ADHESION OF SALMONELLA ENTERITIDIS TO STAINLESS STEEL SURFACES

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ABSTRACT

Adhesion of microorganisms to food processing surfaces and the problems it causes are a matter of strong concern to the food industry. Contaminated food processing surfaces may act as potential sources of transmission of pathogens in food industry, catering and in the domestic environments. Several studies have shown that adhesion of bacteria to surfaces partly depends upon the nature of the inert surfaces and partly upon the bacterial surface properties. The aim of this study was to compare the adhesion of four different strains of Salmonella Enteritidis to stainless steel 304 (SS 304). The effect of surface hydrophobicity and surface elemental composition on the adhesion process was also analysed. Hydrophobicity was evaluated through contact angle measurements using the sessile drop method. All the strains studied showed positive values of the degree of hydrophobicity (ΔGlwl) and so can be considered hydrophilic while stainless steel revealed a hydrophobic character. Bacterial cell surface composition was measured using X-ray photoelectron spectroscopy (XPS). The XPS results corroborated the similarity of the values of the degree of hydrophobicity obtained by contact angles. The different Salmonella strains showed similar elemental composition and cell surface physico-chemical properties. Nevertheless, S. Enteritidis MUSC presented higher adhesion ability to SS 304 (p<0.05). It can be concluded that the physico-chemical properties of the strain does not explain the ability of adhesion to stainless steel. Other factors like the production of polysaccharides must be considered.

Key words: Adhesion, Salmonella Enteritidis, hydrophobicity

INTRODUCTION

Adhesion of microorganisms to food processing equipment surfaces is of great concern to the food industry. Adhered microorganisms to solid surfaces can have the potential to act as a chronic source of microbial contamination, which may compromise food quality and represent a significant health hazard (2). Several studies showed that cross-contamination can result from hands, sponges/clothes and utensils either in domestic kitchens or in any food processing plant (13,16,22,23). For instance, Salmonella spp. is able to colonize different inert food contact surfaces to form biofilms (3, 14, 18, 21). So, it has been recognized that a greater understanding of the interaction between microorganisms and food-processing surfaces is required to control these problems.

Salmonellosis has been one of the most commonly reported food-borne illnesses worldwide. In many countries, including Brazil, Salmonella Enteritidis is the most frequently isolated serotype. Epidemiological evidence has linked the majority of outbreaks in State of Paraná, Brazil, to contaminated poultry products.

Stainless steel has been the material of choice for working surfaces and kitchen sinks for many years due to its mechanical strength, corrosion resistance, longevity and ease of fabrication (17). In the food processing industry most of the surfaces are of stainless steel including, pipelines and tanks (1), machinery
and working surfaces (18,26,28). Moreover, it is relatively resistant to chemical attack by oxidizing and other sanitizing agents used in the food industry, like hypochlorite, peracetic acid and iodophors (5).

The mechanisms governing the adhesion of Salmonella spp. to inert surfaces are not completely understood; several studies have shown that adhesion of bacteria partly depends upon the nature of the inert surfaces and partly upon the bacterial surface properties (7,9,19). Hydrophobicity and surface charge are the most important surface properties in the adhesion process as demonstrated by innumerable studies (18,25,27,32,36).

The understanding of microbial adhesion is of major importance in preventing undesirable biofilm formation. Therefore, the aim of this study was to compare the ability of adhesion of four strains of Salmonella Enteritidis to stainless steel 304 (SS 304), in order to investigate the behavior of different strains of the same species. The effect of surface hydrophobicity and surface elemental composition in the adhesion process was also analysed.

**MATERIALS AND METHODS**

**Media and growth conditions**

The strains used in this study are presented in Table 1. All bacterial isolates were maintained in trypticase soy agar (TSA). Every strain was subcultured twice in trypticase soy broth (TSB) at 37°C in an orbital shaker (130 rpm), overnight. The cells were then harvested by centrifugation at 5000 g for 10 min and washed three times with phosphate buffered saline (PBS 0.1M pH 7). The pellets were resuspended in PBS to an inoculum level of 10^8 CFU/ml, determined by optical density.

**Material used as substratum**

The test surface was stainless steel (304, finish n° 4), commonly present in the food industry and used in domestic kitchens. The coupons were cut in 0.8 x 0.8 cm², washed in a solution of a commercial detergent (Sonasol Pril, Henkel Ibérica S.A., Portugal) in ultrapure water for 30 min and then thoroughly rinsed in ultrapure water (to remove any remaining detergent), followed by immersion in ethanol 90% for 30 min to completely degrease the surface and in sterile water.

