative cells of *G. stearothermophilus* to the native SS than to the modified SS surface, whereas spore attachment was higher on both native and modified surfaces (Figure 3A, 3B, 4A, 4B, 5A,

Figure 3. Scanning electron micrograph of (A) native stainless steel (SS) coupon, and (B) modified SS coupon (Ni-P-polytetrafluoroethylene, Ni-P-PTFE) at 1,000 $\times$ magnification. The total distance between the first and the last tick marks is 50 μ m.

5B). The scanning electron micrographs thus allowed us to visualize and put in perspective the attachment of spores and vegetative cells, which can serve as a reference for future studies in this direction, especially for comparing cleaning and sanitation protocols for residual attachment of low numbers of cells.

The main objective of this study was to analyze the influence of various bacterial cell surface properties, including cell surface hydrophobicity and zeta potential, on the attachment tendency of vegetative cells and spores of common dairy sporeformers. Both cell surface hydrophobicity and zeta potential can affect

the attachment behavior of vegetative cells and spores. Cell surface hydrophobicity was significantly different for vegetative cells and spores of different sporeformers tested, with the highest hydrophobicity measured for *B. sporothermodurans*. In contrast, zeta potential was highest for *B. licheniformis* and lowest for *B. sporothermodurans*. We observed no significant difference between the zeta potential of vegetative cells and spores of *G. stearothermophilus* and *B. licheniformis*. However, the spores of *B. sporothermodurans* exhibited significantly lower zeta potential than its vegetative cells. Overall, the statistical results suggest that zeta potential tended to be higher for vegetative cells than

Figure 4. Scanning electron micrograph of (A) native stainless steel (SS) coupon, and (B) modified SS coupon (Ni-P-polytetrafluoroethylene, Ni-P-PTFE) at 5,000 $\times$ magnification shows attachment of more vegetative cells than spores of *Geobacillus stearothermophilus*. The total distance between the first and the last tick marks is 10 μ m. Arrows in panels A and B demonstrate the attachment of vegetative cells and spores of *G. stearothermophilus*. Black dots in panel B after attachment of vegetative cells represent the Ni-P-PTFE particles.

Figure 5. Scanning electron micrograph of (A) native stainless steel (SS) coupon, and (B) modified SS coupon (Ni-P-polytetrafluoroethylene, Ni-P-PTFE) at 5,000 $\times$ magnification shows higher attachment of spores of *Geobacillus stearothermophilus*. The total distance between the first and the last tick marks is 10 μ m. Arrows in panels A and B demonstrate the attachment of vegetative cells and spores of *G. stearothermophilus*.

for spores. Because spores have a higher tendency to attach than vegetative cells, we conclude that zeta potential has an inverse relation and cell surface hydrophobicity has a direct relation with attachment of bacteria. In fact, cell surface hydrophobicity, zeta potential, and specific bacterial and surface properties determine the adhesiveness of any bacteria. Further studies are necessary to understand the effects of these characteristics, because any pre-conditioning of the dairy processing contact surfaces (e.g., fouling due to residues) before bacterial attachment may modify the contact surface characteristics and thus alter the overall process of bacterial adhesion and biofilm formation.

CONCLUSIONS

This information in this report, coupled with our previously reported results on contact surface properties, could be helpful in designing strategies to minimize the development of biofilms of aerobic sporeformers commonly encountered in the dairy processing industry. These findings should prove useful in the dairy processing environment to ensure dairy products with improved microbial quality, especially with regard to sporeformers and spores.

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