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Evaluation of Silver and Diamond-Like Carbon Coatings for Biofouling Mitigation in the Fresh-Cut Industry

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Abstract

Bacterial attachment to equipment surfaces and further biofilm formation is one of the major problems in the food industry, particularly in the fresh-cut food industry. Silver and two Diamond-Like Carbon (DLC) coatings modified by incorporation of silicon (a-C:H:Si or SICAN) or silicon and oxygen (a-c:H:Si:O or SICON®) were compared to uncoated stainless steel regarding their potential to reduce or inhibit such process. Adhesion experiments were performed using industrial process water from a salad washing line, and the same water spiked with the Gram-positive *Bacillus aryabhattai* that was isolated from that line. Results for the industrial water have shown that the coatings led to reduced initial bacterial adhesion, even though this reduction was not sustained for longer contact times. When the industrial water was spiked with *B. aryabhattai*, the coatings allowed a reduction in both adhesion and initial biofilm formation of about 60%.

Keywords: Biofouling; Bacterial adhesion; Fresh-cut industry; Diamond-Like Carbon (DLC) coatings; Silver; Stainless steel

Abbreviations

Diamond-Like Carbon: DLC; SICAN: a-c:H:Si; SICON®: a-c:H:Si:O; *Escherichia coli*: *E. coli*; Microtiter Plates: MTPs; Computational Fluid Dynamics: CFD; Volume Of Fluid: VOF; *Bacillus aryabhattai*: *B. aryabhattai*; 4,6-Diamino-2-Phenylindole: DAPI; Wall Shear Stress: WSS

Introduction

Bacteria are part of the indigenous flora of raw materials used for food production, hence it is not surprising that they are able to colonize a wide variety of surfaces in food industry settings (e.g. food processing equipment, walls and floors, storage tanks) [1]. Adhesion of microorganisms to such surfaces often results in further biofilm development, which can lead to a wide range of problems such as contamination and spoilage of processed foods, surface corrosion, and deterioration of equipment components [2]. Product contamination is of special concern, especially in the fresh-cut industry, since it presents a hazard to human health [3]. Additionally, it has been shown that most food borne pathogens are able to adhere and form biofilms on food contact surfaces [4]. On the other hand, surface corrosion and equipment damage translate into increased costs for the industry [5].

Stainless steel has become the most commonly used surface in the food industry during the last decades, mainly due to its resistance to extreme temperatures, as well as its good cleanability and corrosion resistance [6]. It has been suggested that reducing initial bacterial adhesion is one of the most promising strategies to reduce biofouling [7]. As a result, demand for engineered surfaces with specific properties that can reduce bacterial adhesion has been the focus of intensive study for the past years.

The antimicrobial properties and low toxicity of silver have been recognized for a long time, and therefore silver deposition onto surfaces that come in contact with food products has been suggested as a good strategy to reduce bacterial adhesion [2]. Hydrogenated amorphous carbon (a-C:H), also called Diamond-Like Carbon (DLC) coatings, have also raised great interest among the food industry community, mainly due to their excellent thermal conductivity, hardness, as well as resistance to wear and corrosion [8,9]. Two types of silicon-doped DLC coatings, SICAN (a-c:H:Si) and SICON® (a-c:H:Si:O) have also been demonstrated to be effective in reducing protein fouling on heat exchanger surfaces [10]. Using a pure *Escherichia coli* culture, it was found on previous studies that none of these surfaces was able to reduce bacterial adhesion, whereas SICON® was able to reduce the amount of biofilm formed over a 24 h period [11, 12]. More recently, SICON® was shown to

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reduce the short-term growth of an *E. coli* strain in single-and dual-species biofilms, as well as the colonization of a *Pseudomonas* isolate after 3 days of incubation [13]. Nevertheless, these studies used either pure cultures or a maximum of two microorganisms, and microbial growth was evaluated using an artificial medium.

The main goal of this study was to evaluate the potential of different surfaces on the reduction of cell adhesion and initial biofilm formation using the natural flora present in a salad washing line and to observe how that performance was affected by the bacterial load of the water (by spiking with a bacterial isolate from that same washing line). To mimic the real industrial settings, adhesion and biofilm formation assays were conducted at the standard factory operation conditions regarding temperature and hydrodynamics. Biofilms were formed in agitated 6-well microtiter plates (MTPs) and the characteristics of the flow inside the wells were determined by Computational Fluid Dynamics (CFD).

