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Influence of free energy on the attachment of *Salmonella Enteritidis* and *Listeria monocytogenes* on stainless steels AISI 304 and AISI 316

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ABSTRACT

Bacterial attachment to stainless steels AISI (American Iron and Steel Institute) 304 and 316, the preferred material choices for food equipment, is a very important food safety consideration. The purpose of the present study was to investigate the attachment of two important food pathogens on commercial samples of these surfaces focusing on the influence of material topography characterized by Atomic Force Microscopy and considering the hydrophobicity and free energy of interaction, determined by measuring contact angle and application of thermodynamic theory principles. Results showed that *S. Enteritidis* and *Listeria monocytogenes* were able to attach to samples of stainless steels, however, initially (t = 0), the number of adhered *S. Enteritidis* (2.55 log CFU/cm^2) was higher than *L. monocytogenes* (1.68 logCFU/cm^2). Corroborating these results, the adhesion was thermodynamically more favorable for *S. Enteritidis*. Even though, the number of adhered cells were similar on both stainless steels samples, negative total energy was higher on AISI 316, for both bacteria. Moreover, the surface's roughness (0.032 μm and 0.021 μm) and hydrophobicity of cells and materials did not show a positive correlation with bacterial adherence.

1. Introduction

Microbial contamination of foods during processing is an issue of great concern to the food industry, because microorganisms may contaminate foods, induce spoilage or cause foodborne diseases. A common route of food contamination is through the contact of food products with contaminated processing equipment surfaces (Sasahara & Zottola, 1993). Gram-positive and Gram-negative microorganisms show different adherence patterns (Pompermayer & Gaylarde, 2000; Sommer, Martin-Rouas, & Mettler, 1999). *S. Enteritidis* is an important Gram-negative food pathogen, responsible for thousands of outbreaks worldwide (EFSA and ECDC, 2015; Lai et al., 2014). Similarly, *Listeria monocytogenes* is recognized as a major Gram-positive foodborne pathogen able to form biofilms on food surfaces (Gandhi & Chikindas, 2007; Pan, Breidt, & Gorski, 2010; Wilks, Michels, & Keevil, 2006). Several studies have shown that these bacteria are capable of adhering and forming biofilms on stainless steel, glass, polyethylene and rubber surfaces (Bong Jae, Travis, & Nehal, 2009; Chia, Goulter, Mcmeekin, Dykes, & Fegan, 2009; Malheiros, Passos, Casarin, Serraglio, & Tondo, 2010; Morita, Komoda, Ono, & Kumagai, 2011; Tondo et al., 2010). Most food processing equipment surfaces are made of stainless steel, traditionally chosen because it is durable, resistant to corrosion, and it is easily cleaned (Holah & Thorpe, 1990; Shi & Zhu, 2009). Stainless Steel AISI 304 and AISI 316 are the most used stainless steel types in the food industry (Hood & Zottola, 1997; EHEDG, 2007). AISI 304 is an austenitic steel with a minimum of 18% chromium, 8% nickel and up to 0.08% carbon, while AISI 316, also an

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austenitic steel, has up to 3% molybdenum and higher nickel content (10–14%) than AISI 304 (Martins, Moreira, & Martins, 2014). What essentially distinguishes type 316 from type 304 is its molybdenum content added to increase corrosion resistance in various environments (brines, bleaching, biofluids, etc.), and, in particular, reduces or inhibits the pitting corrosion induced by chlorides (Martins et al., 2014). Despite its higher chemical resistance, type AISI 304 stainless steel has been the most widely used by manufacturers of equipment for food industry because of its lower cost.

The adhesion of bacteria to the surface equipment is particularly affected by the characteristics of the microorganisms and the surface involved (Teixeira, Lopes, Azeredo, Oliveira, & Vieira, 2005). When bacteria approach a surface, they must overcome an energy barrier in order to establish direct contact with the surface. The repulsive or attractive forces consist of Lifshitz –Van der Waals attractive forces, electrostatic repulsive forces and acid base forces. As an oversimplified rule of thumb, primary adhesion among bacteria and abiotic surfaces is generally mediated by nonspecific interactions (Dunne, 2002). Only when the cells and surfaces are in close proximity the short-range interactions become significant (including hydrogen bonding as well as hydrophobic interactions). The theoretical approaches describing these interactions usually involve DLVO (Derjaguin, Landau, Verwey and Overbeek) or XDLVO (Extended DLVO) theory (van Oss, Chaudhury, & Good, 1988; van Oss, 2003). These theories have been applied in investigations of bacterial adhesion on surfaces in controlled environments, by taking into account the surface contact angle, roughness and surface charge, as well as the bacteria cell wall properties (Sinde & Carballo, 2000; Teixeira et al., 2005; Subramani & Hoek, 2008; Szlavik et al., 2012).

Therefore, the purpose of the present study was to investigate the attachment of *S. Enteritidis* and *L. monocytogenes* on AISI 304 and AISI 316 stainless steels, and also to evaluate the influence of the material topographies, hydrophobicity and free energy of interaction of the attachment process.

