Adhesion of *Salmonella* Enteritidis and *Listeria monocytogenes* on stainless steel welds

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**Abstract**

Pathogenic microorganisms are able to adhere on equipment surfaces, being possible to contaminate food during processing. *Salmonella* spp. and *Listeria monocytogenes* are important pathogens that can be transmitted by food, causing severe foodborne diseases. Most surfaces of food processing industry are made of stainless steel joined by welds. However currently, there are few studies evaluating the influence of welds in the microorganism's adhesion. Therefore the purpose of the present study was to investigate the adhesion of *Salmonella* Enteritidis and *L. monocytogenes* on surface of metal inert gas (MIG), and tungsten inert gas (TIG) welding, as well as to evaluate the cell and surface hydrophobicities. Results demonstrated that both bacteria adhered to the surface of welds and stainless steel at same levels. Despite this, bacteria and surfaces demonstrated different levels of hydrophobicity/hydrophilicity, results indicated that there was no correlation between adhesion to welds and stainless steel and the hydrophobicity.

1. Introduction

The adhesion of microorganisms to equipment surfaces has the potential to transmit pathogens to food, and this is a serious concern to food processing industry (Giaouris et al., 2005). The surfaces of equipment used in food processing are recognized as one of the major sources of microbial contamination (Chmielewski and Frank, 2003; Wong, 1998). *Salmonella* spp. and *Listeria monocytogenes* are important pathogenic bacteria, which can be transmitted by food. Numerous studies have shown that these bacteria are capable of adhering and form biofilms on metal, glass, polyethylene, and rubber surfaces (Bong Jae et al., 2009; Chia et al., 2009; Malheiro et al., 2010; Morita et al., 2011; Tondo et al., 2010). Most surfaces of the food processing industry, such as machinery, pipelines, and working surfaces are made of stainless steel. This material is traditionally selected for food equipment because it is durable, resistant to corrosion, and it is easily cleaned (Holah and Thorpe 1990; Shi and Zhu, 2009). Due to their diverse projects and designs, food equipment frequently present corners and sharp angles, and the stainless steel plates are often joined by welds. Gas tungsten arc welding (GTAW), also called tungsten inert gas (TIG), and gas metal arc welding (GMAW), sometimes referred as metal inert gas (MIG), are the conventional welding techniques used for food equipment fabrication and maintenance (Lewan, 2014; Tide et al., 1999).

TIG welding is a process in which the joining of metal parts is produced by heating and melting of a non-consumable tungsten electrode arc, and the parts to be joined (EHEDG, 1993). The cost of the inert gas makes TIG welding more expensive than other stainless steel weldments, but it provides welds with very high quality and surface finish. In MIG welding process electric arc is struck between a continuously fed wire and the metallic surface (EHEDG, 1993). The advantages of this process are: 1) easy operation, 2) high productivity, 3) the possibility of automation, 4) low cost, and 5) no slag formation, resulting in a weld bead relatively well finished (EHEDG, 1993). However, there are several limitations in performing MIG welding, such as the need of a complex regulation of the weld equipment; it cannot be performed in the presence of flowing air, limited welding position, and allowance of extensive porosity in the weld bead (EHEDG, 1993). Also it produces frequent spils and the welds are difficult to maintain (O’Brien, 1991; Rao et al., 2011). Welding process commonly provides greater roughness on stainless steel surfaces welded. Several studies have reported correlation between surface roughness and bacterial accumulation on surfaces (Whitehead et al., 2009; Wirtanen and Mattila-Sandholm, 1992). However, other studies have reported a lack of correlation between surface irregularities or roughness and the number of bacteria attached (Silva et al., 2008; Szlavik et al., 2012). In order to reduce the roughness caused...
by welding, stainless surfaces need to be ground and polished, achieving compliance with industry surface roughness standards.