**Hydrophobicity and surface free energy**

Hydrophobicity was evaluated through contact angle measurements and using the approach of van Oss and co-workers (37-39). In this approach, the degree of hydrophobicity of a given material (1) is expressed as the free energy of interaction between two entities of that material when immersed in water (w) - ΔG_{int}. If the interaction between the two entities is stronger than the interaction of each entity with water (ΔG_{int} < 0) the material is considered hydrophobic. Conversely, if ΔG_{int} > 0 the material is hydrophilic. ΔG_{int} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{int} = -2\left(\sqrt{\gamma_{LW}^{LW}} - \sqrt{\gamma_{LW}^{LW}}\right)^2 + 4$$

where γ_LW accounts for the Lifshitz-van der Waals component of the surface free energy and γ_L and γ_W are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ_{AB}), with γ_{AB} = 2γ_Lγ_W.

The surface tension components of a solid material are obtained by measuring the contact angles of three pure liquids (one apolar and two polar), with well known surface tension components, followed by the simultaneous resolution of three equations of the form:

$$\left(1 + \cos \theta\right)\gamma_{T}^{TOT} = 2\left(\sqrt{\gamma_{LW}^{LW}\gamma_{LW}^{LW}} + \sqrt{\gamma_{LW}^{LW}\gamma_{LW}^{LW}} + \sqrt{\gamma_{LW}^{LW}\gamma_{LW}^{LW}}\right)$$

where θ is the contact angle and γ^{TOT} = γ_L^{AB} + γ_W^{AB}.

Contact angle measurements (at least 25 determinations with each liquid on stainless steel and on each microbial strain) were performed automatically with the aid of an image analysis system (G2/G40) installed in a standard contact angle apparatus (Kruss-GmbH). The images were transmitted by a video camera to a personal computer for evaluation. All the measurements were performed at room temperature. In the case of bacterial cells, the measurements were performed on a cell lawn using the sessile drop method described by Busscher et al. (6). Briefly, bacteria were deposited on a 0.45 µm cellulose acetate membrane filter by filtration of the suspension using negative pressure. To standardize the moisture content, the filters were then transferred onto Petri dishes containing 1% (w/v) agar with

<table>
<thead>
<tr>
<th>Strains</th>
<th>Source</th>
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<tbody>
<tr>
<td>Salmonella Enteritidis EMB¹</td>
<td>Water from poultry</td>
</tr>
<tr>
<td>Salmonella Enteritidis MUSC¹</td>
<td>Breast meat of poultry</td>
</tr>
<tr>
<td>Salmonella Enteritidis AL²</td>
<td>Food sample related to</td>
</tr>
<tr>
<td>Salmonella Enteritidis PC²</td>
<td>Fecal human sample</td>
</tr>
</tbody>
</table>

The bacterial isolates were obtained from:

¹Food Microbiology Lab., Depart. of Food and Dry Technology, University of Londrina, Pr, Brazil; ²LACEN (Central Paraná Public Health Laboratory Service).
10% (v/v) glycerol. Measurements of advancing water contact angles were carried out at 25°C and three liquids with different polarities were used, water (W), formamide (F) and α-bromonaphtalene (α-B). Their surface tension components were obtained from literature (20).

Hydrophobicity of the stainless steel was estimated by the same technique, with direct measurements of contact angles on stainless steel surface, after degreasing and cleaning.

X-ray photoelectron spectroscopy
Bacterial cell surface composition was measured using X-ray photoelectron spectroscopy (XPS). The bacterial cells were grown in 200 ml TSB at 37°C under 120 rpm for 18 h and washed three times in deionized water by centrifugation (10 min at 5000 g and 4°C). A volume of 200 ml of a cellular suspension (10^6 cells/ml) was vacuum filtered through an acetate cellulose membrane of 45 µm. The membrane, completely covered with cells, was immediately frozen with liquid nitrogen and then stored at -80°C until the subsequent step of lyophilization. Freeze drying was performed at 10 Pa, overnight. The samples were placed in a desiccator, at room temperature and immediately analyzed by XPS. The XPS analysis was performed using an apparatus ESCALAB 200A, with a VG5250 software and data analysis. The spectrometer used monochromatized Mg Kα X-ray radiation (13.000 eV). The constant pass energy of the analyzer was 20 eV and it was calibrated with reference to Ag 3d_{5/2} (368.27 eV). The pressure during analysis was under 1x10^-6 Pa. The spectra were recorded following the sequence C 1s, O 1s, N 1s, P 2p. The elemental composition was defined as the ratio between oxygen and carbon (O/C), nitrogen and carbon (N/C) or phosphorous and carbon (P/C).

