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Listeria monocytogenes Repellence by Enzymatically Modified PES Surfaces

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ABSTRACT: The effect of enzyme-catalyzed modification of poly(ethersulfone) (PES) on the adhesion and biofilm formation of two *Listeria monocytogenes* strains is evaluated under static and dynamic flow conditions. PES has been modified with gallic acid, ferulic acid and 4-hydroxybenzoic acid. The surfaces modified with any of these compounds show up to 70% reduced adhesion of *L. monocytogenes* under static conditions and up to 95% under dynamic flow conditions compared with unmodified surfaces. Also, under static conditions the formation of biofilms is reduced by ~70%. These results indicate that the brush structures that are formed by the polymers on the PES surface directly influence the ability of microorganisms to interact with the surface, thereby reducing attachment and biofilm formation of *L. monocytogenes*. Based on these results, it is expected that enzyme-catalyzed surface modification is a promising tool to reduce microbial adhesion and biofilm formation. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41576.

KEYWORDS: adsorption; functionalization of polymers; grafting; monolayers and polymer brushes

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INTRODUCTION

Listeria monocytogenes is a gram-positive food-borne human pathogen that can cause a disease known as listeriosis,[1,2] which can lead to miscarriage, meningitis, and septicaemia. *Listeria* species are able to attach to various surfaces including plastics, rubber, stainless steel, and glass.[3,4] This pathogen can survive and grow at a very wide temperature range (below freezing point up to 46°C), high salinity (grows up to 13% and remains alive up to 30%), and wide pH range (below 5 and up to 9).[5,6] The ability of *L. monocytogenes* to survive and grow in such severe conditions is attributed to their ability to adapt themselves to changing environments.[5,6]

L. monocytogenes is associated with foods like raw milk, cheese, ice cream, and raw and smoked fish.[7,8] It has also been isolated from sea, sewage, and river water.[9–13] The factors that affect adhesion of *L. monocytogenes* to surfaces are not completely understood, but bacterial cell surface properties, the properties of the substratum (inert) surface, and local conditions have been suggested.[5,14] Although hydrophilic, negatively charged, and smooth surfaces have been shown to be effective in reducing the initial adhesion of live cells of several bacterial species,[15–17] it has also been reported that neither initial adhesion of *L. monocytogenes* nor biofilm formation depend on the surface roughness.[18] Further, the effect of surface hydrophilicity on attachment of *Listeria* to polymeric surfaces is limited (less than one order of magnitude).[19]

Poly(ethersulfone) (PES) membranes are widely used in the food industry and for water treatment (separation and purification purposes). However, the drawback of this type of membranes is the significant adhesion of foulants including proteins and living cells, resulting in a severe reduction in membrane performance (flux and selectivity).[20] The resulting membrane replacement that needs to take place regularly forms the largest operating cost in any membrane separation process.[21]

