Effects of natural antimicrobials on bacterial cell hydrophobicity, adhesion, and zeta potential

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Interactions between bacterial cells and contact materials play an important role in food safety and technology. As bacterial strains become ever more resistant to antibiotics, the aim of this study was to analyse adhesion of selected foodborne bacterial strains on polystyrene surface and to evaluate the effects of natural antimicrobials on bacterial cell hydrophobicity, adhesion, and zeta potential as strategies of adhesion prevention. The results showed strain-specific adhesion rate on polystyrene. The lowest and the highest adhesion were found for two B. cereus lines. Natural antimicrobials ferulic and rosmarinic acid substantially decreased adhesion, whereas the effect of epigallocatechin gallate was neglectful. Similar results were found for the zeta potential, indicating that natural antimicrobials reduce bacterial adhesion. Targeting bacterial adhesion using natural extracts we can eliminate potential infection at an early stage. Future experimental studies should focus on situations that are as close to industrial conditions as possible.

KEY WORDS: epigallocatechin gallate; ferulic acid; polystyrene; rosmarinic acid

Food spoilage bacteria and pathogens are increasingly resistant to constantly changing environments and antimicrobials, which compromises their control in food production. Bacteria that form biofilms have several advantages over the free-floating ones (1) and have greater potential to contaminate and spoil food (2, 3), as they stick to the surfaces of equipment used for food handling, storage, or processing (4, 5) such as those made of polystyrene, glass, rubber, and stainless steel (6).

Adhesion of bacterial cells to surfaces and biofilm formation depend on the properties of bacterial cells, environmental factors influencing their mode of growth, and on the properties of the materials to which they adhere (7) but is mainly governed by the electrostatic, van der Waals, hydrophobic, and contact interactions (8).

In the early adhesion stages, these interactions between the cell and substrate surfaces are weak and reversible. Anti-adhesion strategies seek to delay or even block these early interactions by changing bacterial and/or surface properties (9). An alternative strategy is the use of low-dose natural antimicrobial agents, preferably derived from plants generally recognised as safe (GRAS) that do not affect the sensory quality of food or provoke resistance. Several plant-derived extracts or active compounds can prevent attachment of pathogens, but surprisingly, little is known about their effects on bacterial adhesion, with a few exceptions (10, 11).

The aim of this study was therefore to address this gap by: i) characterising polystyrene surface as one of the most common materials used in food processing; ii) determining cell surface hydrophobicity, adhesion to polystyrene surface, and zeta potential of foodborne bacterial strains; and iii) evaluating the effect of natural antimicrobials ferulic and rosmarinic acid and epigallocatechin gallate, for which we determined antibacterial efficiency on the adhesion properties of the selected pathogens in an earlier study (12).

MATERIALS AND METHODS

Polystyrene surface roughness

To assess bacterial adhesion we used a flat-bottomed polystyrene microtiter plate (Nunc®, Roskilde, Denmark), and to characterise plate surface on the sub-micrometer scale we used atomic force microscopy (AFM, VEECO Dimension 3100, Town of Oyster Bay, NY, USA) in contact mode. With AFM it is possible to image surface topography and measure root mean squared roughness $R_q$.

Bacterial strains

Strains used in this study were selected from two culture collections (with designations ZM and ZMJ) kept at the
Food Microbiology Laboratory of the Food Science Department, Biotechnical Faculty (Table 1). The bacteria were preserved in tryptic soy broth (TSB, Oxoid CM0129, Hampshire, UK) with 15 % glycerol as frozen stock at -80 °C. Cultures for all tests were revitalised on tryptic soy agar (TSA, Oxoid CM0131) by overnight incubation at 37 °C and further inoculated in TSB where they grew overnight at 37 °C. Bacterial cultures that were used for hydrophobicity testing were then cultivated in TSB until early log phase (6 h at 37 °C and at 25 °C for L. monocytogenes). Staphylococcus aureus ŽMJ72 was used to optimise measuring bacterial cell surface hydrophobicity. The preparation of cultures for adhesion measurements included inoculation of a single colony from TSA in 5 mL TSB and growth at 37 °C with shaking (75 rpm) for 30 hours for Gram-positive bacteria and for 24 h for Gram-negative bacteria. Staphylococcus aureus ŽMJ72 and Pseudomonas aeruginosa ŽMJ87 were used to optimise the crystal violet (CV) assay.