Beyond the surface roughness, other factors may influence microorganism adherence to surfaces, such as surface properties (electric charge and hydrophobicity), the microorganism characteristics (hydrophobicity, electric charge, production of extracellular substances), and environmental factors (pH, temperature, nutrients, etc.) (Araújo et al., 2009; Hood and Zottola, 1997; Sinde and Carballo, 2000). Currently, there are few studies evaluating the adherence of microorganisms on welds, and many doubts still exist concerning which is the most suitable weld to be used in food equipment. Therefore, the purpose of the present study was to investigate adhesion of S. Enteritidis and L. monocytogenes on the surface of MIG and TIG welds and also to evaluate the influence of both cells and weld surface hydrophobicity in the bacterial attachment.

2. Materials and methods

2.1. Bacterial strains

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

Before each experiment, S. Enteritidis was cultivated in Brain Infusion Broth – BHI (OXOID, Basingstoke, England) at 37 °C for approximately 18 h and the L. monocytogenes was grown in BHI with 0.6% yeast extract (OXOID, Basingstoke, England), incubated at 37 °C for approximately 30 h, both were diluted to 10^3 CFU/ml using 0.1% peptone water (OXOID, Basingstoke, England) for artificial contamination of stainless steel coupons.

2.2. Preparation of coupons

Coupons of stainless steel type 304 (2 cm × 2 cm and 0.2 cm) joined with MIG or TIG welds were kindly provided by Sulmaq Equipamentos™ (Sulmaq, Guaporé, Brazil). Welds were carried out on the transversal central area of each coupon (Fig. 1), and 12 coupons joined by MIG weld (6 polished and 6 unpolished) and 12 coupons joined by TIG weld (6 polished and 6 unpolished) were used. Coupons (n = 6) without any weld were prepared in order to be used as control. The technical characteristics of coupons are described in Table 1. Roughness (Ra) was determined by the weld supplier using roughness tester.

2.3. Contamination of coupons and evaluation of bacterial adhesion

The coupons were immersed in 100 ml of BHI broth (Oxoid, Basingstoke, UK) containing each bacterial culture at a concentration of 10^8 CFU/ml, kept at room temperature (25 ± 1 °C). Six coupons of each kind of weld were immersed in the culture of each microorganism during the following times: 0, 1, and 4 h (adapted from Kusumaningrum et al., 2003). After that, coupons were rinsed with 1 ml of sterile distilled water to remove the weakly adhered cells. The coupons were subsequently immersed in 25 ml of 0.1% peptone water (Oxoid) and immediately treated in ultrasonic bath – sonicator (Unique Group, Indaiatuba, Brazil), in order to remove adhered cells (Sinde and Carballo, 2000). The survival of adhered cells to the ultrasonic treatment was tested before the experiments. To do that, the number of bacterial cells was counted (around 10^8 CFU/ml) before and after ultrasonic treatment and no significant (P > 0.05) reduction was verified. Two decimal dilutions of this solution containing the cells of each treated coupon were prepared (10^-1 and 10^-2). Then 20 μl of each dilution was plated on Triptic Soy Agar (TSA, Oxoid) and on TSA added with 0.6% yeast extract (Oxoid). Plates of TSA were used to count S. Enteritidis, while TSA containing 0.6% yeast extract was used to enumerate L. monocytogenes. Enumerations were carried out by the drop method (Milles and Misra, 1938). TSA plates were incubated at 37 °C for 18 h and 30 h for S. Enteritidis and L. monocytogenes, respectively. Stainless steel coupons without welding (control) were submitted to the same procedure described above.

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. After, 20 μl of these dilutions was plated on TSA and TSA with 0.6% yeast extract (to S. Enteritidis and L. monocytogenes, respectively) by the drop method (Milles and Misra, 1938) and were incubated at 37 °C for 18 h and 30 h for S. Enteritidis and L. monocytogenes, respectively, and then colonies were enumerated.

The experiments were performed in duplicate and each experiment was repeated three times.