2. Materials and methods

2.1. Bacterial strains

In this study, two bacterial strains previously isolated by the Laboratory of Food Microbiology and Food Control of Federal University of Rio Grande do Sul (Porto Alegre, Brazil) were used: *S. Enteritidis* (SE86) isolated from cabbage involved in a Rio Grande do Sul (Southern Brazil) foodborne outbreak and *L. monocytogenes* (J11) isolated from a bovine slaughterhouse located in the same state.

S. Enteritidis was cultivated in Brain Infusion Broth – BHI (OXOID, Basingstoke, England) at 37 °C, for approximately 18 h and *L. monocytogenes* was grown in BHI supplemented with 0.6% yeast extract (OXOID, Basingstoke, England), incubated at 37 °C, for approximately 30 h. After cultivation, both microorganisms were diluted to about 10^5 CFU/ml using 0.1% peptone water (OXOID, Basingstoke, England) for artificial contamination of stainless steel surface-coupons.

2.2. Preparation of coupons

Stainless steel AISI 304 and AISI 316 mechanically brushed and polished, also known as number 4 finish were kindly provided by Metalúrgica Ralf Winter™ (Alvorada, Brazil). Prior to bacterial adhesion assays, stainless steel were cut into 2 cm × 2 cm and 0.2 cm coupons and then immersed for 1 min in a 3% (v/v) solution of neutral detergent (Kalykim, Porto Alegre, Brazil), rinsed with distilled water and immersed for 2 min in 70% (v/v) ethanol in order to remove grease. Then, the coupons were autoclaved at 121 °C for 15 min and dried at 60 °C, for 2 h (adapted from Rossoni & Gaylarde, 2000). The coupons were kept inside sterile Petri dishes and remained at room temperature (25 ± 1 °C) until testing.

2.3. Contamination of coupons and evaluation of bacterial adhesion

The coupons were immersed at room temperature (25 ± 1 °C) in 100 ml of BHI broth (Oxoid, Basingstoke, UK) containing 10^5 CFU/ml. Twelve coupons of each kind of stainless steel were immersed in the culture of each microorganism for 0, 1, 2, 4, 6, and 8 h (adapted from Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). Coupons were rinsed with 1 ml of sterile distilled water in order to remove weakly adhered cells. The coupons were subsequently immersed in 25 ml of 0.1% peptone water (Oxoid) and immediately treated inside a ultrasonic bath – sonicator (Unique Group, Indaiatuba, Brazil), aiming to remove adhered cells (Sinde & Carballo, 2000). Decimal dilutions of this solution containing the cells removed from each coupon were prepared, using 0.1% peptone water (Oxoid). Then, 20 μl of each dilution were plated on Tryptic Soy Agar (TSA, Oxoid) and on TSA added with 0.6% of yeast extract (Oxoid) to enumerate *S. Enteritidis*, and *L. monocytogenes,* respectively (Milles & Misra, 1938). TSA plates were incubated at 37 °C, for 18 h and 30 h for *S. Enteritidis* and *L. monocytogenes* cultivation, respectively. In parallel with each assay, decimal dilutions were prepared in order to determine the amount of cells in the suspensions used for immersion of the coupons. Experiments were performed in duplicate (i.e. two coupons per contact time) and each was repeated three times.

2.4. Scanning electron microscopy

The cells were fixed with 12% (v/v) glutaraldehyde (Merck, Darmstadt, Germany) for 7 days, and washed with 0.2 M phosphate buffer (pH 7.2 ± 0.2). Subsequently, the coupons were dehydrated with increasing concentrations of acetone (Labsynth, Diadema, Brazil), ranging from 30 to 100%, during 10–20 min. After drying at room temperature (25 ± 1 °C), the samples were subjected to critical point drying in liquid CO_2 and were coated with gold using a sputter coater (Balzers Union Ltd, Balzers, Liechtenstein). After coating, the samples were observed in a scanning electron microscope model 6060 (Jeol Ltd., Tokyo, Japan) at the Electron Microscopy Center of Federal University of Rio Grande do Sul (Porto Alegre, Brazil).

2.5. Hydrophobicity and free energy of adhesion

Bacterial hydrophobicity was measured by the contact angle method as described by Busscher et al. (1984). Bacteria suspensions (10^8 CFU/ml) were washed three times with phosphate buffered saline solution (PBS) and filtered using 0.45 mm pore diameter filters (Sartorius, Goettingen, Germany). Filters with bacteria were maintained for 1 h in Petri dishes containing 1% (w/v) agar–agar Type I (Himedia Laboratories, Mumbai, India) with 10% (v/v) glycerol (CAQ - Casa da Química, Diadema, Brazil) in order to establish constant moisture content. After that, filters were transferred to microscope glass slides. Five-microliter drops of two polar solvents (water and formamide, Vetec – Sigma Aldrich, Duque de Caxias, Brazil) and one nonpolar solvent (α-bromonapthalene, Vetec – Sigma Aldrich) were deposited on bacterial layer, using a goniometer (Krüss DSA30, Hamburg, Germany), according to the recommendations of van Oss (1995).

Hydrophobicity of the materials was estimated by the same technique, using the measurement of the contact angle formed by the three solvents with different polarities on the surface of