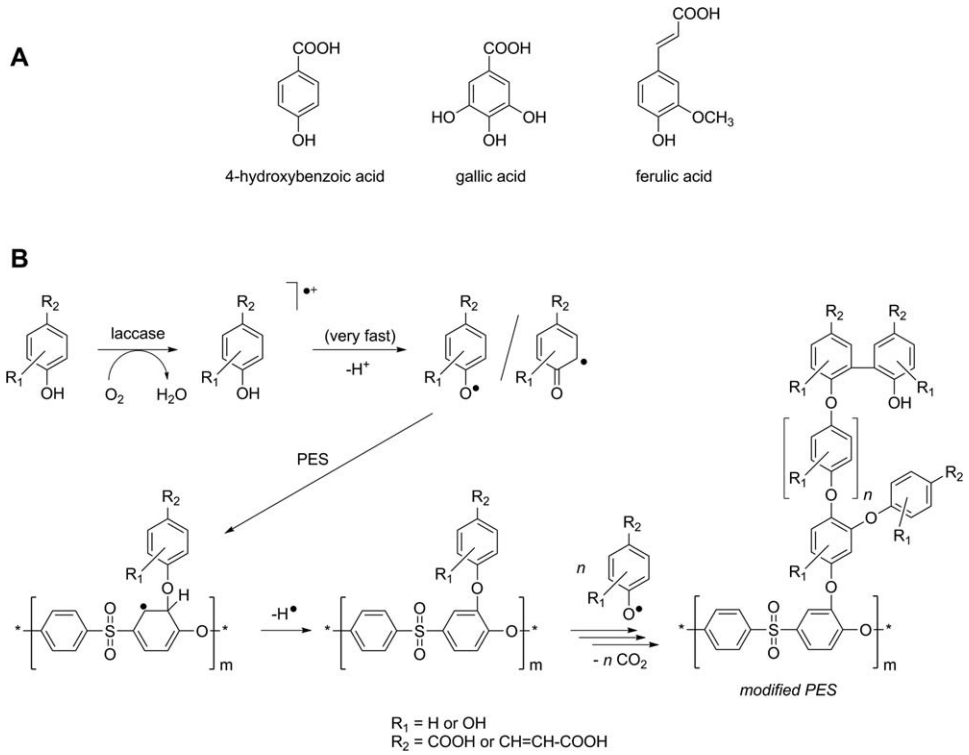

Figure 1. (A) Chemical structure of the used modifiers of PES. (B) Tentative mechanism for the reaction of laccase-generated radicals with PES, and subsequent formation of grafted brushes. N.B: in case of ferulic acid, coupling can also occur *via* the side chain.

In previous research,[22] it was shown that it is possible to covalently link phenolic compounds such as 4-hydroxybenzoic acid and gallic acid via their oxygen atoms to PES surfaces using the laccase enzyme from *Trametes versicolor*. This enzyme is able to oxidize phenolic compounds to their corresponding free radicals that are subsequently grafted onto PES membranes, introducing polar groups (OH, COOH) on the surface (see Figure 1). This modification, that is carried out in aqueous medium under mild conditions (room temperature and pH 5), leads to a remarkable suppression of protein adsorption on both modified "real membranes"[23] and modified laminated PES on silicon dioxide surfaces (model PES surfaces).[24]

In this article, PES surfaces modified with 4-hydroxybenzoic acid, gallic acid, and ferulic acid were tested for their ability to reduce surface adhesion and biofilm formation of the model organism *L. monocytogenes*. The 4-hydroxybenzoic acid and gallic acid modified PES surfaces have previously been reported to show protein repellence[24] and adsorption of cellular surface proteins is often proposed to act as an initial step in microbial adhesion. In addition we report on surfaces modified with ferulic acid, a compound that is reported to have anti-bacterial action.[25–27]

EXPERIMENTAL

Chemicals

Brain heart infusion (BHI) broth was purchased from Becton Dickinson, Le Pont de Claix, France, and agar bacteriological from Oxoid, Hampshire, England. Potassium phosphate monobasic anhydrous (99.5%) and sodium phosphate dibasic anhydrous (99.5%) were obtained from Merck - Germany, and sodium chloride was received from VWR international BVBA, Belgium. Sodium acetate (anhydrous, ≥99%), acetic acid (99.9%), potassium chloride (99%), catechol (>99%), 4-hydroxybenzoic acid (99%), gallic acid (>97.5%), ferulic acid (99%), dichloromethane (ACS, stabilized, 99.9%), and laccase from *Trametes versicolor* (>20 U mg^{-1}) were obtained from Sigma–Aldrich. Calbiochem provided 2,2′-Azobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). BASF (Ludwigshafen, Germany) provided poly(ethersulfone) (PES) (Ultrason, E6020P), Wafer Net Inc (San Jose, CA) delivered prime grade 150 mm silicon wafers of type P/B <100> orientation, thickness 660–700 μm, and 2.5 nm native oxide layer. All chemicals were used as received. Phosphate buffered saline (PBS) was prepared by dissolving 80 g NaOH, 2 g KCl, 14.4 g anhydrous Na_2HPO_4, 2.4 g anhydrous KH_2PO_4 (0.1*M* final concentration) in 1000 mL reverse osmosis (R.O.) water, which was adjusted to pH 7.4 if needed, and autoclaved at 121°C for 15 min. Milli-Q water was used throughout the preparation of the model PES surfaces and sterilized R.O. water was used in all the biofouling tests.

Laccase Assay

Laccase activity was determined with 2,2′-azobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. The assay mixture contained 0.33 mL of 1*M* ABTS solution, 2.67 mL of 0.1*M* sodium acetate buffer (pH 5), with 0.05 U mL^{-1} laccase. Oxidation of ABTS is monitored by following the increase in absorbance at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$).[23,28] The reaction time taken is 1 min. One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min at 25°C.