Colonies were counted after 24 h of incubation at 37 °C on TSA. The total number of bacteria in each suspension was calculated using the equation [1] according to the ISO standard 4833 (13).

\[ N = \frac{\sum C_i}{(n1 + 0.1 \times n2) \times d} \]  

where \( N \) is the number of bacteria per millilitre, \( \sum C \) is the sum of colonies counted on all the dishes retained, \( n1 \) is the number of the dishes retained in the first dilution, \( n2 \) is the number of the dishes retained in the second dilution, and \( d \) is the dilution factor corresponding to the first dilution.

### Table 1 Bacterial surface hydrophobicity, adhesion, and zeta potential

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain designation</th>
<th>Source of isolation</th>
<th>Hydrophobicity ± SD (%)</th>
<th>Δȳ ± SD</th>
<th>ζ ± SD (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive bacteria</td>
<td>Bacillus cereus ŽMJ3</td>
<td>Apple vinegar</td>
<td>20.6±1.6</td>
<td>0.020±0.12</td>
<td>-35.14±1.00</td>
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<td></td>
<td>Bacillus cereus ŽMJ91</td>
<td>Laboratory type strain</td>
<td>16.0±0.4</td>
<td>0.076±0.20</td>
<td>-42.07±1.52</td>
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<tr>
<td></td>
<td>Bacillus cereus ŽMJ116</td>
<td>Condensed milk</td>
<td>34.5±1.3</td>
<td>0.121±0.26</td>
<td>-43.70±0.54</td>
</tr>
<tr>
<td></td>
<td>Bacillus cereus ŽMJ123</td>
<td>Chocolate syrup</td>
<td>10.8±3.0</td>
<td>1.862±1.18</td>
<td>-52.97±1.78</td>
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<tr>
<td></td>
<td>Listeria monocytogenes ŽM58</td>
<td>IHM; reference strain</td>
<td>29.0±1.8</td>
<td>0.055±0.90</td>
<td>-43.62±1.26</td>
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<tr>
<td></td>
<td>Listeria monocytogenes ŽM69</td>
<td>Human isolate</td>
<td>32.6±1.0</td>
<td>0.060±0.13</td>
<td>-41.11±1.23</td>
</tr>
<tr>
<td></td>
<td>Listeria monocytogenes ŽM80</td>
<td>Human isolate</td>
<td>14.1±1.5</td>
<td>0.082±0.14</td>
<td>-40.97±1.88</td>
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<td>Listeria monocytogenes ŽM407</td>
<td>Chicken meat</td>
<td>37.0±1.3</td>
<td>0.096±0.25</td>
<td>-42.95±0.49</td>
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<td>Listeria monocytogenes ŽM520</td>
<td>DMRICC 3633</td>
<td>7.2±0.9</td>
<td>0.120±0.15</td>
<td>-37.45±1.62</td>
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<td>Staphylococcus aureus ŽMJ72</td>
<td>ATCC2 5923</td>
<td>42.9±14.4</td>
<td>1.396±0.72</td>
<td>-28.75±1.19</td>
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<td></td>
<td>Staphylococcus aureus ŽM504</td>
<td>Cream cake</td>
<td>13.2±0.4</td>
<td>0.225±0.18</td>
<td>-31.49±1.85</td>
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<td></td>
<td>Staphylococcus aureus ŽM518</td>
<td>ATCC 24213</td>
<td>23.9±6.6</td>
<td>0.042±0.08</td>
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<td>Escherichia coli ŽMJ135</td>
<td>Human isolate</td>
<td>0.0±0.0</td>
<td>0.659±0.09</td>
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<td>Escherichia coli ŽM370</td>
<td>ATCC 11229</td>
<td>0.4±0.7</td>
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<td>Escherichia coli ŽM513</td>
<td>Tartar beefsteak</td>
<td>2.6±0.5</td>
<td>0.067±0.08</td>
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<td></td>
<td>Pseudomonas aeruginosa ŽMJ87</td>
<td>Laboratory type strain</td>
<td>35.5±0.3</td>
<td>1.331±0.79</td>
<td>-22.86±2.28</td>
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<td>Pseudomonas aeruginosa ŽM517</td>
<td>ATCC 15442</td>
<td>31.8±17.6</td>
<td>0.404±0.10</td>
<td>-41.11±0.95</td>
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<td>Pseudomonas aeruginosa ŽM519</td>
<td>ATCC 27853</td>
<td>1.9±1.4</td>
<td>0.113±0.04</td>
<td>-36.65±1.29</td>
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<td>Salmonella Enteritidis ŽM348</td>
<td>Egg yolk</td>
<td>14.1±0.6</td>
<td>0.062±0.06</td>
<td>-11.32±2.11</td>
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<td></td>
<td>Salmonella Infantis ŽM350</td>
<td>Egg</td>
<td>8.2±1.7</td>
<td>0.530±0.27</td>
<td>-13.36±2.00</td>
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<tr>
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<td>Salmonella Hadar ŽM378</td>
<td>Chicken meat</td>
<td>8.2±2.0</td>
<td>0.166±0.19</td>
<td>-10.37±1.50</td>
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<tr>
<td></td>
<td>Salmonella Infantis ŽM390</td>
<td>Chicken meat</td>
<td>8.0±0.6</td>
<td>0.180±0.07</td>
<td>-10.83±2.14</td>
</tr>
</tbody>
</table>

