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Research Note

Inhibition of Initial Attachment of Injured *Salmonella* Typhimurium onto Abiotic Surfaces

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ABSTRACT

Following sanitation interventions in food processing facilities, sublethally injured bacterial cells can remain on food contact surfaces. We investigated whether injured *Salmonella* Typhimurium cells can attach onto abiotic surfaces, which is the initial stage for further biofilm development. We utilized heat, UV, hydrogen peroxide, and lactic acid treatments, which are widely utilized by the food industry. Our results showed that heat, UV, and hydrogen peroxide did not effectively change populations of attached *Salmonella* Typhimurium. Cells treated with hydrogen peroxide had a slightly higher tendency to adhere to abiotic surfaces, although there was no significant difference between the populations of control and hydrogen peroxide–treated cells. However, lactic acid effectively reduced the number of *Salmonella* Typhimurium cells attached to stainless steel. We also compared physicochemical changes of *Salmonella* Typhimurium after application of lactic acid and used hydrogen peroxide as a positive control because only lactic acid showed a decreased tendency for attachment and hydrogen peroxide induced slightly higher numbers of attached bacteria cells. Extracellular polymeric substance produced by *Salmonella* Typhimurium was not detected in any treatment. Significant differences in hydrophobicity were not observed. Surface charges of cell membranes did not show relevant correlation with numbers of attached cells, whereas autoaggregation showed a positive correlation with attachment to stainless steel. Our results highlight that when lactic acid is applied in a food processing facility, it can effectively interfere with adhesion of injured *Salmonella* Typhimurium cells onto food contact surfaces.

Key words: Attachment inhibition; Biofilm; Inactivation; Injured cell

Biofilm is a well-organized structure of microorganisms embedded in extracellular polymeric substance (EPS). EPS consists of diverse materials such as eDNA, polysaccharides, and proteins secreted by microorganisms. EPS serves as a physical barrier against environmental stress, and it is well known that bacteria that form biofilms have greater resistance to antibiotics, sanitizers, and other treatments intended to remove biofilms *(7, 15, 29)*. Foodborne pathogens are also able to form biofilms, according to several researchers. Among them, *Salmonella* Typhimurium, a major causative agent of foodborne diseases, gives rise to salmonellosis, which produces diarrhea, abdominal cramps, and fever. *Salmonella* Typhimurium is also known to develop biofilms on food contact surfaces such as steel or PVC and, thus, has greater resistance to sanitizers *(25)*.

In the food industry, microorganisms that adhere to food contact surfaces can bring about several problems, including food safety issues. Bacteria that attach to surfaces can form biofilms, and biofilm-forming bacteria can be a source of cross-contamination. Thus, inactivation of biofilms is essential for the food industry to maintain consistent product quality and to ensure food safety. However, the higher resistance of biofilms necessitates more frequent treatments that require much higher doses of sanitizers, which can lead to damage of food processing surfaces.

This implies that appropriate doses and applications are needed to suppress microorganisms before they can develop into biofilms. However, in previous studies, several researchers showed that, under certain circumstances, subinhibitory concentrations may actually induce biofilm formation *(19, 35)*. This becomes a food safety concern in that a food processing facility, if not adequately sanitized, can be a source of foodborne illness outbreaks. Also, bacteria can be problematic if they are shielded behind crevices or in cavities in food contact surfaces. Thus, even though sanitization is conducted periodically, there are opportunities for microorganisms to become attached within food facilities and develop into biofilms.

After microorganisms are subjected to a given inactivation method, the population of bacteria can be divided into three groups: healthy cells (live), injured cells (live), and dead cells *(6, 14)*. Under a suitable environment, sublethally injured cells can recover from damage and develop into colonies, even on selective agar. Even after treating food facilities, there is always the possibility that injured cells

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may remain on food contact surfaces. This indicates that the ability of injured cells to attach onto food contact surfaces and form biofilms should be analyzed.

Although several research studies have investigated how sanitizer concentrations induce biofilm formation, none has elucidated what effect cell injury has on microorganism attachment to abiotic surfaces, which is a very crucial stage in biofilm development. In this study, we investigated how several interventions, including heat treatment, UV, hydrogen peroxide, and lactic acid, which are widely used in the food industry, could effectively interfere with initial attachment of *Salmonella* Typhimurium onto abiotic surfaces.