Table I. Characterization of Modified PES Slides[a]

Substrate	Thickness of PES layer (nm)	Thickness of modification layer (nm)	Contact angle of PES layer (°)	Contact angle of modification layer (°)
4-Hydroxybenzoic acid	20 ± 1	6.3 ± 0.14	78.9 ± 1.0	63.2 ± 1.2
Gallic acid	20 ± 1	6.3 ± 1.1	78.9 ± 1.0	64.1 ± 1.4
Ferulic acid	20 ± 1	3 ± 0.2	78.9 ± 1.0	60 ± 1.5

[a] Data for 4-hydroxybenzoic acid and gallic acid are taken from Ref. 24.

Preparation of Modified PES Surfaces

Silicon wafers with a silicon dioxide top layer of about 70 nm were cut into strips of 1 × 5 cm (for experiments under static conditions) or 1.5 × 5.5 cm (for dynamic conditions). The strips were sonicated in ethanol for 15 min, washed with water and ethanol, and dried in a flow of nitrogen. Subsequently the strips were plasma cleaned (PDC-32G, Harrick at RF-level high, 10 min) and after removal of any dust (nitrogen flow) the strips were immediately spin-coated with a 0.25 wt % PES solution in dichloromethane for 10 s at 2500 rpm. The spin-coated PES surfaces were immersed in 20 mL (1 × 5 cm strips) or 33 mL (1.5 × 5.5 cm strips) of 0.1*M* sodium acetate buffer pH 5 containing phenolic acids and laccase. Air was bubbled through the solution for mixing and as a source of oxygen for the enzyme. As phenolic acids were used 4-hydroxybenzoic acid (28 m*M*, 2 h modification), ferulic acid (4.8 m*M*, 1 h), and gallic acid (4.8 m*M*, 7 min).[22–24] The enzyme concentration was 0.5 U mL^{-1} and the reaction was carried out at room temperature (23°C ± 2°C). After the indicated incubation time the strips were recovered, washed by strong flushing with Milli-Q water and dried for 24 h in glass-covered dishes in desiccators charged with silica gel.

The characterization of the modified PES surfaces has been reported before, the most significant data are listed in Table I.

L. monocytogenes Attachment and Biofilm Formation

L. monocytogenes strains EGD-e and LR-991[29,30] were cultured and stored as described previously.[31]

Determination of the number of bacteria attached to a surface was performed by swabbing the surface and allowing the thus obtained cells to produce colonies. As it is generally unknown how many bacteria are in a sample, it is almost always necessary to prepare a dilution series to ensure that a dilution is obtained containing a reasonable number of bacteria to count. Dilutions in the range 10^{-1} to 10^{-9} have been tested. For each testing condition, a suitable dilution is used. Serial dilutions of swapped microorganisms from each surface were plated onto a suitable growth medium. The suspension was spread onto the surface of agar plate (spread plate method). The plates were subsequently incubated under conditions that permit microbial reproduction. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Thus, the number of cfu in the original sample is determined by multiplying the number of colonies on a dilution plate by the corresponding dilution factor. Also, the used sample area is included to finally obtain cfu cm^{-2}.

More precisely, for the attachment of *L. monocytogenes* under static conditions, silicon slides spincoated with PES[24] (modified or unmodified) were placed in petri dishes and covered with 20 mL PBS. After addition of the bacterial suspension (~10^9 total colony-forming units [cfu]), the system was incubated for 2.5 h at room temperature (23°C ± 2°C). The slides were washed with PBS twice after which the adhered bacteria were collected using a sterile cotton swab. Subsequently, the swab was placed in 1 mL PBS and vigorously vortexed. The suspended bacteria were serially diluted in PBS and plated on agar plates. The plates were incubated for 48 h at 30°C and colonies were counted. The average adsorbed amounts on blank surfaces were about 8 × 10^7 cfu cm^{-2} for the EGD-e strain and 9 × 10^7 cfu cm^{-2} for the LR-991 strain. The difference between duplicate measurement was in general below 15% with only few exceptions that amounted up to around 25%. Experiments were performed in duplicate, using two different cell batches.

A similar procedure was used for the determination of biofilm formation on modified and unmodified slides, with the exception that BHI was taken as a medium and the incubation time was 24 h.