Materials and Methods

CFD analysis

The flow behaviour inside the wells was determined using Ansys Fluent CFD package (version 14.5). A cylindrical well (diameter of 34.8 mm and height of 48.3 mm) containing liquid (4 mL of water) and gas (air, filling the remaining volume) was built in Design Modeller 14.5 and discretized into a grid of 62,748 hexahedral cells by Meshing 14.5. The simulation was performed at a shaking frequency of 160 rpm and an orbital radius of 4 mm at 5ºC. The two-phase flow in the vessel was simulated by the volume of fluid (VOF) methodology [14], and the air/water interface was determined by the Geo-Reconstruct method [15]. The simulation was performed as previously described [16]. After the stabilization of the flow, the average instantaneous shear stress was obtained by integrating an instantaneous solution over the bottom surface of the well (corresponding to the coupon surface). The time-averaged shear stress was calculated by averaging the instantaneous shear stress throughout a complete rotation cycle.

Bacteria and culture preparation

The process water was collected from the washing line and transported in refrigerated conditions (for 4 h at 4ºC) to the laboratory. The bacterial composition of the water was determined as previously described [17] and included *Rahnella aquatilis, Pseudomonas poae, Enterobacteriaceae bacterium, Bacillus aryabhattai,* and *Pseudomonas* sp. The bacterial load of that water was of 1.12×10^5 cells ml^{-1}. From the different isolates that were obtained from the washing line, *B. aryabhattai* was selected for this study since it produced the highest number of virulence factors, including siderophores, proteases and gelatinases [17] and because it may be an early colonizer. A starter culture of this microorganism was prepared by inoculating 0.2 l of inoculation media [5.5 g l^{-1} glucose, 2.5 g l^{-1} peptone, 1.25 g l^{-1} yeast extract in phosphate buffer (1.88 g l^{-1} KH_2PO_4 and 2.60 g l^{-1} Na_2HPO_4) at pH 7.0] with 500 μL of a glycerol stock (kept at -80ºC), as described in [18]. Cultures were grown in a 1 l shake-flask and incubated overnight at 30ºC with orbital agitation (120 rpm). A volume of 50 ml from the overnight grown cultures was used to harvest cells by centrifugation (10 min, 3202 *g*). Cells were then washed with saline solution (8.5 g l^{-1} NaCl), and the pellet was resuspended in the process water to yield a final bacterial load of 3.4×10^7 cells ml^{-1}.

Surfaces and cleaning procedure

Round coupons (1 cm diameter) made from electropolished stainless steel and stainless steel coupons coated with SICAN (a-c:H:Si), SICON® (a-c:H:Si:O) and silver were prepared by the Fraunhofer Institute for Surface Engineering and Thin Films (Braunschweig, Germany) and a detailed description of the coatings preparation method is given in references [19].

Surfaces were disinfected using a protocol that mimics the equipment disinfection procedure used in the salad washing industry from where the process water was retrieved. Briefly, coupons were first placed in a glass beaker containing 150 ml of distilled water and stirred for 20 min at 150 rpm (CERTOMAT® BS-1, Sartorius, Goettingen, Germany). Next, coupons were soaked with 2% (v/v) TEGO® (JohnsonDiversey, Goldschmidt AG, Germany), a commercial disinfectant used in the washing line, for 20 min under agitation at 150 rpm (typical contact time in the washing line). Coupons were then aseptically rinsed and immersed in sterile distilled water for 20 min with agitation to completely remove the disinfectant, thus simulating the industrial rinsing.

Adhesion assays

The disinfected coupons were aseptically fixed at the bottom of 6-well MTPs (VWR Internacional, Carnaxide, Portugal) using double-sided tape and covered with 4 ml of (i) process water only or (ii) *B. aryabhattai*-spiked process water. Industrial process water was used to mimic the processing conditions present in the salad washing industry and the *B. aryabhattai*-spiked process water was used to evaluate the performance of the different surfaces in a situation of increased microbial contamination of the vegetables (that can occur during cultivation or harvest). Plates were then incubated at 5ºC (the working temperature in the industry where the water was collected from) with agitation (160 rpm, IKA KS 130 basic, Staufen, Germany). Coupons were removed from the MTPs at defined time points (0.5 and 6 h) and gently dipped into sterile saline solution (8.5 g l^{-1} NaCl) to remove loosely attached cells prior to enumeration. Results originated from three independent experiments with process water samples collected at three consecutive days.