2.4. Scanning electron microscopy

The coupons with adhered 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). After, the samples were dehydrated with increasing concentrations of acetone (Labsynth, Diadema, Brazil) in 30 to 100% with a range of 10 to 20 min. After drying at room temperature (25 ± 1 °C), the samples were subjected to critical point drying in liquid CO2 in the equipment Balzers (Balzers Union Ltd, Balzers, Liechtenstein) then, the samples were coated with gold in Balzers equipment (Balzers) (Tondo et al., 2010). After coating, the samples were observed in a scanning electron microscope Jeol 6060 (Jeol Ltd., Tokyo, Japan) at the Electron Microscopy Center of Federal University of Rio Grande do Sul (Porto Alegre, Brazil).

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Materials used in the assays and their characteristics.</td>
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<tr>
<td>Welding type/sample identification</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>MIG 07</td>
</tr>
<tr>
<td>MIG 13</td>
</tr>
<tr>
<td>TIG 21</td>
</tr>
<tr>
<td>TIG 31</td>
</tr>
</tbody>
</table>

⁣* Roughness (Ra) was determined by the weld supplier using roughness tester.
2.5. Evaluation of material hydrophobicity

Contact angle measurements were carried out in order to determine the surface hydrophobicity as described by Van Oss (1995). The surface hydrophobicity is determined as the free energy of interaction between molecules (i) immersed in water (w) and can be expressed as \( \Delta G_{iwi} \)

\[
\Delta G = -2 \gamma_{il}L
\]

where \( \gamma_{il} \) is the interfacial tension between the interface of the surface and water, and is given by the following equation:

\[
\gamma_{il} = \left( \sqrt{\gamma_{lw} - \gamma_{iwl}^2} \right)^2 + 2 \left( \sqrt{\gamma_{iw} \gamma_{il} - \gamma_{iwl} \gamma_{iwl}} \right) \sqrt{\gamma_{iw} \gamma_{il} + \gamma_{iwl} \gamma_{iwl} - \gamma_{lw} \gamma_{il}}.
\]

To calculate the interfacial tension, contact angle measurements were made using two polar solvents: water and formamide (Vetec – Sigma Aldrich, Duque de Caxias, Brazil) and one nonpolar solvent: \( \alpha \)-bromonaphthalene (Vetec – Sigma Aldrich, Duque de Caxias, Brazil), according to the recommendations of van Oss (1995), using a goniometer (Krus DSA30, Hamburg, Germany). The contact angle measurements were used to determine the Lifshitz-van der Waal compound of the surfaces \( \gamma_{st} \), the electron acceptor parameter of the polar surface tension component \( \gamma_{sL} \) and the electron donor parameter of the polar surface tension component \( \gamma_{sL} \).

\[
1 + \cos \theta = \frac{2(\sqrt{\gamma_{il} \gamma_{lw}} + \sqrt{\gamma_{iw} \gamma_{il}} + \sqrt{\gamma_{lw} \gamma_{il}})}{\sqrt{\gamma_{lw} - \gamma_{iwl}^2}}
\]

The surface tension parameters for the apolar and polar liquids used in the calculations are given in Table 2. The interfacial tension was calculated using the surface tension parameters for the surface and the polar and apolar solvents using the following equation:

\[
\gamma_{il} = \gamma_{iwl}^L + \gamma_{AB}^L
\]

where \( \gamma_{sL} \) is the apolar Lifshitz-van der Waal compound of the surface and the liquid and \( \gamma_{sL} \) is the Lewis acid base compound of the surface and the liquid. The Lifshitz-van der Waal compound and the Lewis acid base compound were calculated using the surface tension parameters calculated by Eq. (3) and are given in Table 2.

The surface hydrophobicity was calculated based on four experimental measurements giving a total of eight contact angle estimates (left and right angle of the droplet) and presented as the mean ± standard deviation for each measured value.