The bacterial attachment under dynamic conditions was investigated using a flow cell, depicted in Figure 2. A PES slide (1.5 × 5.5 cm) was placed on the sample support inside the flow cell (sample support size: 1.6 cm × 5.7 cm × 1 mm w × l × d). The bacterial suspension was diluted in 500 mL PBS. The complete system (i.e., the connection tubes and the flow cell) was first washed with PBS during 10 min and subsequently filled with the bacterial suspension (~10^9 total cfu). The bacterial suspension was circulated through the system for 2.5 h at a speed of 0.038 m s^{-1} (equivalent to a Reynolds number of 38 at a water temperature of 20°C). After removal of the PES slide, bacteria were collected and counted as described above for static conditions. The average adsorbed amounts on blank surfaces were about 8 × 10^8 cfu cm^{-2} for the EGD-e strain and 5 × 10^9 for the LR-991 strain. The difference between duplicate measurements was in general below 15% with only few exceptions that amounted up to around 25%. Experiments were performed using three different cell batches.

Fluorescence Microscopy

Fluorescence microscopy experiments were performed on a BX41 microscope (Olympus, Zoeterwoude, The Netherlands). Images were acquired using a XC30 camera (Olympus) and Olympus Cell^B software. After washing the modified PES slide twice with PBS, it was placed on a microscope slide (76 × 26 mm^2), and a square cover glass (18 mm) was placed on top of the sample. Green fluorescent protein (GFP) was visualized using a MNIBA3 filter (Olympus).

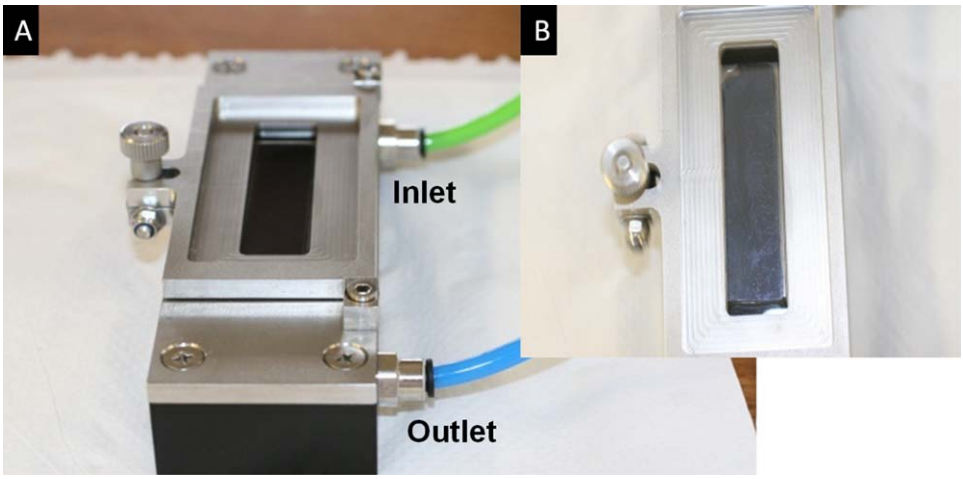

Figure 2. (A) Flow cell (made in the mechanical workshop of Wageningen University) used in this study. (B) Plain view of the flow cell during flowing of the bacteria suspension over the fixed modified model PES surface. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

The overall purpose of this study was to examine the effect of laccase-catalyzed modification of PES surfaces on the adhesion and biofilm formation of *L. monocytogenes* under both static conditions and dynamic conditions.

Static Conditions

When cell attachment is studied under static conditions, the cells are simply brought into contact with the surface without any flow or shear. For our studies we used two *Listeria* strains, namely EGD-e, which is the most commonly used research strain worldwide, and LR-991, which is known for its high tendency to biofilm formation.[30] Laccase-mediated modification of the PES surfaces resulted in about 60% reduction in the number of attached *Listeria* cells from both strains, when compared to the unmodified surface [= pure PES; see Figure 3(A)]. Treatment of PES with laccase and 4-hydroxybenzoic acid leads to the formation of brushes[22,24] and the high reduction of cell adhesion found on these surfaces (63%, $P < 0.004$) might be attributed to these brushes. This effect has also been observed in other studies,[32–34] where it was shown that bacterial adhesion was diminished by the attachment of brush-like structures to surfaces like stainless steel, glass, polyamide and polyester. It is thought that bacteria are repelled because of steric hindrance by the brushes, thereby preventing the formation of favorable van der Waals interactions between the cells and the surface.[35,36] In addition, presence of polar groups on the membrane surface may create repellence between the modifying layer and proteins and polysaccharides on the surface of the bacterial cells[23] or the surface in general.[36] This interpretation is also plausible for gallic acid-modified PES; at the chosen reaction conditions, a brush-like structure is formed (Figure 1).[23,24] PES surfaces modified with ferulic acid also show inhibition of bacterial adhesion, although the structure of the layer is in need of further investigation. In contrast to 4-hydroxybenzoic and gallic acid, bond formation to ferulic acid is also possible via the side chain, just as in lignin formation.[37] It is likely however that also