Adhesion assays
The coupons of stainless steel were immersed in 2 ml of each bacterial suspension containing 10^6 CFU/ml. After 1 h at 25°C with constant shaking at 100 rpm, the coupons were rinsed twice with PBS to remove poorly adhered bacteria. An aliquot of 20 µl/ml of a 4',6-diamidino-2-phenylindole (DAPI) solution was added to each coupon containing the plates and incubated for 30 min in the dark. After this time, the coupons were rinsed with sterile distilled water and the adherent microorganisms were quantified by automatic enumeration using epifluorescence microscopy. Thirty fields per coupon were scanned and the fluorescent cells were enumerated. Computerized image analysis software (Image-Pro Plus, Media Cybernetics) was used for the quantitative estimation of the adherent cells. All experiments were done in triplicate.

Statistical analysis
The resulting data were analysed using SPSS software (Statistical Package for the Social Sciences). One-way ANOVA with Bonferroni test was used to compare the number of adhered cells. All tests were performed with a confidence level of 95%.

RESULTS AND DISCUSSION

The contact angles formed by the three liquids (water, formamide, and α-bromonaphtalene) on stainless steel and on bacterial lawns are present in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>θ&lt;sub&gt;W&lt;/sub&gt; (°) (±SD)</th>
<th>θ&lt;sub&gt;f&lt;/sub&gt; (°) (±SD)</th>
<th>θ&lt;sub&gt;α-B&lt;/sub&gt; (°) (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel</td>
<td>81.2 (±0.9)</td>
<td>60.0 (±1.1)</td>
<td>23.4 (±0.5)</td>
</tr>
<tr>
<td>S. Enteritidis EMB</td>
<td>10.8 (±2.2)</td>
<td>15.6 (±1.8)</td>
<td>26.1 (±4.2)</td>
</tr>
<tr>
<td>S. Enteritidis MUSC</td>
<td>13.5 (±1.6)</td>
<td>15.9 (±2.3)</td>
<td>27.6 (±1.7)</td>
</tr>
<tr>
<td>S. Enteritidis PC</td>
<td>14.0 (±4.4)</td>
<td>17.0 (±3.2)</td>
<td>31.7 (±2.8)</td>
</tr>
<tr>
<td>S. Enteritidis AL</td>
<td>9.7 (±1.9)</td>
<td>14.8 (±2.6)</td>
<td>27.2 (±2.5)</td>
</tr>
</tbody>
</table>

SD - standard deviation.

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<thead>
<tr>
<th></th>
<th>γ&lt;sup&gt;W&lt;/sup&gt; (mJ/m²)</th>
<th>γ&lt;sup&gt;f&lt;/sup&gt; (mJ/m²)</th>
<th>γ&lt;sup&gt;α-B&lt;/sup&gt; (mJ/m²)</th>
<th>ΔG&lt;sub&gt;mol&lt;/sub&gt;</th>
<th>lipid</th>
<th>protein</th>
<th>carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel</td>
<td>40.81</td>
<td>0.00</td>
<td>5.84</td>
<td>-59.80</td>
<td>34.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Enteritidis EMB</td>
<td>39.89</td>
<td>0.97</td>
<td>55.99</td>
<td>32.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Enteritidis MUSC</td>
<td>39.49</td>
<td>1.07</td>
<td>54.41</td>
<td>32.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Enteritidis PC</td>
<td>38.06</td>
<td>1.22</td>
<td>54.48</td>
<td>33.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Enteritidis AL</td>
<td>39.50</td>
<td>1.05</td>
<td>55.84</td>
<td>33.79</td>
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</table>
The $\Delta G_{\text{int}}$ values obtained were very similar for all the strains tested being all strains hydrophilic ($\Delta G_{\text{int}} > 0$). From Table 3, it can be observed that all cell surfaces were predominantly electron donors (higher values of $\gamma$), with low electron acceptor parameters ($\gamma^+$).