2.6. Evaluation of cell hydrophobicity

To measure the contact angles of the cell surface the measurements on a layer of vegetative cells using the drops method described by Busscher et al. (1984) were performed. First, cells were cultivated in BHI (Oxoid) broth and BHI broth with 0.6% yeast extract (Oxoid) to cells of S. Enteritidis and L. monocytogenes, respectively, in order to obtain a suspension of 30 ml of each culture with approximately \( 10^8 \) CFU/ml. Subsequently, the suspension was centrifuged at 12,000 g for 10 min and then washed three times in phosphate buffered saline (PBS). Cells in pellet were resuspended in the same buffer being deposited on a filter membrane of cellulose acetate with pore size of 0.45 μm (Sartorius, Goettingen, Germany) by filtration using negative pressure.

The membranes were placed in a Petri dish containing agar–agar Type I (1% v/v) (Himedia Laboratories, Mumbai, India) and glycerol (10% v/v) (CAQ — Casa da Química, Diadema, Brazil), and were kept to dry for 1 h at room temperature (25 ± 1 °C). Immediately, membranes were cut into three strips of 1 cm and then 5.0 μl drops of each solution (water, formamide and \( \alpha \)-bromonaphthalene) and placed on the bacteria layer (Araújo et al., 2009; Busscher et al., 1984). Measures of four drops on each surface were performed using a goniometer (Krus DSA30, Hamburg, Germany) and the average of these measurements was considered as a result. The calculation of the interfacial tension was performed as described above, for evaluation of material hydrophobicity.

2.7. Statistical analysis

For statistical evaluation of the data obtained in adhesion tests (CFU/ml and CFU/cm² to cells in suspension and adhered cells, respectively), values were converted to \( \log_{10} \) colony-forming units. In all cases duplicate samples were used and the experiment was repeated at least three times. Analysis of variance was performed using the GLM (general linear model), with the SPSS software version 18.0. The Tukey’s test was used to compare the averages and a \( P < 0.05 \) was considered statistically significant.

3. Results

The adhesion of Salmonella Enteritidis on MIG and TIG welds is illustrated in Fig. 2. It can be observed that adhesion occurred in both surfaces and the cells tend to form clusters.

The results presented in Fig. 3 demonstrate that there are no significant differences between the two types of welding (MIG and TIG) for adhesion of S. Enteritidis (SE86) and L. monocytogenes (J11). Also, no significant differences between the polished and unpolished welds were observed for S. Enteritidis (SE86) and L. monocytogenes (J11) adhesion. In addition, no significant differences were observed in adhesion of S. Enteritidis (SE86) and L. monocytogenes (J11) on the welded surfaces (MIG and TIG) and not welded surfaces used as controls (stainless steel without welds). Results also revealed that S. Enteritidis (SE86) initially adhered (time 0) significantly more than L. monocytogenes (J11) on all materials, and there is significant difference (\( P < 0.05 \)) between contact times for all welds.

The hydrophobicity (\( \Delta G_{iwi} \)) of the investigated surfaces was determined based on contact angle measurements (Table 3). Surfaces are considered hydrophobic when \( \Delta G_{iwi} < 0 \), and hydrophilic when \( \Delta G_{iwi} > 0 \) (van Oss, 1995). Further, when the water contact angle is \( < 50° \) the surface is considered hydrophilic, whereas the surface is considered hydrophobic when the angle is \( > 50° \) (van Oss and Giese, 1995). According to the data presented in Table 3, only the bacteria had a water contact angle \( < 50° \) and a positive \( \Delta G_{iwi} \) value and thus present hydrophilic characteristics. Values obtained for bacteria were significantly different from the weld surfaces (\( P < 0.05 \)), which are all hydrophobic.