MATERIALS AND METHODS

Bacterial strains. *Salmonella* Typhimurium strains (ATCC 19585, ATCC 43971, DT 104) were obtained from the bacterial culture collection of Seoul National University (Seoul, Republic of Korea) for study. Each strain of *Salmonella* Typhimurium was grown in 5 mL of tryptic soy broth (Difco, Sparks, MD) at 37°C for 24 h. The three strains were combined, and bacterial cells were collected by centrifugation at 4,000 × *g* at 4°C for 20 min and washed three times with phosphate-buffered saline (PBS; pH 7.4). Bacterial cells from the final pellet were then treated with heat, lactic acid, hydrogen peroxide, or UV to induce 1 log CFU/mL of injured cells.

Preparation of stainless steel coupons. Stainless steel 304, which is widely used in the food industry, was cut into coupons (2 by 5 cm); they were immersed in 70% alcohol to disinfect the surfaces and then were rinsed with sterile distilled water. Next, stainless steel coupons were dried in a laminar flow biosafety hood (22 ± 2°C) for 3 h and then were sterilized by autoclaving (121°C, 15 min).

1-log injury of bacterial cell population. After collecting the cell pellets, each treatment was performed. Untreated control bacteria were resuspended in 30 mL of sterile PBS, corresponding to 10^7 CFU/mL.

Heat treatment. Heat treatment was performed in a water bath (Jeio Tech Co., Ltd., Daejeon, Korea) adjusted to 55°C. The cell pellet was resuspended in 30 mL of sterile PBS preheated to 55°C in a sterile 50-mL conical centrifuge tube (SPL Lifescience, Pocheon, Korea). The tube containing the cell suspension was tightly closed and completely immersed in the water bath for 4 min. The heated sample tube was then removed and cooled in ice water for 3 min.

Treatment with lactic acid. Lactic acid solution (1.0%, v/v) was prepared by adding sterile distilled water to extra pure lactic acid (Dslab, Seoul, Korea). Cell pellets were resuspended in 5 mL of lactic acid solution and treated for 20 s. Treated cell suspensions were immediately transferred to 50-mL conical centrifuge tubes containing 25 mL of Dey-Engley broth to neutralize lactic acid.

Treatment of hydrogen peroxide. Thirty percent hydrogen peroxide solution (Junsei Chemical Co. Ltd., Tokyo, Japan) was used to prepare hydrogen peroxide solution (0.1%, v/v) by adding sterile distilled water. Cell pellets were resuspended in 5 mL of hydrogen peroxide solution and treated for 210 s. Treated cell suspensions were immediately transferred to 50-mL conical centrifuge tubes containing 25 mL of Dey-Engley broth.

Treatment with UV. A low-pressure mercury vapor lamp (357 nm; G10T5/4P, Sankyo, Tokyo, Japan) with a peak wavelength of 254 nm was utilized. The nominal power output of this germicidal UV lamp was 8 W, and the light intensity was 0.58 mW/cm^2 at the sample location. A sample pellet was resuspended in a 50-mL conical centrifuge tube containing 5 mL of PBS buffer, and this was poured into a sterile petri dish (60 by 25 mm; SPL Lifescience, Pocheon, Korea). The UV lamp system was located 13.5 cm vertically and directly above the petri dish containing the bacterial cell suspension, which was irradiated at a dose of 2.9 mJ/cm^2 for 5 s.

