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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 Mikroorganisem und Zellkulturen (DSM, 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 × *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