*P < 0.05*

ŽM, ŽMJ: designations for bacterial culture collections of the Laboratory for Food Microbiology, Dept. of Food Science and Technology, Biotechnical Faculty; Δȳ: average strain absorbance obtained with the CV assay; ζ: zeta potential; IHM: Institute for Hygiene and Microbiology, Wuerzburg, Germany; DMRICT: Danish Meat Research Institute, Roskilde, Denmark
maximum speed of 2500 twiddles per min. After the separation of two layers (time to separation was 20 min), we measured optical density (OD) of the aqueous phase. The percentage of cells in the xylene layer was calculated as the percentage of hydrophobicity using the equation [2].

\[
\text{Percentage of hydrophobicity} = \left(1 - \left(\frac{A}{A_0}\right)\right) \times 100 \quad [2]
\]

where \(A_0\) is the OD of cell suspension before the addition of xylene (before separation), and \(A\) is the OD of the aqueous phase (after separation).

**Crystal violet assay**

Crystal violet (CV) assay was first described by Christensen et al. (16) and has since been modified many times. We studied the influence of selected parameters (different initial number of bacteria from log or stationary growth phase, different concentration of CV, different solvent) on the quantification accuracy of the adhered biomass. For each experiment we inoculated a flat-bottomed polystyrene 96-well microtiter plate (Nunc®) with 200 µL of bacterial culture diluted in sterile TSB to the desired concentration (10^5 CFU mL^-1) for Gram-positive bacteria from log growth phase or 10^6 CFU mL^-1 for Gram-negative bacteria from stationary growth phase). The total number of bacteria in each suspension was counted in Plate Count Agar (PCA CM0463, Oxoid) at 37 °C after 24 h. As negative control we used 200 µL of sterile TSB added to 12 wells of each microtiter plate. After incubation (24, 48, or 72 h) at 37 °C the supernatant with free-floating cells was removed from each well and the plate rinsed with 150 µL of sterile distilled water three times and dried with a hair dryer at 60 °C for 10 min. To observe polystyrene microtiter wells microscopically we used a Jeol SEM 840A (Akishima, Tokyo, Japan).

**Zeta potential determination**

Bacterial surfaces are also characterised by their electric charge, which allows the measurement of zeta potential through electrophoretic mobility of the bacteria (18, 19). In the experiment we used the bacterial strains listed in Table 1. The bacteria were cultured as previously described. Briefly, 24-hour bacterial cultures were harvested by centrifugation at 9500 g, and the cells washed twice with phosphate buffer solution (pH 7) with the ionic strength of 1 mmol L^-1 (0.026 g KH₂PO₄, 0.047 g K₂HPO₄ per litre) and finally resuspended in the same buffer to the final concentration of 10^9 to 10^10 CFU mL^-1. For resuspension, the samples were exposed to ultrasound (40 kHz) for one minute to achieve fine colloidal suspension (20). Zeta potential was measured with a Zetasizer Nano ZS (Malvern, Worcestershire, United Kingdom) equipped with a universal dip cell.