Bacterial cell attachment and enumeration. The method for bacterial cell attachment was adapted from Kim et al. *(22)*. After each treatment was performed, the pathogens were collected by centrifugation at 4,000 × *g* at 4°C for 20 min and were washed twice with PBS. The final pellets were resuspended in 30 mL of sterile PBS in 50-mL conical centrifuge tubes, and sterile stainless steel coupons were immersed in the bacterial cell suspensions. Coupons were incubated for 24 h at 4°C to allow bacterial cell attachment, and then adhered bacterial cells were detached and enumerated as follows: coupons were removed with sterile forceps, transferred to 1,200 mL of sterile distilled water (22 ± 2°C), and gently stirred for 5 s to remove unattached or loosely attached cells. Washed coupons were deposited in 50-mL centrifugation tubes containing 3 g of glass beads (425 to 600 μm; Sigma-Aldrich, St. Louis, MO) and 30 mL of sterile PBS and then were vortexed at maximum speed for 1 min. After vortexing, cell suspensions were 10-fold serially diluted in peptone water (0.2%; Difco), and 0.1 mL of diluted cell suspension was spread plated onto xylose lysine desoxycholate agar (XLD; Difco). To enumerate injured cells, the overlay (OV) method was also used *(23)*. Tryptic soy agar (TSA) was used as a nonselective agar to resuscitate injured bacterial cells. Aliquots (100 μL) of appropriate dilutions were spread plated onto TSA medium, incubated for 2 h at 37°C, and then overlaid with 10 mL of selective medium (XLD). After solidification, the plates were further incubated for 22 h at 37°C.

Quantification of EPS. The method for quantification of EPS was adapted from Jung et al. *(21)*. Stainless steel coupons were inoculated as described previously. After incubation for 24 h at 4°C, coupons were transferred to 30 mL of sterile PBS containing 3 g of glass beads and then were vortexed as described above. Portions (20 mL) of samples were removed, poured into 50-mL conical centrifugation tubes, and then placed in boiling water (100°C) for 15 min. After cooling at room temperature for 20 min, 80 μL of Pronase E (Sigma-Aldrich, St. Louis, MO) was added to the sample and incubated at 37°C for 1 h. Trichloroacetic acid solution (200 μL) was added, and the mixture was then placed in ice water for 30 min and centrifuged at 15,000 × *g* for 20 min. The supernatant containing EPS was decanted, combined with an equal volume of 95% ethanol, and kept at –20°C for 1 h; it was then centrifuged at 15,000 × *g* for 20 min. After washing twice in 95% ethanol with centrifugation (15,000 × *g* for 20 min), the final precipitate was dissolved in 1 mL of sterile distilled water. The relative amount of EPS was assayed by measuring absorbance, following the phenol–sulfuric acid method *(13)*.

Measurement of surface charge. Bacterial cell surface charge was estimated by measuring zeta potential of the microorganisms. After performing treatments, bacterial cell

TABLE 1. *Log reduction of* Salmonella *Typhimurium after heat, lactic acid, hydrogen peroxide, and LP UV lamp treatments of planktonic cells*

Treatment	Log reduction [log(N_0/N)] by selective medium[a]	
	XLD	OV-XLD
Heat treatment	1.06 ± 0.09 a A	0.05 ± 0.17 a B
Lactic acid	1.05 ± 0.08 a A	0.06 ± 0.16 a B
Hydrogen peroxide	1.10 ± 0.04 a A	0.08 ± 0.16 a B
LP UV lamp (254 nm)	0.99 ± 0.11 a A	0.07 ± 0.15 a B

[a] Values are means ± standard deviations. Means followed by same uppercase letter in the same row are not significantly different ($P > 0.05$). Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$). XLD, xylose lysine desoxycholate agar; OV-XLD, XLD agar overlay on TSA.

suspensions were centrifuged at 4,000 × g for 20 min, and collected cell pellets were resuspended in 10 mL of sterile PBS. The pathogen suspension was 10-fold diluted in sterile PBS buffer. Zeta potential measurement was conducted with a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK).

Measurement of hydrophobicity. Hydrophobicity of the bacterial cell surface was estimated by using the MATH test *(8)*. After treatment with 3 mL of 1.0% lactic acid or 3 mL of 0.1% hydrogen peroxide solution, samples were combined with 27 mL of PBS. After collection of bacterial cells by centrifugation at 4,000 × *g* at 4°C for 20 min, 3 mL of sterile PBS was used to resuspend the cell pellet and an equal volume (3 mL) of hexadecane was added, followed by vortexing at maximum speed for 1 min and incubation at 30°C for 10 min. After incubation, the mixture was again vortexed at maximum speed for 2 min and then was further incubated at room temperature for 20 min. The absorbance of the aqueous phase was measured at 600 nm, and hydrophobicity was determined by the following equation: hydrophobicity (%) = (*Aí* − *Af*) / *Aí* × 100, where *Aí* is absorbance of the bacterial suspension and *Af* is absorbance of the aqueous phase after mixing with hexadecane.

