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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 ŽM and ŽMJ) kept at the

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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 five 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).

$$N = \frac{\sum_i C_i}{(n1 + 0.1 \times n2) \times d} \qquad [1]$$

where N is the number of bacteria per millilitre, ΣC 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.

Bacterial cell surface hydrophobicity

To optimise bacterial cell hydrophobicity measurements, in our preliminary experiment (20) we used *Staphylococcus aureus* ŽMJ72 to evaluate the effect of different wavelengths (400 nm, 600 nm, and 650 nm), time of mixing (from 20 s to 2 min), time of water and organic phase separation (1 min, 10 min, and 15 min), and the use of plastic *vs.* glass tubes on absorbance measurements. Our optimal choices were the 650 nm wavelength, 1 min of mixing, and 15 min of phase separation, whereas the choice of plastic or glass tubes made no difference.

Surface hydrophobicity of bacterial cells was determined using a slightly modified method described by Rosenberg (14) and Tahmourespour et al. (15) as follows: 2 mL of bacterial culture was centrifuged at 5000 *g* for 4 min and washed twice with phosphate buffer saline (PBS, Oxoid). The cells were then re-suspended in 15 mL of PBS, and absorption was measured (A_o) with a spectrophotometer (Tecan Männedorf, Zürich, Switzerland) at the 620 nm wavelength. Absorbance was measured in each of the 96 wells of the microtiter plate with a microplate reader (Tecan Männedorf). Then 0.5 mL of xylene (Kemika, Zagreb, Croatia) was added to 3.5 mL of bacterial suspension in PBS, and the mixture was agitated on a vortex at the

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

Group	Strain designation	Source of isolation	Hydrophobicity ± SD (%)	$\Delta\bar{A}$ ± SD	ζ ± SD (mV)
Gram-positive bacteria	*Bacillus cereus* ŽMJ3	Apple vinegar	20.6±1.6	0.0206±0.12	-35.14±1.00
	Bacillus cereus ŽMJ91	Laboratory type strain	16.0±0.4	0.0763±0.20	-42.07±0.52
	Bacillus cereus ŽMJ116	Condensed milk	34.5±1.3	0.1212±0.26	-43.70±0.54
	Bacillus cereus ŽMJ123	Chocolate syrup	10.8±3.0	1.8622±1.18	-52.97±1.78
	Listeria monocytogenes ŽM58	IHM; reference strain	29.0±1.8	0.0553±0.90	-43.62±1.26
	Listeria monocytogenes ŽM69	Human isolate	32.6±1.0	0.0603±0.13	-41.11±1.23
	Listeria monocytogenes ŽM80	Human isolate	14.1±1.5	0.0824±0.14	-40.97±1.88
	Listeria monocytogenes ŽM407	Chicken meat	37.0±1.3	0.0696±0.25	-42.95±0.49
	Listeria monocytogenes ŽM520	DMRICC 3633	7.2±0.9	0.1201±0.15	-37.45±1.62
	Staphylococcus aureus ŽMJ72	ATCC2 5923	42.9±14.4	1.3966±0.72	-28.75±1.19
	Staphylococcus aureus ŽM504	Cream cake	13.2±0.4	0.2256±0.18	-31.49±1.85
	Staphylococcus aureus ŽM518	ATCC 24213	23.9±6.6	0.0420±0.08	-23.18±2.07
Gram-negative bacteria	*Escherichia coli* ŽMJ135	Human isolate	0.0±00	0.6596±0.09	-22.11±1.38
	Escherichia coli ŽM370	ATCC 11229	0.4±0.7	0.1312±0.11	-23.20±1.50
	Escherichia coli ŽM513	Tartar beefsteak	2.6±0.5	0.0676±0.08	-27.80±2.08
	Pseudomonas aeruginosa ŽMJ87	Laboratory type strain	35.5±0.3	1.3314±0.79	-22.86±2.28
	Pseudomonas aeruginosa ŽM517	ATCC 15442	31.8±17.6	0.4043±0.10	-41.11±0.95
	Pseudomonas aeruginosa ŽM519	ATCC 27853	1.9±1.4	0.1139±0.04	-36.65±1.29
	Salmonella Enteritidis ŽM348	Egg yolk	14.1±0.6	0.0626±0.06	-11.32±2.11
	Salmonella Infantis ŽM350	Egg	8.2±1.7	0.5304±0.27	-13.36±2.00
	Salmonella Hadar ŽM378	Chicken meat	8.2±2.0	0.1663±0.19	-10.37±1.50
	Salmonella Infantis ŽM390	Chicken meat	8.0±0.6	0.1804±0.07	-10.83±2.14
	$P_{strain} < 0.05$		$P_{strain} < 0.05$	$P_{strain} < 0.05$	

ŽM, ŽMJ: designations for bacterial culture collections of the Laboratory for Food Microbiology, Dept. of Food Science and Technology, Biotechnical Faculty; ΔĀ: average strain absorbance obtained with the CV assay; ζ: zeta potential; IHM: Institute for Hygiene and Microbiology, Wuerzburg, Germany; DMRICC: 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].