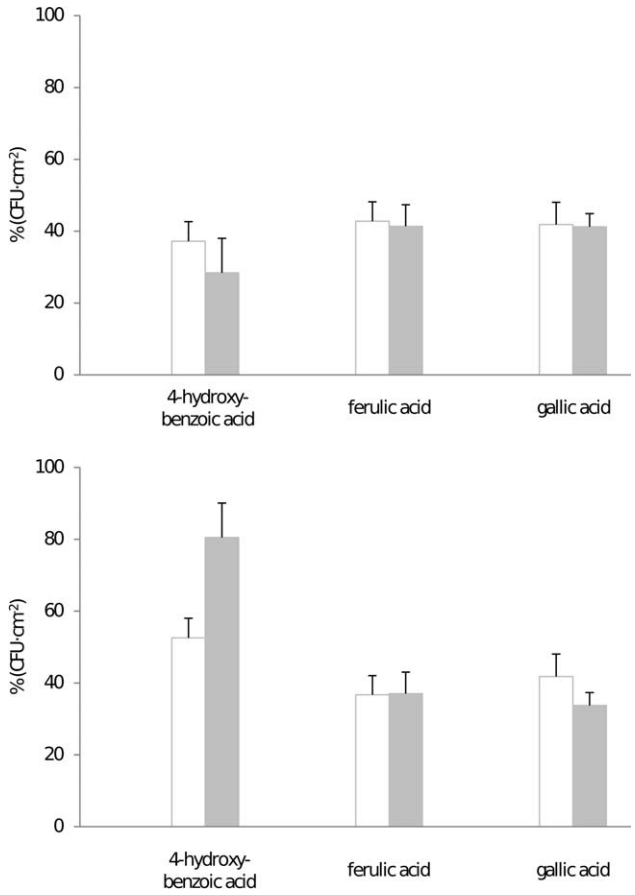

Figure 3. Effect of PES modification on attachment (A) and biofilm formation (B) of *L. monocytogenes* EGDe (white bars) and LR-991 (grey bars) under static conditions. The graph represents the average and standard deviation of four model surfaces from experiments performed on two occasions. The *y* axis shows the percentage of cfu per cm^2 for each condition compared with the unmodified surface. All treatments with the exception of biofilm growth on a 4-hydroxybenzoic acid-modified surface are significantly different from the unmodified surface ($P < 0.05$, *t* test).

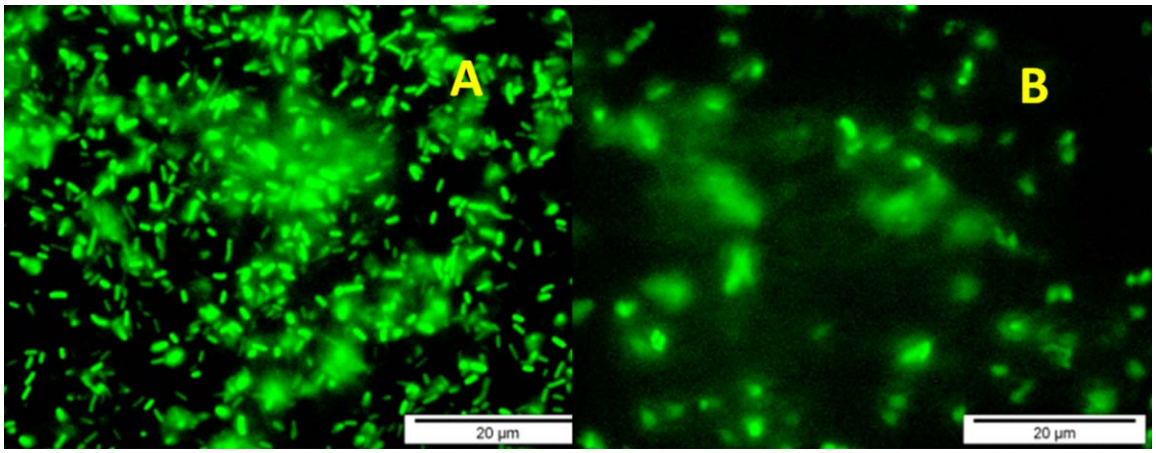

Figure 4. Representative fluorescence microscopy images of *L. monocytogenes* biofilms grown on PES surfaces. The images present *L. monocytogenes* LR-991 grown under static conditions on unmodified (A) and ferulic acid-modified (B) model PES surfaces. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

for this substrate, at short modification time and low concentration, a brush type layer is formed.