Measurement of autoaggregation. Evaluation of autoaggregation ability was carried out to determine cell-to-cell surface interaction according to Del Re et al. *(10)*. After treatment with 3 mL of 1.0% lactic acid or 3 mL of 0.1% hydrogen peroxide, samples were combined with 27 mL of PBS. After collecting the bacterial cells by centrifugation at 4,000 × *g* for 20 min at 4°C, 2 mL of sterile PBS was used to resuspend the cell pellet. The bacterial suspension absorbance was measured at 600 nm (A_{0h}). Also, the bacterial cell suspension was incubated at 37°C for 2 h and then the absorbance was again measured at 600 nm (A_{2h}). Then, autoaggregation was determined: autoaggregation (%) = $(1 - A_{2h}/A_{0h}) \times 100$.

Statistical analysis. All experiments were repeated at least three times with independently prepared duplicate samples. Data were analyzed by analysis of variance and *t* test (least significant difference) using statistical analysis software (SAS Institute, Cary, NC). A *P* value of <0.05 was utilized to indicate significant difference.

RESULTS

1-log injury. Table 1 shows the level of *Salmonella* Typhimurium injury induced by each treatment. Heat treatment for 4 min in a 55°C water bath reduced the cell population by 1.06 log as revealed on selective agar, but it recovered by 1.01 log when plated with the overlay method. Lactic acid, hydrogen peroxide, and UV irradiation (254 nm) resulted in 1.05-, 1.10-, and 0.99-log reductions, respectively, but there was no significant difference between the control and each treatment in resuscitated cell numbers.

Attached bacterial cell numbers after 24 h of storage at 4°C. Table 2 shows populations of *Salmonella* Typhimurium attached to stainless steel coupons after 24 h of storage at 4°C. After 24 h of storage, the control cell population was 4.86 log CFU per coupon. All treatments showed reduced bacterial populations compared to the control. Attached bacterial cell numbers were reduced by 0.71,0.93, 0.44, and 0.61 log after heat, lactic acid, hydrogen peroxide, and UV treatment, respectively. On the other hand, sublethally injured *Salmonella* Typhimurium showed a different attachment trend. Heat, hydrogen peroxide, and UV treatment yielded similar bacterial cell populations, but lactic acid treatment showed dramatically reduced numbers of cells attached to stainless steel. This suggests that cells injured by each treatment, except for lactic acid, retain the ability to adhere to stainless steel.

Physicochemical property of bacterial cell. We analyzed several physicochemical properties (Table 3) that are important for adhesion and biofilm formation to investigate the greater reduction shown when *Salmonella* Typhimurium was treated with lactic acid. EPS production by bacterial cells was not observed in the control, lactic acid, or hydrogen peroxide treatments. Cell membrane charge measured by zeta potential of bacterial cells was –4.34 mv in the control, whereas lactic acid– and hydrogen peroxide–treated cells showed –9.20 and –9.11 mv, respectively. When measuring hydrophobicity, no significant difference was observed among the control, lactic acid, or hydrogen peroxide treatments. Autoaggregation ability was highest in bacterial cells treated with hydrogen peroxide (28.3%), although there was no significant difference between it and the control (25.4%). However, lactic acid showed reduced autoaggregation ability (17.5%) compared with the control and hydrogen peroxide treatments.

DISCUSSION

Because biofilm-forming bacteria have higher resistance to inactivation, more frequent and stronger doses of sanitizers must be applied. However, if food contact surfaces are exposed to repeated harsh treatments, these surfaces can deteriorate. Dror-Ehre et al. *(12)* pointed out that prevention of biofilm formation is needed to solve this problem. However, improper treatment can generate injured cells defined as sublethally damaged bacterial cells. Although healthy cells have the ability to grow on selective media containing inhibitory agents, sublethally damaged cells

TABLE 2. *Population of* Salmonella *Typhimurium on stainless steel coupons after initial attachment in sterile PBS for 24 h at 4°C*