Considering the values of water contact angle (81.2º) and $\Delta G_{\text{int}} = -59.8 \text{ mJ/m}^2$, the stainless steel assayed was hydrophobic, which is in accordance with several authors (12,30,33). A point to be noted is that stainless steel does not have an electron acceptor parameter but is only electron-donor ($\gamma^{-}$).

The chemical composition of microbial cells surface obtained by XPS spectra is usually expressed in terms of N/C, O/C and P/C ratios (35). The corresponding values for the microorganisms assayed are presented in Table 4. All strains used in this study exhibited high O/C values, ranging from 0.465 to 0.584, and low P/C values, ranging from 0.008 to 0.0137.

Microbial surface thermodynamics is a reflection of the physico-chemistry of bacterial surfaces, which is controlled by macromolecular components, e.g., lipo-polysaccharides, proteins and exopolymers, varying in quantity with growth conditions and from strain to strain. The amount of the macromolecular components can be represented by a variety of different functional groups (31,34). In previous works, cell surface hydrophobicity, assessed by water contact angle, was directly correlated with the concentration of nitrogen or carbon involved in hydrocarbon form and inversely correlated with the oxygen concentration (4,11,29). In this study, the water contact angle was directly correlated with the N/C ratio whereas hydrophobicity expressed as $\Delta G_{\text{int}}$, was inversely correlated with the oxygen concentration. The XPS results corroborated the similarity of the hydrophobicity values. Cerca et al. (8) correlated the N/C ratio of S. epidermidis strains with cell surface hydrophobicity, with the less hydrophobic cells exhibiting the lower N/C ratio. The presence of proteinic appendages is often reflected in a high nitrogen concentration at the cell surface (29).

The number of cells of different strains of Salmonella Enteritidis adhered to stainless steel are presented in Fig. 1. The extent of adhesion of Salmonella serovars can be considered a factor of virulence.

The different extent of adhesion of four Salmonella Enteritidis strains to stainless steel 304 could not be explained in terms of cell surface physico-chemical properties. Other factors might be governing the process of adhesion, namely the production of exopolysaccharides is worthy to be investigated.

This study proves that adhesion is strongly strain dependent and in this sense the adhesion ability of Salmonella serovars can be considered a factor of virulence.

**CONCLUSIONS**

**ACKNOWLEDGEMENTS**

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RESUMO
Adesão de Salmonella enteritidis a superfícies de aço inoxidável

A adesão de microrganismos a superfícies de processamento de alimentos e os problemas que daí resultam são matéria de grande preocupação para a indústria alimentar. Superfícies de processamento de alimentos contaminadas podem actuar como uma potencial fonte de transmissão de patogénicos na indústria alimentar, restauração e em ambientes domésticos. Diversos estudos têm demonstrado que a adesão de bactérias a superfícies depende, por um lado, da natureza das superfícies inertes e, por outro, das propriedades superficiais das bactérias. O objectivo deste trabalho consistiu na comparação da capacidade de adesão de 4 cepas diferentes de Salmonella Enteritidis ao aço inoxidável 304 (SS 304). Analisou-se também o efeito da hidrofobicidade e da composição elementar no processo de adesão. A hidrofobicidade foi determinada através da medição de ângulos de contacto usando o método da gota séssil. Todas as cepas apresentaram valores positivos do grau de hidrofobicidade (ΔG_{s,a}) podendo, assim, ser consideradas hidrofílicas enquanto o aço inoxidável revelou um carácter hidrofílico. A composição elementar da superfície das células bacterianas foi medida através de espectroscopia de fotoelectrões X (XPS). Os resultados do XPS corroboraram a similaridade de valores do grau de hidrofobicidade obtidos por ângulos de contacto. As diferentes cepas de Salmonella apresentaram uma composição elementar e propriedades físico-químicas semelhantes. No entanto, a Salmonella MUSC apresentou uma capacidade de adesão ao aço inoxidável mais elevada (p<0.05). Pode então concluir-se que as propriedades físico-químicas das cepas não explicam a capacidade de adesão ao aço inoxidável, devendo ser considerados outros factores tais como a produção de exopolissacáridos.

Palavras-chave: Adesão, Salmonella Enteritidis, hidrofobicidade

REFERENCES
Adhesion of *Salmonella* to surfaces