Bacterial cell enumeration

In order to quantify the number of adhered cells, the coupons were stained with 4,6-diamino-2-phenylindole (DAPI; MerckMillipore, USA) at 0.1 $mgml^{-1}$ and left in the dark for 10 min. Cells were then visualized under an epifluorescence microscope (Nikon Eclipse LV100, Japan) incorporating a camera (Nikon digital sight DS-RI 1, Japan). Images were acquired using a ×100 oil immersion fluorescence objective, and a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). At least 10 fields from each coupon were acquired and the number of adhered cells per field was counted. The number of bacterial cells was then divided by the surface area of the field of view to obtain the number of cells per square centimeter.

Data analysis

The total number of adhered cells on each surface was compared to the number of cells adhered to stainless steel since it is the most widely used surface material in the salad washing industry [20]. Results are presented as mean ± standard deviation of three independent assays. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.). Comparisons were made using unpaired Student's t-test (two-tailed) to evaluate if statistically significant differences were obtained between stainless steel and modified surfaces (Figure 2). Statistical analysis corresponding to each time point is represented with an asterisk for a confidence level greater

than 90% (P<0.1) and with double asterisks for a confidence level greater than 95% (P<0.05). Two-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparison test between groups.

Results and Discussion

Agitated 6-well MTPs were used for the adhesion and initial biofilm formation assays. The flow inside the wells containing the surface coupons was analyzed by CFD. The simulation shows that the shear stress in the coupon surface is not uniform at any given time with maximum shear stresses around 0.2 Pa (Figure 1). However, the shear stress field was very uniform during a complete cycle (not shown), and the time-averaged shear stress in the coupon surface was 0.14 Pa. This range of shear stresses can be found in critical zones (washing tank corners, valves, pumps, etc.) in industrial plants [21-25], which is a further confirmation that the conditions used in this work mimic the industrial settings.

Bacterial adhesion experiments were performed with industrial process water in order to simulate the processing conditions present in a salad washing industry. Two time points (0.5 and 6 h) were assayed so that distinct phases of bacterial adhesion process, i.e., primary, reversible adhesion [26], and initial biofilm formation could be observed. Longer contact times were not tested as a 6-8 h cleaning interval is typical of the salad washing industry [11].

Cell adhesion results are shown in Figure 2. When process water was tested alone (Figure 2a), it was found that the total number of adhered cells after 0.5 h was lower on the three surfaces tested than on stainless steel (although statistically significant differences were only obtained for SICON, P<0.1). The adhesion results at this point exhibited a large variability between independent experiments. This may be explained by the weak physicochemical interactions between bacterial cells and the substratum surface at this early stage of adhesion [27] and the fact that the initial adhesion process is reversible [26]. Additionally, the large diversity of bacterial species competing for the colonization of the surface may result in such variability as on different occasions, different types of bacteria may reach the surface first and prevent adhesion by other bacteria. At a later time point (6 h), only silver seemed to exhibit slightly better performance at inhibiting cell adhesion, even though not at a statistically significant level. At this point, the reversible character of the bacterial-surface interaction is decreased, and this may explain the lower variability in adhered cells obtained at this stage.

Besides testing the natural flora from the process water, we also wanted to evaluate the performance of the surfaces when challenged with a much higher microbial load (300-fold higher). For this test, we have spiked the process water with a *B. aryabhattai* isolate from the same process line. The results shown in Figure 2b demonstrate that all the surfaces performed better than the standard stainless steel at both time points, although the differences obtained at 0.5 h are not statistically significant. After 6 h, reductions in the number of adhered bacteria were of approximately 60% when compared to stainless steel. The results suggest that the performance of the modified surfaces is increased at higher bacterial loads, and since *B. aryabhattai* is present at a much higher concentration in this spiked water, it is likely that it may be an early colonizer in these conditions. If this is true, this isolate may protect the surfaces from colonization by other organisms, which may also be competing with the isolate for nutrients and space, thus reducing the overall cell count in these