4. Discussion

The results of this work show that Salmonella Enteritidis is able to adhere to stainless steel surface and welds at a rate of about \( 3 \log_{10} \) CFU/cm², considering an initial artificial contamination of 5 \( \log_{10} \). These results are in agreement with findings by Bae et al. (2012) and Chia et al. (2009), who had demonstrated the same adhesion rate of Salmonella Typhimurium and L. monocytogenes to stainless steel.

L. monocytogenes J11 also adhered to stainless steel and welds, but at initial rates significantly lower than S. Enteritidis SE86. Similar results

Table 2

<table>
<thead>
<tr>
<th>Liquid</th>
<th>( \gamma_{il} )</th>
<th>( \gamma_{iwl}^L )</th>
<th>( \gamma_{il}^L )</th>
<th>( \gamma_{il}^C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>72.8</td>
<td>21.8</td>
<td>25.5</td>
<td>25.5</td>
</tr>
<tr>
<td>Formamide</td>
<td>58</td>
<td>39</td>
<td>2.28</td>
<td>39.6</td>
</tr>
<tr>
<td>( \alpha )-Bromonaphthalene</td>
<td>44.4</td>
<td>44.4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
were found by Stepanovic et al. (2004), evaluating biofilm formation by *L. monocytogenes* and *Salmonella* spp. on plastic surfaces. The number of attached *L. monocytogenes* ranged between $10^2$ and $10^3$ CFU/cm². Same levels of adherence of *L. monocytogenes* to glass surfaces were observed by Chae and Schraft (2000).

Results of the present study also revealed that there is significant difference ($P < 0.05$) between adhesion on different contact times for all welds, and that *S. Enteritidis* SE86 demonstrated the largest numbers of adhered cells after 1 h of contact with the surfaces. *L. monocytogenes* had demonstrated the same levels of adherence only after 4 h. The higher adhesion levels showed by *Salmonella* spp. than that by *L. monocytogenes* could be explained by the superiority of Gram-negative bacteria to form biofilm on inert surfaces (Pompermayer and Gaylarde, 2000; Sommer et al., 1999).

Unlike expectations, results shown in this work demonstrated that there were no significant differences in adhesion levels between the two types of welding (MIG and TIG) for *S. Enteritidis* (SE86) and *L. monocytogenes* (J11). These results were observed despite the very distinctive difference in appearance of MIG and TIG welds. TIG welds present a surface with a distinct undercut, maintaining an acceptable surface roughness, whereas MIG welds had a surface lifted and very poor surface finish. Moreover, polished welds exhibited surfaces with smooth profile and unpolished welds had irregular surfaces.

The MIG and TIG welds also exhibit significant differences in the values of roughness (Table 1) between the polished and unpolished surfaces, and both showed mean surface roughness values higher than 1.0 maximum Ra value defined by New Zealand’s Code of Practice for Dairy Food Manufacturing (New Zealand Dairy Industry Manual, 1995) and 0.8 Ra maximum values defined by the Dairy German Standard – DIN 11480 (DIN, 1978).

Our results showed no relation between bacteria adhesion and the Ra values. Flint (1996) also found no correlation between surface roughness, the presence or absence of welds, and the attachment of a heat-resistant *Streptococcus* responsible for spoilage problems in dairy manufacturing plants. The lack of correlation between surface roughness and adhesion of *Listeria monocytogenes* in different weld surfaces was also described by Tide et al. (1999).

In one of the few studies concerning bacterial colonization on welded material, Walsh et al. (1993) have presented evidence that initial bacterial attachment was random, but that colonies formed more frequently and developed more rapidly at the attached zones than on parent metal surfaces. Based on these independent observations, welds have been proposed to be sites for preferential bacterial accumulation and entrapment (Notermans et al., 1991). However, the present study demonstrated no significant differences in adherence levels between the welded surfaces (MIG and TIG) and not welded.
surfaces (stainless steel without welds) to S. Enteritidis (SE86) and L. monocytogenes (J11). Similar results were observed by Tide et al. (1999), when assessing the adhesion of L. monocytogenes to welded and not welded surfaces and observed no significant differences between the density of bacterial cells associated with the weld and adjacent parent metal surface. The same authors have also demonstrated that L. monocytogenes and the 3-species consortium composed by bacteria recovered from stainless steel weldments in a seafood processing plant, colonized the unpolished surface of different welds and the parent metal (stainless steel without weld), even when these welds had scratches and pits (Tide et al., 1999). Similar behavior was observed in our study through the images of scanning electron microscopy (Fig. 2), where it was observed that the bacteria adhered similarly in all areas of the coupons.

