Bacterial Adherence to Different Inert Surfaces Evaluated by Epifluorescence Microscopy and Plate Count Method

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ABSTRACT

The adherence of Listeria innocua L6a and Staphylococcus aureus ATCC6538 was evaluated on stainless steel (SS), polycarbonate (PC) and polypropylene (PP) chips. The EP results showed a significant difference (p<0.05) among the microorganism species interactions, the surfaces and the contact time. The number of adhered cells on the chip surfaces increased as contact time increased and the number of L. innocua or S. aureus on the surface of SS, PC and PP chips reached 1.0x10⁵ CFU/cm² after 12 h of contact, in both methods. The CP method showed a better sensitivity to detect low number of adhered cells. The EP was better when the average number of adhered cells was between 10 and 100 per microscopy field.

Key words: Bacterial adherence, Listeria innocua and Staphylococcus aureus, stainless steel, polycarbonate and polypropylene

INTRODUCTION

Bacterial adherence on inert food contact surfaces could lead to food contamination by undesirable microorganisms, resulting in food spoilage or transmission of disease (Bower et al., 1996; Hood, 1996; Jeong and Frank, 1994; Kumar and Anand, 1997; Stickler, 1999; Zottola and Sasahara, 1994). Bacterial adherence may trigger the process of microbial growth and biofilm formation and is a complex process that can be initiated with one bacterial cell and be affected by several factors such as microorganism species, growth conditions and polysaccharide production. The species that have cell appendages (fimbriae, pili and flagella) can bring the cells closer to the substrate and help the bacteria adhere to the surface (Austin et al., 1997; Dalton and March, 1998; O’toole, 1998).

The hydrophobicity and charge surface of microorganisms affect their capacity to adhere to the surface (Hood 1996; Hood and Zottola, 1995). Growth conditions such as culture medium, pH, salt concentration, organic compounds, time and temperature contact, agitation and substrate hydrophobicity and electric charge and its microtopography play an important role in bacterial adherence (Jeong and Frank, 1994; Smoot and Pierson, 1998; Zottola and Sasahara, 1994). Other important factor is the ability of the microorganisms to produce extracellular adhesive material that helps the cells to anchor to surface (Sticlér, 1999; Zottola and Sasahara, 1994; Zottola, 1997).

A considerable number of surfaces used as food contact surfaces can promote microbial adherence process such as stainless steel, glass, cast iron, rubber, polypropylene, low-density polyethylene and polycarbonate (Andrade et al., Assanta et al.,...
Sahara, 1994). Several techniques such as impedance measurement (Andrade et al., 1998a; Rule, 1997; Siley and Forshiyte, 1996), ATP bioluminescence (Stewart et al., 1997) and microscopy can be used to evaluate the bacterial adherence and biofilm formation. Microscopy techniques allow better visualization of the adherence process, growth and biofilm formation in food processing systems. Different microscopic techniques such as optical, epifluorescence, phase contrast, scanning electron, transmission electron and force atomic microscopy can be applied to evaluate cell adherence to the surfaces (Zottola, 1997). Epifluorescence microscopy (EP) is a good choice to determine the number of adhered cells on transparent or nontransparent surfaces since the cells become fluorescent after absorption of acridine orange permitting their microscopic observation. In this study, the efficiency of Staphylococcus aureus and Listeria innocua adherence on stainless steel, polypropylene and polycarbonate was evaluated by plate count (CP) and epifluorescence microscopy methods.

MATERIALS AND METHODS

Bacterial suspensions and culture media
The studies on adherence were conducted using suspensions of Listeria innocua L6a and Staphylococcus aureus ATCC 6538. One hundred µL of these cultures were maintained at -18°C on semi-solid media. For L. innocua growth, Tryptic Soy Broth (TSB-DIFCO) was used, with 0.6% of yeast extract and for S. aureus growth, Brain Heart Infusion (BHI-DIFCO) was used. A working culture was prepared by inoculating 100µL of frozen culture into 10mL of TSB or BHI broth and by incubating at 30°C for 24h. The culture was sub-cultured twice before use.