$$Percentage\ of\ hydrophobicity = \left(1 - \left(\frac{A}{Ao}\right)\right) x100 \quad [2]$$

where A_o 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 (10^3 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. The plate was then air-dried or dried with a hair dryer at 60 °C for 10 min and 100 µL of a crystal violet (CV, Merck, Darmstadt, Germany) solution (1%) added to all wells. After 15 min, the CV solution was removed by washing each well with 150 µL of sterile distilled water three times and the plate was dried with a hair dryer at 60 °C for 10 min. Bound CV was released by adding 200 µL of ethanol (>99.9 %, Merck) for Gram-negative bacteria or acetic acid (33 %, Merck) for Gram-positive bacteria. The absorbance was measured at 584 nm on a microplate reader. The average absorbance as a measure for strain adhesion was calculated using the equation [3] (17).

$$\Delta\bar{A} = \sum_{i=1}^{n} \frac{(A_i - \bar{A}o)}{n} \quad [3]$$

where $\Delta\bar{A}$ is the average strain absorbance, A is the absorbance of a particular well, $\bar{A}_o$ is the arithmetic mean of absorbance of 12 wells with negative control, and n is the number of wells (12 to 24) inoculated with bacterial strains.

Microscopy

P. aeruginosa ŽMJ87 was used to assess bacterial morphology on polystyrene using scanning electron microscopy (SEM). The bacteria were inoculated into polystyrene microtiter plates as previously described and incubated at 37 °C for 3, 6, 12, and 24 h. After incubation, the supernatant with free-floating cells was removed from each well and the plate was 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 at low-magnification (up to 2000x) 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 KH_2PO_4, 0.047 g K_2HPO_4 per litre) and finally resuspended in the same buffer to the final concentration of 10^7 to 10^8 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 (MIC_{50}). The reduction of 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:

$$Percentage\ of\ inhibition = \left(1 - \left(\frac{T}{C}\right)\right) x100 \quad [4]$$

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.

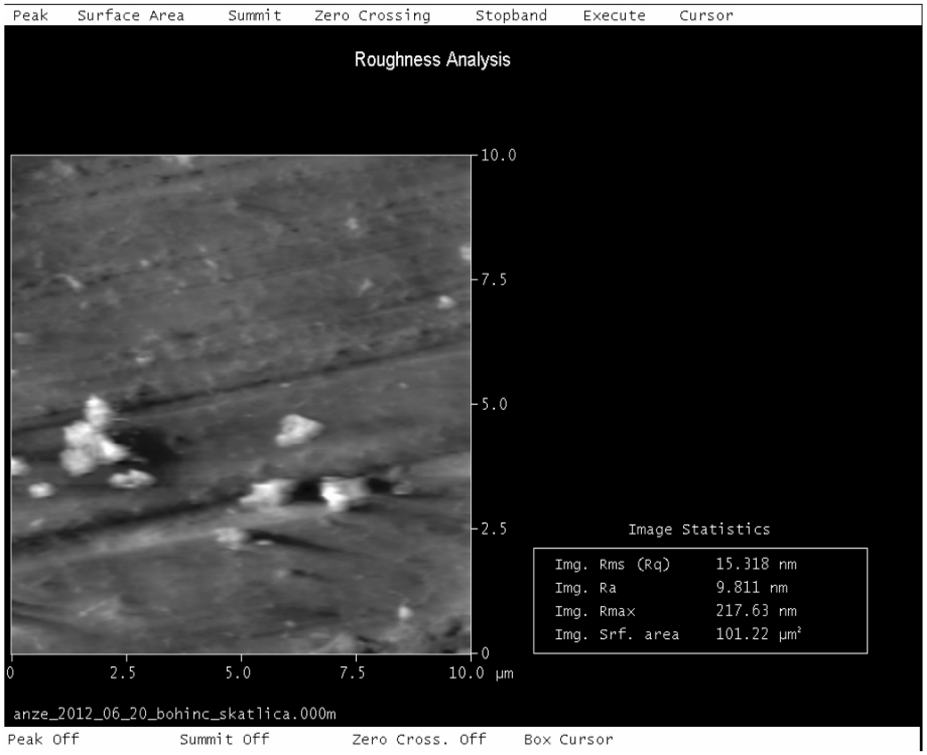

Figure 1 *AFM image of polystyrene surface*

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 Biazzar 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