**Effect of natural antimicrobials on bacterial hydrophobicity, adhesion, and zeta potential**

The inhibitory activities of ferulic acid (Sigma-Aldrich) rosmarinic acid (Chromadex, Santa Ana, CA, USA), and epigallocatechin gallate (Sigma-Aldrich) were assessed by measuring adhesion, hydrophobicity, and zeta potential of Bacillus cereus ŽMJ123, Staphylococcus aureus ŽMJ72, and P. aeruginosa ŽMJ87 exposed to the antimicrobials for 24 h at half the minimal inhibitory concentration (MICₕ₅₀). The reduction in bacterial hydrophobicity, adhesion, and zeta potential in the presence of natural antimicrobials was calculated as the percentage of inhibition of each parameter using equation [4] (21), as follows:

\[
\text{Percentage of inhibition} = \left(1 - \left(\frac{T}{C}\right)\right) \times 100 \quad [4]
\]

where C is the average value for control samples that contained bacteria in TSB with no addition of antimicrobial component and T is the average value for treated samples that contained bacteria in TSB supplemented with antimicrobials.
Statistical analysis

For statistical analysis of the interactions between all factors included in the optimisation of the CV assay we used the analysis of variance (ANOVA). For correlations between hydrophobicity and adhesion to polystyrene we used the regression model. All tests were performed at the 95 % confidence level.

RESULTS AND DISCUSSION

Figure 1 shows a typical AFM image of polystyrene surface. Average surface roughness (Rq) was 14.2 nm, which is comparable to the results of Biazzer et al. (22).

Table 1 shows surface hydrophobicity of the tested strains. The strains varied in hydrophobicity, ranging from 0 to 42.9 %. Most bacteria (16 out of 22) were hydrophilic, with hydrophobicity lower than 30 %, irrespective of the source of isolation [for hydrophobicity classification see Martin et al. (23) and Scheneider and Reiley (24)].

The highest adhesion to polystyrene surface was observed for B. cereus ŽMJ123, S. aureus ŽMJ72, and P. aeruginosa ŽMJ87. Gram-negative bacteria showed significantly higher adhesion to polystyrene surface (p<0.05) than Gram-positive bacteria. Differences in adhesion were not related to the source of isolation, but rather to the strain, which confirms earlier findings (17, 25-27). In general, the strains showed low adhesion potential, which could be related to their hydrophilic properties. However, studies investigating the relationship...
between hydrophobic and adhesive properties of *Escherichia coli* (28) are inconclusive, as they show both positive and negative correlation. Our findings are also inconclusive because two of the strains that adhered well to polystyrene surface were hydrophobic (*S. aureus* ŽMJ72 and *P. aeruginosa* ŽMJ87) and the one with the highest adhesion (*B. cereus* ŽMJ123) was hydrophilic. Perhaps this result was affected by the use of xylene. In our study, we measured bacterial surface hydrophobicity using 0.5 mL of xylene, and Nwanyanwu and Abu (29) showed that hydrophobicity in *Bacillus* sp. cells decreased from 95% to less than 20%, when they increased xylene from 0.1 to 0.5 mL.

All bacteria were negatively charged, with zeta potentials ranging from -10.37 to -52.97 mV in a 1 mmol L⁻¹ solution of PBS. Even though the results vary considerably, same bacterial species show a similar zeta potential. Soni et al. (30) also found a large variability of zeta potential among bacterial species in drinking water, from -16.6 mV for *Salmonella* sp. to -47.8 mV for *E. coli*.

To find the locations of preferential adhesion of the bacteria we scanned the surfaces of samples with attached microorganisms. Figure 2 shows control measurements of bacterial adhesion using SEM (31). In the beginning only a small part of the 2890 μm² polystyrene surface area was covered with bacteria, whereas at the end, bacteria covered nearly the entire surface.

We tested the effects of ferulic acid, rosmarinic acid, and epigallocatechin gallate on the bacteria that showed highest adhesion, namely *B. cereus* ŽMJ123, *S aureus* ŽMJ72, and *P. aeruginosa* ŽMJ87. Figure 3 shows that epigallocatechin gallate was uniformly successful in reducing adhesion with all three bacterial strains and that all antimicrobial substances had great effect on the zeta potential of *S. aureus*. However, rosmarinic acid was the only able to affect all three species, which suggests that it readily permeates the cell membrane and binds electrostatically with anionic groups within the cell and on the cell surface, which results in zeta potential drop.
CONCLUSIONS

Contact material and bacterial surface properties play an important role in food safety and technology. Our findings could help to prevent bacterial adhesion and consequently the formation of biofilm on food contact materials and reduce the risk of food poisoning.

Future research should go in two directions. The first is to understand the interaction between particular bacteria and material surface (32). The second includes food as an intermediate between surface, natural antimicrobials, and bacteria in order to come up with applicable findings for food industry.

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REFERENCES

Vpliv naravnih protimikrobnih snovi na bakterijsko hidrofobnost, adhezijo in zeta potencial


**KLJUČNE BESEDE:** epigalokatehin galat; ferulična kislina; polistiren; rožmarinska kislina