Attachment of cells
The attachment of L. innocua and S. aureus on stainless steel, AISI 304, finish # 4, on low-density polypropylene and on polycarbonate chips with dimensions of 10x10x1 mm, 10x10x4 mm and 10x10x1 mm, respectively, was evaluated. The stainless steel and polypropylene chips were first immersed in aceton for 30 min followed by immersion in 1% NaOH solution for 1h. After that they were rinsed with distilled water and sterilized at 121°C for 15min. The polycarbonate chips were immersed in ethyl alcohol for 1h, rinsed with distilled water, followed by UV exposure of 100µW/cm² for 1h. The sterile clean chips were added to flasks containing 100 mL of TSB or BHI, which was previously inoculated with suspensions of L. innocua and S. aureus. The initial number of cells ranged from 10³ to 10⁴ CFU/mL. The flasks were incubated, statically in a water bath at 30°C. The number of adhered cells on the different surfaces was evaluated after 0, 2, 4, 6, 8, 10 and 12 h of contact time. The results were expressed in CFU/cm² of surface.

Enumeration of adhered cells
At each period of time, four chips were removed and rinsed for 1 min in two tubes - two chips/tube - containing 10mL of sterilized phosphate buffer (K₂HPO₄, 0.31M, pH 7.2) to remove the non-adhered cells. Each chip was placed into a tube containing 2 mL of phosphate buffer solution. Then, two tubes each containing a chip, were swirled with a vortex mixer for 2 min. The cells released from the chip surface during the vortex mixing were diluted and spread on TSB or BHI agar plates, according to the type of microorganism, and incubated at 30°C for 48h. The other two chips were submitted to EP technique. Adhered cells found on chip surfaces after 1min rinse were considered to be irreversibly attached to the surfaces. These attached cells were fixed to the chip surface with Kirkpatrick’s solution (isopropyl alcohol: chloroform:formaldehyde-6:3:1), stained with 0.04% of acridine orange solution, and the chips were analyzed by epifluorescence microscope (FLUOVAR (Car/Zien - IENA). Each individual cell or group of cells was counted as DMC (Direct Microscopy Count). In this experiment, it was established that 1 DMC was equal to 1 CFU. The average of cells per field was obtained from 5 or 10 observation fields. The CFU/cm² was determined by multiplying of the average number of cells per field the microscopy factor (4,500). The experiment was conducted in triplicates.

Photomicrography
The photomicrography of the microorganisms adhered to different surfaces was obtained by using Olympus BX 60 microscopy, coupled with an epifluorescence objective and an Olympus photo camera.
Statistical analyses

Data were analyzed as a split-split plot design with the split - split plots consisting of two treatments: 1 - microorganism and surface types and 2 - time. The results were analyzed by regression and variance analysis using the Statistical Analysis System (SAS) (1988).

RESULTS

Bacterial adherence to surfaces

Fig. 1 shows the ability of _L. innocua_ and _S. aureus_ to adhere on stainless steel, polypropylene and polycarbonate surfaces at different contact times. Table 1 shows the analysis of variance of the log$_{10}$ of the number of _L. innocua_ and _S. aureus_ adhered to surfaces, as determined by EP and CP methods. Significant differences (p<0.05) were observed for the EP method in the interactions between microorganisms, surfaces and contact times.

Epifluorescence Microscopy versus count plate after vortex mixing

The results showed different numbers of adhered cells at 0 (zero) min contact time (Fig. 2) on the surface types when EP or CP techniques were used. For _L. innocua_, the log$_{10}$ of the initial numbers determined by EP were 3.39; 3.42; and 3.63 on stainless steel, polypropylene and polycarbonate, respectively. For the CP method, these values were 1.23, 1.76 and 1.47. For _S. aureus_, the log$_{10}$ of the initial numbers determined by the EP technique were 3.75, 3.46 and 3.16 on the surfaces mentioned above. The values found by the CP method were 1.62, 1.0 and 0.54. After 10 h, the log$_{10}$ of the number of cells of _L. innocua_ per cm$^2$, as determined by EP, were 5.13, 5.10 and 4.68, for stainless steel, polypropylene and polycarbonate, respectively. These values, as determined by CP, were 4.0, 4.34 and 3.97. If the same incubation time and surfaces had been considered, the log$_{10}$ of the number of cells of _S. aureus_ adhered were 5.35, 4.95 and 4.90 by EP and 4.5, 4.6 and 4.5, by CP. The results showed that the number of _L. innocua_ adhered to stainless steel and _S. aureus_ to polycarbonate was higher when counted by EP. However, the number of _S. aureus_ adhered to polycarbonate and polypropylene was higher when determined by CP.