When the cell and material surface hydrophobicities were investigated, we observed that the bacteria were hydrophilic and all surfaces were hydrophobic. Despite the different levels of hydrophobicity/hydrophilicity there was no relation between adhesion and the surface hydrophobicity. This is in agreement with the results of Mafu et al. (1990) and Silva et al. (2008), carried out under static conditions, and also with Szlak et al. (2012), which were accomplished under liquid flow conditions.

4.1. Conclusions

Our results demonstrated that both bacteria adhered to the surface of welds and stainless steel at same levels and demonstrated similar patterns during 4 h of contact. Further, the type of welding, its polishing and roughness did not influence on the bacterial adhesion. The hydrophobicity of cells and materials did not demonstrate a positive relation with the bacterial adherence, indicating that the adhesion process is likely to involve other physicochemical and/or biological factors not investigated by the present study.

Acknowledgments

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References


Table 3

Water contact angle (in degrees) of the cells and surfaces with their respective hydrophobicity parameters ($\Delta G_m$).

<table>
<thead>
<tr>
<th>Surface</th>
<th>Water contact angle</th>
<th>$\gamma_i^{LV}$</th>
<th>$\gamma_s$</th>
<th>$\gamma_i$</th>
<th>$\Delta G_m$ (mJ·m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. monocytogenes</td>
<td>27.8 ± 2</td>
<td>42.12 ± 1.6</td>
<td>0.44 ± 0.2</td>
<td>47.80 ± 3.6</td>
<td>26.04 ± 3.2</td>
</tr>
<tr>
<td>S. Enteritidis</td>
<td>23.4 ± 3</td>
<td>41.97 ± 2.0</td>
<td>0.71 ± 0.1</td>
<td>48.78 ± 3.8</td>
<td>25.98 ± 3.1</td>
</tr>
<tr>
<td>MIG weld unpolished</td>
<td>71.5 ± 3</td>
<td>39.04 ± 0.5</td>
<td>0.08 ± 0</td>
<td>11.67 ± 2.2</td>
<td>−36.03 ± 4.2</td>
</tr>
<tr>
<td>MIG weld polished</td>
<td>65.4 ± 5</td>
<td>41.44 ± 0.7</td>
<td>1.48 ± 0.5</td>
<td>9.22 ± 2.1</td>
<td>−37.09 ± 3.9</td>
</tr>
<tr>
<td>TIG weld unpolished</td>
<td>49.9 ± 6</td>
<td>42.27 ± 1.3</td>
<td>1.02 ± 0.3</td>
<td>23.29 ± 3.1</td>
<td>−10.31 ± 1.0</td>
</tr>
<tr>
<td>TIG weld polished</td>
<td>75.6 ± 3</td>
<td>40.27 ± 1.1</td>
<td>0.31 ± 0.1</td>
<td>6.60 ± 1.0</td>
<td>−50.12 ± 7.8</td>
</tr>
</tbody>
</table>

Fig. 3. Salmonella Enteritidis and Listeria monocytogenes adhesion to stainless steel without welding (■) and welds: unpolished MIG welds (▲), polished MIG welds (Δ), unpolished TIG welds (●) and polished TIG welds (○) after 0, 1 and 4 h of contact. Each point represents the mean ± SD of three independent experiments. a, b compared values were significantly different (P < 0.05).


