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Predicting adhesion and biofilm formation boundaries on stainless steel surfaces by five *Salmonella enterica* strains belonging to different serovars as a function of pH, temperature and NaCl concentration

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ABSTRACT

This study aimed to assess the capability of 97 epidemic *S. enterica* strains belonging to 18 serovars to form biofilm. Five strains characterized as strong biofilm-producers, belonging to distinct serovars (*S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177, *S.* Heidelberg 281 and *S.* Corvallis 297) were assayed for adhesion/biofilm formation on stainless steel surfaces. The experiments were conducted in different combinations of NaCl (0, 2, 4, 5, 6, 8 and 10% w/v), pH (4, 5, 6 and 7) and temperatures (8 °C, 12 °C, 20 °C and 35 °C). Only adhesion was assumed to occur when S. enterica counts were ≥3 and < 5 log CFU/cm^2, whereas biofilm formation was defined as when the counts were ≥5 log CFU/cm^2. The binary responses were used to develop models to predict the probability of adhesion/biofilm formation on stainless steel surfaces by five strains belonging to different *S. enterica* serovars. A total of 99% (96/97) of the tested *S. enterica* strains were characterized as biofilm-producers in the microtiter plate assays. The ability to form biofilm varied ($P < 0.05$) within and among the different serovars. Among the biofilm-producers, 21% (20/96), 45% (43/96), and 35% (34/96) were weak, moderate and strong biofilm-producers, respectively. The capability for adhesion/biofilm formation on stainless steel surfaces under the experimental conditions studied varied among the strains studied, and distinct secondary models were obtained to describe the behavior of the five *S. enterica* tested. All strains showed adhesion at pH 4 up to 4% of NaCl and at 20 °C and 35 °C. The probability of adhesion decreased when NaCl concentrations were > 8% and at 8 °C, as well as in pH values ≤ 5 and NaCl concentrations > 6%, for all tested strains. At pH 7 and 6, biofilm formation for *S.* Enteritidis, *S.* Infantis, *S.* Typhimurium, *S.* Heidelberg was observed up to 6% of NaCl at 35 °C and 20 °C. The predicted boundaries for adhesion were pH values < 5 and NaCl ≥ 4% and at temperatures < 20 °C. For biofilm formation, the predicted boundaries were pH values < 5 and NaCl concentrations ≥ 2% and at temperatures < 20 °C for all strains. The secondary models obtained describe the variability in boundaries of adhesion and biofilm formation on stainless steel by five strains belonging to different *S. enterica* serovars. The boundary models can be used to predict adhesion and biofilm formation ability on stainless steel by *S. enterica* as affected by pH, NaCl and temperature.

1. Introduction

Salmonella enterica is a foodborne pathogen of significant importance worldwide. *Salmonella* is prevalent in raw materials (Denis et al., 2016; Komitopoulou and Peñaloza, 2009; Maharjan et al., 2006; Wierup and Kristoffersen, 2014), but the environment of food processing has also been described as a source of this pathogen (Cossi et al., 2014; Finn et al., 2013; Trimble et al., 2013).

The ability of *S. enterica* to form biofilms is one of the main contributing factors for its survival and persistence in food processing

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environments (Spector and Kenyon, 2012; Steenackers et al., 2012). Besides, the ability of *S. enterica* to form biofilms increases the risks of salmonellosis outbreaks because the cells of this bacterium are released from biofilms resulting in recontamination of foods during processing (Ribaudo et al., 2017; Villa-Rojas et al., 2017; Wang et al., 2016).

S. enterica biofilms are communities of cells bound to each other and the surface of a self-produced matrix highly capable of resisting stress for extended periods of time (O'Leary et al., 2015). Biofilm-associated *S. enterica* cells exhibit increased resistance to traditional antimicrobial agents used to clean surfaces and utensils in food processing environments limiting their effectiveness (Lianou and Koutsoumanis, 2012). During the industrial food processing, the biofilm formation on surfaces may be favored by the availability of nutrients, pH, water activity (a_w), temperature and moisture on industrial equipment surfaces (Bridier et al., 2015). Particularly, stainless steel, one of the most common materials used in equipment surfaces and utensils in the food industry has been considered a suitable site of bacterial adhesion and biofilm formation (Rodrigues et al., 2017; Srey et al., 2013). The preceding stage in biofilm formation is bacterial adhesion. Initially, the cell adhesion is a reversible process because the bacterial cell comes to this position by its motility or Brownian motion. The continuous attachment of the bacterial cells to the surface followed by their growth and production of exopolysaccharides originate the biofilm (Srey et al., 2013; Steenackers et al., 2012).

It has been suggested that the cell adhesion with subsequent biofilm formation in *S. enterica* is a virulence factor related to specific characteristics of some serovars (Díez-García et al., 2012; Patel and Sharma, 2010). However, only a few studies have assessed the biofilm-forming ability of multiple *S. enterica* strains belonging to different serovars under distinct environmental conditions (Díez-García et al., 2012; Lianou and Koutsoumanis, 2012; Steenackers et al., 2012). Also, there is a lack of information about the ability of epidemic *S. enterica* strains to form biofilms. Thus, the development of predictive models accounting for the variability of *S. enterica* strains to adhere and form biofilms on stainless steel may provide insightful information to control the environmental prevalence of this pathogen, avoid recontamination and consequently to safeguard public health.

Considering these aspects, the first aim of this study was to assess the capability of biofilm formation by 97 strains of *S. enterica* belonging to 18 different serovars. Then, the *S. enterica* strains were classified from weak to strong according to their adhesion and biofilm formation abilities. Finally, models to predict the boundaries of adhesion and biofilm formation by *S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177, *S.* Heidelberg 281 and *S.* Corvallis 297 on stainless steel surfaces as a function of pH, temperature, and NaCl concentration were developed.