DISCUSSION

In this experiment, the highest number of _L. innocua_ or _S. aureus_ adhered cells was between $10^3$ and $10^6$ CFU per cm$^2$, regardless of the surface studied. To be considered a biofilm, the number of adhered cells should range between $10^6$ and $10^7$CFU per cm$^2$, which means 10-100 folds above the values found. The results showed that the CP method has a better sensitivity to detect a low number of adhered cells than the EP method. This difference could be explained since by EP it was more difficult to count cells when the number on surfaces was still low in the early log-phase growth and initial adherence process. The number of adhered cells counted by CP was found to be lower than the number determined by EP. A likely explanation for these results was the fact that CP was not able to remove all irreversibly adherent cells from the surfaces after vortex mixing the chips immersed in the phosphate buffer solution contained in the tubes. The vortex method failed to totally remove all attached cells. It was also possible that in this experiment, the growth conditions for a considerable number of cells were not enough.

The results showed that the EP was better than CP, particularly when the average number of adhered cells per microscopic observation field range between 10 and 100. In these values, the count range by EP was between $4.5 \times 10^5$ and $4.5 \times 10^6$ CFU per cm$^2$.

The number of adhering cells on the surface of the chips increased as a function of contact time and depended on the method used for enumeration. For example, the log$_{10}$ of the _L. innocua_ numbers attached after 10h, determined by EP, were 5.13, 5.1 and 4.68 in stainless steel, polypropylene and polycarbonate, respectively. For CP, these numbers were 4.0, 4.34, and 3.97. These values indicated an adherence process of the microorganisms on the surfaces studied. The use of microscopy to count adhered cells on surface chips is a viable technique, since, on a microscopic scale, surfaces can be found to have cracks and crevices, quite unlike macroscopic appearance. These surface imperfections protect the microorganisms against removal by swab or rinse, for example. Thus, they will not be enumerated by plate count methods.
Table 1 - Analysis of variance of the log_{10} of the number (CFU/cm^2) of Staphylococcus aureus ATCC 6538 and Listeria innocua L6a, adhered to different surfaces

<table>
<thead>
<tr>
<th>Epifluorescence Method</th>
<th>Plate Count Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>VF</td>
<td>MS</td>
</tr>
<tr>
<td>Bacteria (B)</td>
<td>1</td>
</tr>
<tr>
<td>Surface (S)</td>
<td>2</td>
</tr>
<tr>
<td>Interaction BxS</td>
<td>2</td>
</tr>
<tr>
<td>Error (a)</td>
<td>12</td>
</tr>
<tr>
<td>Time (T)</td>
<td>6</td>
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<tr>
<td>SxT</td>
<td>12</td>
</tr>
<tr>
<td>BxT</td>
<td>6</td>
</tr>
<tr>
<td>BxSxT</td>
<td>12</td>
</tr>
<tr>
<td>Error (b)</td>
<td>72</td>
</tr>
</tbody>
</table>

*Significant at 5% probability (p<0.05).

Figure 2 - Log_{10} of the number of bacterial cells adhered on different surfaces as a function of contact time. (A) and (C): adherence of Listeria innocua L6a, as determined by EPM and CPM, respectively. D and B: adherence of Staphylococcus aureus ATCC 6538, as determined by EPM and CPM, respectively.
Microscopes coupled with image analysis systems can help the count process of adhered cells on surfaces by EP. The use of image analysis systems renders the EP a viable alternative to quantify adherence. Currently, the determination of the percentage area covered by bacterial growth on surfaces by software, combined with epifluorescence microscopy, has improved the evolution of the adherence process and biofilm formation.

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REFERENCES