Under static conditions, also biofilm formation of *L. monocytogenes* EGD-e and LR-991 was determined [Figure 3(B)]. The PES surfaces modified with ferulic acid and gallic acid exhibit percentages of reduction in adhesion close to their percentages of reduction in the attachment test (~60%). This may be attributed to the same phenomena, i.e., steric hindrance and electrostatic interactions, preventing cell adhesion and subsequent biofilm formation. However, biofilm formation of the strain LR-991 was hardly affected by modification of PES with 4-hydroxybenzoic acid (19.5% reduction).

Both attachment and biofilm formation was microscopically verified using *L. monocytogenes* EGDe and LR-991 expressing EGFP.[29,31] Figure 4 shows representative images of biofilm formation for the strain LR-991 on unmodified and ferulic acid-modified PES surfaces. The images were in concordance with the cfu counts under all conditions. No significant differences in static attachment and biofilm formation between the modified surfaces were observed i.e. all the modified surfaces have ability to resist the attachment of bacteria cells and consequently alter biofilm formation. While this aspect is currently still poorly understood, recently reported repellence of fungi and (non) pathogenic bacteria by laccase-catalyzed modified surfaces using different phenolic compounds[26,27,38–40] shows the potential of this approach, which is worthy of further investigation.

Dynamic Conditions

The effect of flowing *L. monocytogenes* suspensions in PBS over the model surfaces was studied for both strains. Modification of the surfaces led to a substantial reduction of the adhered cells compared with the unmodified surfaces (Figure 5). This could be a direct effect of prevention of adhesion, or a delay of adhesion (less fast) that allows lift forces to remove loosely adhered bacteria.[41]

Compared to the results previously obtained with BSA repellence[22–24] on similarly modified surfaces, the repellence of microorganisms is less, but one has to keep in mind that the modification layers were tuned to repel a specific protein, BSA. For the proteins present on the cell wall (or even those present in the fermentation broth) and/or other surface components including specific sugar residues or polymers, adjusted modification methods may be needed, that are tuned to the specific targets; but the results obtained here have shown the proof-of-principle that these layers can be effective in reducing adhesion and colonisation by microorganisms under static and flowing conditions.

CONCLUSIONS

The obtained results indicate that enzyme-catalyzed modification of PES surfaces affects *L. monocytogenes* attachment and biofilm formation. Depending on the used substrate a reduction of 40–60% is found under static conditions, while this percentage increases up to 95% under dynamic conditions.

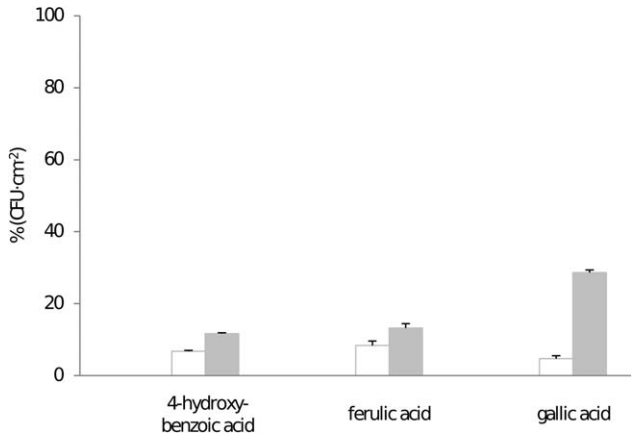

Figure 5. Effect of PES modification on attachment of *L. monocytogenes* EGDe (white bars) and LR-991 (grey bars) under dynamic flow conditions. The graph represents the average and standard deviation of three model surfaces from experiments performed on three occasions. The *y* axis shows the percentage of cfu per cm^2 for each condition compared with the unmodified surface. All treatments are significantly different from the untreated surface ($P < 0.05$, *t* test).

Because the enzyme-catalyzed modification method is an eco-friendly method to reduce biofouling on PES membranes there might be an application of such modified membranes in food processing as well as in water treatment, because the anti-biofouling effects can strongly reduce the membrane replacement costs that are currently a major hurdle. However, further optimization of our system is still necessary.

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