2. Materials and methods

2.1. S. enterica strains

Ninety-seven strains of *S. enterica* belonging to *S.* Enteritidis ($n = 48$), *S.* Infantis ($n = 9$), *S.* London ($n = 7$), *S.* Typhimurium ($n = 6$), *S.* Johannesburg ($n = 4$), *S.* Derby ($n = 6$), *S.* Heidelberg ($n = 2$), *S.* Ohio ($n = 1$), *S.* Anatum ($n = 2$), *S.* Newport ($n = 2$), *S.* Pomona ($n = 2$), *S.* Mvester ($n = 2$), *S.* Bredeney ($n = 1$), *S.* Mbandaka ($n = 1$), *S.* Schawarzengrund, ($n = 1$) *S.* Albany ($n = 1$), *S.* Agona ($n = 1$) and *S.* Corvallis ($n = 1$) serovars were evaluated in this study (Supplementary Table 1). The *S. enterica* strains were isolated from human patients or foods involved in salmonellosis outbreaks occurred in the South of Brazil (Reference Collections of epidemic *Salmonella* strains of Central Laboratory of the Parana State, Brazil; Adolfo Lutz Institute, São Paulo, Brazil and State University of Londrina, Paraná State, Brazil). Stock cultures of the *S. enterica* strains were maintained in cryovials at −80 °C. The inoculum of each strain was obtained after preparing suspensions in sterile saline solution (0.85% NaCl, w/v) from cultures grown in Tryptic Soy Broth (TSB; HiMedia, Mumbai, India) at 37 °C for 24 h (late exponential growth phase). Cells were harvested by centrifugation (4500 × *g*, 15 min, 4 °C) and washed twice with a sterile saline solution (NaCl, 0.85%) and re-suspended in sterile saline solution to obtain standard cell suspensions. Optical density of 0.1 at 630 nm (OD_{630}) corresponded to viable counts of 6 (± 0.2) log CFU/mL (Melo et al., 2017; Sant'Ana et al., 2012).

2.2. Assessment of biofilm formation

The ability of the ninety-seven *S. enterica* strains to form biofilms was evaluated using a microtiter plate method (MtP) as previously described by Lianou and Koutsoumanis (2012). Initially, a 20 µL-aliquot of the bacterial suspension (~6 log CFU/mL) of each *S. enterica* strain was inoculated in each one of the ninety-six wells of a microplate containing 180 µL of trypticase soy broth (TSB; Himedia, Mumbai, India). The microplates were covered with a lid and incubated aerobically under static conditions for 48 h at 35 °C. Then, the wells were emptied, and 200 µL of phosphate-buffered saline (PBS) was used to wash each well for three times. During each wash step, the microplates were agitated for 5 min. After fixation with 150 µL of methanol for 15 min, the microplates were dried at room temperature for 20 min. The wells were stained with crystal violet (0.5% w/v; Himedia, Mumbai, India) and incubated for 5 min. Then, the content of the wells was discarded and, and each well was washed three times with 200 µL of PBS. After three vigorous tapping on absorbent paper, the microplates were air dried at room temperature, the dye bound to the cells was eluted from attached cells with 200 µL of 99.8% ethanol for 30 min. In each plate, six control wells were included containing non-inoculated TSB (negative control). The optical density of each well was measured at 580 nm (OD_{580}) using FlexStation 3 plate reader furnished with SoftMax Pro software (Molecular Devices). The microplates were agitated for 10 s at average amplitude before the measurements. The measurements of the control negative (OD_{580C}) wells in each microplate was subtracted from the OD_{580nm} of each well in the same plate, and this difference (ΔOD580), was used for the characterization of the biofilm-forming ability of the tested strains (Lianou and Koutsoumanis, 2012). The interpretation criteria were as follow: $OD_{580} \leq OD_{580}c$ = no biofilm producer; $OD_{580}c < OD_{580} \leq (2 \times OD_{580}c)$ = weak biofilm producer; $(2 \times OD_{580}c) < OD_{580} \leq (4 \times OD_{580}c)$ = moderate biofilm producer and $(4 \times OD_{580}c) < OD_{580}$ = strong biofilm producer (Stepanović et al., 2000). Three (independent) experimental replicates were performed and six samples (i.e. wells) were analyzed in each replicate. The results were expressed as the average (± standard deviation) of 18 OD_{580} readings for each strain and submitted to ANOVA followed by Scott-Knott test, considering $P < 0.05$.

2.3. Development of models for prediction of boundaries of S. enterica adhesion/biofilm formation

2.3.1. S. enterica serovars, experimental conditions, and test surfaces

The strains *S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177, *S.* Heidelberg 281 and *S.* Corvallis 297, were selected for assays of adhesion and biofilm formation on stainless steel as these were characterized as strong biofilm-producers in Mtp assays. Besides, these strains belong to serovars frequently involved in salmonellosis outbreaks from 2012 to 2016 according to reports of Brazilian, European and USA International Surveillance Programs (Centers for Disease and Prevention, 2017; European Centre for Disease Prevention and Control, 2017; National Health Surveillance Agency, 2016). A total of 480 different combinations of pH values (4, 5, 6 and 7), NaCl concentration (0%, 2%, 4%, 6%, 8%, 10% w/v) and temperature (8 °C, 12 °C, 20 °C and 35 °C) comprising a broad range of conditions commonly found during food processing were assayed (96 distinct combinations each serovar). The above salt concentrations were attained by adding, when needed, to the medium (which has a NaCl concentration (w/v) of 0.5% as part of its basal composition) appropriate amounts of NaCl