Treatment	Survival by selection medium[a]	
	XLD	OV-XLD
Control	4.86 ± 0.18 a A	5.29 ± 0.12 ab B
Heat treatment	4.15 ± 0.13 bc A	5.23 ± 0.03 b B
Lactic acid	3.83 ± 0.10 c A	4.26 ± 0.38 c B
Hydrogen peroxide	4.42 ± 0.18 b A	5.54 ± 0.19 a B
LP lamp (254 nm)	4.25 ± 0.27 b A	5.14 ± 0.26 ab B

[a] Populations are expressed as log CFU per coupon; values are means ± standard deviations. Means followed by the same uppercase letter in the same row are not significantly different ($P > 0.05$). Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$). XLD, xylose lysine desoxycholate agar; OV-XLD, XLD agar overlay on TSA.

cannot survive on selective agar *(33)*. However, on nonselective agar such as TSA, injured bacterial cells resuscitate and resume growth. This means that injured microorganisms also have metabolic activity, which implies that they can participate in biofilm formation. Therefore, we investigated how different kinds of injury affect the initial attachment of *Salmonella* Typhimurium.

We utilized several methods to evaluate the efficacy of retarding biofilm development. Table 2 shows that lactic acid treatment effectively reduced the number of attached *Salmonella* Typhimurium cells both on XLD selective agar and on OV-XLD, whereas other treatments did not show significant differences on OV-XLD. These results reveal that injuries caused by these methods do not impact the ability to participate in initial attachment on abiotic surfaces, except for damage caused by lactic acid. According to several articles, the physicochemical properties of bacterial cells influence their attachment to abiotic surfaces and biofilm formation *(17, 26, 31)*. Because lactic acid treatment produced the lowest number of attached *Salmonella* Typhimurium cells and hydrogen peroxide yielded the greatest number of attached bacterial cells, despite no significant difference among the other treatments, we chose these two treatments for further study of changes in physicochemical properties.

EPSs play a significant role in the formation of biofilms. They are a major constituent of biofilms and contribute to bacterial cell aggregation and biofilm structure. Moreover, it is known that adhesion onto solid surfaces is also affected by EPS *(2, 9, 30)*. Because the majority of EPS consists of polysaccharides, we chose to investigate the amount of polysaccharides attached to stainless steel coupons. However, our results show that we cannot sufficiently detect EPS even in the control and hydrogen peroxide treatments. This might be due to the composition of the suspending medium. We used PBS as a suspending medium for adhering bacterial cells to stainless steel, and PBS has no glucose or other carbon sources, which are required for polysaccharide synthesis *(2)*. PBS is composed of disodium hydrogen phosphate, sodium chloride, potassium chloride, and potassium dihydrogen phosphate. Also, because we performed our experiments at 4°C, *Salmonella* Typhimurium might have been in a metabolically dormant state. Thus, we can assume that levels of EPS would be too small to detect, even though attached cells produce it.

Also, we investigated the bacterial cell surface charge by assessing zeta potential. It is commonly reported that stainless steel has a negative charge at neutral pH *(18)*. Because bacterial cells have a negative charge on their surface membrane, the electric repulsive force influences the adhesion of bacterial cells *(26)*. In a previous study, a slightly positive correlation between the surface charge of bacterial cells and initial attachment was shown *(11)*. However, Parkar et al. *(27)* reported that there is no clear proof that membrane surface charges of spores and vegetative cells have any influence on attachment. In this study, hydrogen peroxide– and lactic acid–treated cells showed a higher negative charge, but our results revealed that hydrogen peroxide treatment resulted in a greater number of attached cells. Thus, this implies that other factors are also involved in the attachment process.

Hydrophobicity also participates in bacterial cell attachment. It is reported that hydrophobic cells have a greater tendency to adhere than do hydrophilic cells *(32)*. Some researchers have shown that hydrophobicity of bacterial cells can change after certain treatments. Annuk et al. *(3)* studied the effect of plant extracts on cell surface hydrophobicity. It was also revealed that bile can change the hydrophobicity of the bacterial cell surface *(4)*. However, in the present study, no significant differences in hydrophobicity were observed after treatment with lactic acid or hydrogen peroxide. The study of Jacobs and Chenia *(20)* implied that there might be no correlation between hydrophobicity and cell attachment. Also, it has been shown that hydrophobicity has little impact on biofilm formation *(35)*. Thus, we can easily assume that hydrophobicity did not impact the initial attachment onto stainless steel in our study.