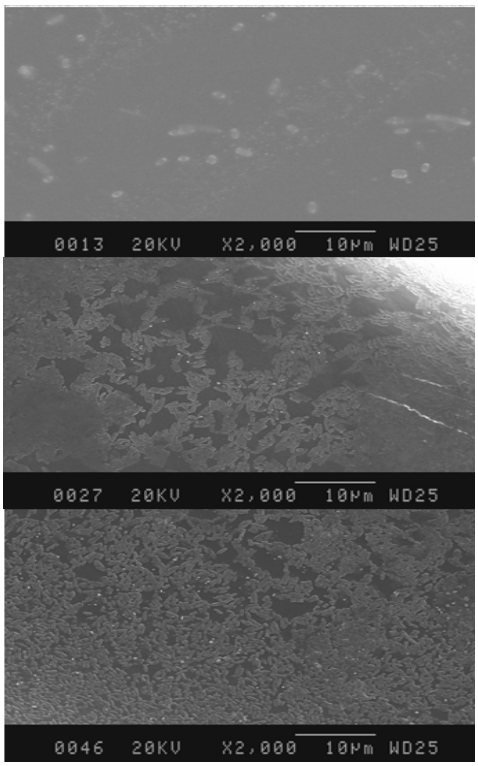

Figure 2 *SEM images of polystyrene surface covered with the microorganisms (*P. aeruginosa*) after 3 h (A), 12 h (B), and 24 h (C)*

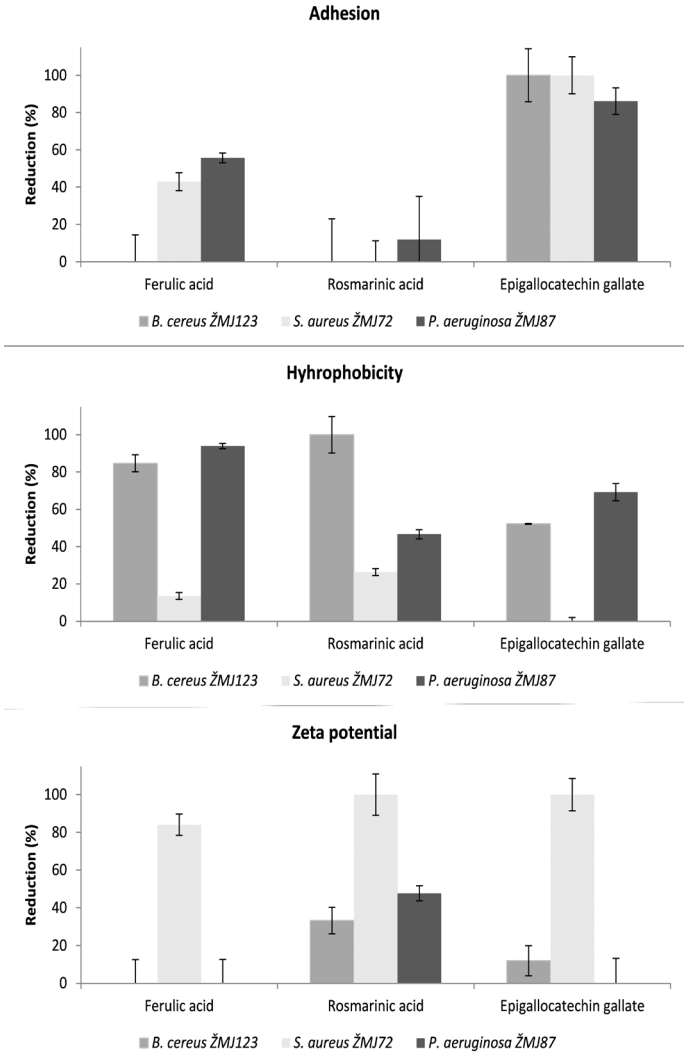

Figure 3 *Reduction of hydrophobicity (A), adhesion (B), and zeta potential (C) in bacteria cultivated with ferulic acid, rosmarinic acid, and epigallocatehin gallate*

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 μm^2 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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Vpliv naravnih protimikrobnih snovi na bakterijsko hidrofobnost, adhezijo in zeta potencial

Interakcije med bakterijskimi celicami in površinami delovnih materialov imajo pomembno vlogo v živilski tehnologiji pri zagotavljanju varnih živil. Poznano je, da različni bakterijski sevi postajajo bolj in bolj odporni proti antibiotikom in drugim biocidom. Zato je bil namen naših raziskav analizirati adhezijo izbranih patogenih bakterij, ki se prenašajo z živili. Proučevali smo njihov oprijem na polistirensko površino in ovrednotili vpliv naravnih protimikrobnih snovi na bakterijsko hidrofobnost, adhezijo in zeta potencial, v smislu možnih strategij za preprečevanje adhezije. Rezultati so pokazali, da je adhezija sevno specifična lastnost, saj je bila najmanjša in največja stopnja adhezije določena za različna seva bakterij vrste *Bacillus cereus*. Naravni protimikrobni snovi, ferulična in rožmarinska kislina, sta zmanjšali stopnjo adhezije na polistiren, medtem ko je bil vpliv epigalokatehin galata zanemarljiv. Podobne rezultate smo dobili pri zeta potencialu, kar nakazuje na možnosti delovanja naravnih snovi kot protiadhezivnih komponent. Uporaba naravnih protimikrobnih snovi lahko prepreči oziroma zmanjša stopnjo adhezije bakterijskih celic in s tem eliminira možnosti kontaminacij ali okužb v začetni fazi. Nadaljnje eksperimentalno delo bo potrebno za ovrednotenje razmer, ki so čim bolj podobne industrijskemu okolju.

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