TABLE 3. *EPS production, zeta potential, hydrophobicity, and autoaggregation of* Salmonella *Typhimurium treated with lactic acid or hydrogen peroxide*

Treatment	Physicochemical properties of *Salmonella* Typhimurium[a]			
	EPS (absorbance at 470 nm)	Zeta potential (mv)	Hydrophobicity (%)	Autoaggregation (%)
Control	0.0163 ± 0.0228 a	−4.34 ± 0.54 a	29.9 ± 2.26 a	25.4 ± 0.57 a
Lactic acid	0.0190 ± 0.0082 a	−9.20 ± 0.93 b	29.6 ± 2.75 a	17.5 ± 0.72 b
Hydrogen peroxide	0.0105 ± 0.0107 a	−9.11 ± 0.86 b	27.7 ± 2.10 a	28.3 ± 3.51 a

[a] Values are means ± standard deviations. Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$).

Autoaggregation is one of the key physicochemical indicators involved in initial attachment and biofilm formation. Several researchers have shown a correlation between autoaggregation and biofilm formation ability. Sorroche et al. *(28)* found that autoaggregation correlates positively with biofilm development. Also, autoaggregation can be affected when bacterial cells are subjected to certain treatments. In a previous study, *Salmonella* Typhimurium cells treated with essential oils had different autoaggregation abilities depending on the kind of treatment *(35)*. They found that, with higher autoaggregation ability, more biofilm was formed. In this study, hydrogen peroxide induced higher autoaggregation of cells, whereas lactic acid reduced autoaggregation ability. Our results reveal a positive correlation between autoaggregation ability and initial attachment of bacterial cells.

Lactic acid damages bacterial cell membranes, liberating lipopolysaccharides (LPS) from the outer membrane of *Salmonella* Typhimurium *(1)*. LPS consist of lipids and polysaccharides, including the O-antigen, outer core, and inner core. It is known that LPS play a role in biofilm development *(31)*. In a previous study, *E. coli* mutants involved in lipopolysaccharide synthesis tended to produce lower populations of adhered cells than the wild-type *(16)*. Whereas a *Porphyromonas gingivalis* strain having a mutation in *gtfB* related to glycosyltransferase expressed enhanced autoaggregation, a *gtfA* mutant exhibited reduced autoaggregation *(24, 34)*. Also, Barak et al. *(5)* demonstrated that LPS are required for *Salmonella enterica* to attach to and colonize plants. Thus, we can reasonably assume that *Salmonella* Typhimurium cells injured by lactic acid exposure have lower attachment and autoaggregation ability owing to loss of LPS in their outer membranes.

Many factors affect initial attachment and biofilm formation of microorganisms, such as bacterial species, bacterial cell conditions, surface properties, nutritional conditions, pH, and temperature. In this study, we conducted our experiment using a three-strain cocktail of *Salmonella* Typhimurium. Each strain could have different sensitivities or behave differently from other strains with respect to attachment. Also, we utilized stainless steel for abiotic surfaces; however, other surfaces such as PVC, which has greater hydrophobicity, might show a different tendency, and cells grown as biofilms or in a sessile form may behave differently. Therefore, these varying aspects need to be addressed in future studies.

In conclusion, we investigated the types of cellular injuries that affect attachment onto abiotic surfaces, which is a crucial stage for biofilm development. In this study, several bactericidal interventions widely used in the food industry were utilized to induce 1-log injury in the cell population. Heat, UV, and hydrogen peroxide did not significantly affect the attachment property of injured cells, whereas lactic acid treatment effectively decreased the population of bacterial cells attached to abiotic surfaces. It was found that membrane surface charge and autoaggregation properties of *Salmonella* Typhimurium were changed after lactic acid and hydrogen peroxide treatments. However, surface charge did not correlate with attachment. Lactic acid affects the bacterial cell membrane by liberation of LPS, and it is strongly assumed that it is the main cause of reduced populations of attached cells. In a food processing line, there are many opportunities for injured cells to survive and attach onto abiotic surfaces; thus, this study demonstrates that lactic acid efficiently retards their initial attachment and biofilm formation.

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