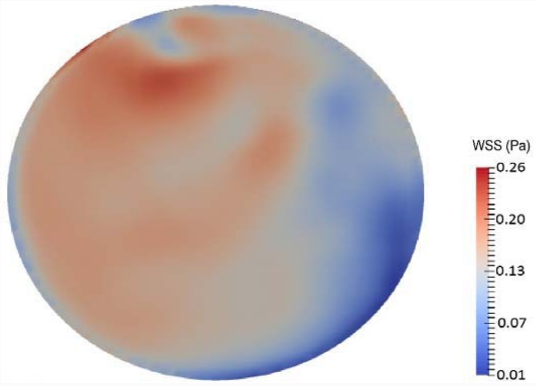

Figure 1: Wall shear stress (WSS) field at the coupon surface obtained by CFD simulation.

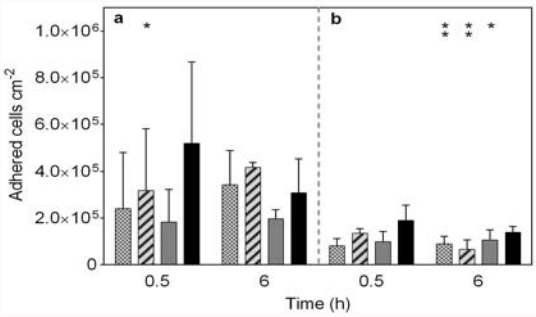

Figure 2: Number of adhered cells on each tested surface [SICAN ( ); SICON® ( ); silver ( ); and stainless steel ( )] after 0.5 and 6 h in: a) industrial process water, b) *B. aryabhattai*-spiked industrial process water. Results are shown as mean ± standard deviation of three independent experiments. Significant differences from the control surface (stainless steel) for the same time point are depicted as *(P<0.1) or **(P<0.05).

conditions as it can be seen when comparing Figure 2a and Figure 2b. This protective effect was already demonstrated with another industrial isolate (*Pseudomonas grimontii*) using the same DLC coatings and *E. coli* as a model pathogen [13]. Although the bacterial load used in this spike test is much higher than one can typically find in the washing line, such level of bacterial concentration has actually been reported on the factory from where the process water was retrieved, especially during periods of intense rainfall when mud accumulation in the vegetables leaves is higher. Additionally, the flow velocity in critical process areas like corners or joints is very low [25], which can lead to a several-fold increase in cell concentration due to sedimentation [28] in quasi-stagnant flow.

Conclusion

To the best of our knowledge, this is the first study that evaluates silver and DLC coatings in conditions that mimic the settings of a standard salad washing industry in terms of bacterial composition, growth conditions, and hydrodynamic regime. Given the cost of these coatings [29], it is likely that their use may be restricted to specific critical process areas (e.g., crevices, corners, joints and valves) which are harder to clean and where lower fluid velocities may be found, making these zones suitable niches for biofilm accumulation and growth [11]. The results presented in this work also show that such a capital investment may be more appropriate for processes subjected to high microbial loads where significant reductions in bacterial counts were observed, leading to a higher hygienic level of the process.

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References

1. Srey S, Jahid IK, Ha SD. Biofilm formation in food industries: A food safety concern. Food Control. 2013; 31: 572-585.
2. Araújo EA, Andrade NJ, Silva LHM, Bernardes PC, Teixeira ÁVN, Júnior JFQ, *et al.* Modification of stainless steel surface hydrophobicity by silver nanoparticles: strategies to prevent bacterial adhesion in the food processing. Journal of Adhesion Science and Technology. 2013; 27: 2686-2695.
3. Pompermayer DMC, Gaylarde CC. The influence of temperature on the adhesion of mixed cultures of *Staphylococcus aureus* and *Escherichia coli* to polypropylene. Food Microbiology. 2000; 17: 361-365.
4. Bridier A, Sanchez-Vizuete P, Guilbaud M, Piard JC, Naitali M, Briandet R. Biofilm-associated persistence of food-borne pathogens. Food Microbiology. 2015; 45: 167-178.
5. Brooks JD, Flint SH. Biofilms in the food industry: problems and potential solutions. International Journal of Food Science and Technology. 2008; 43: 2163-2176.
6. Detry JG, Sindic M, Deroanne C. Hygiene and cleanability: a focus on surfaces. Critical Reviews in Food Science Nutrition. 2010; 50: 583-604.
7. Busscher HJ, van der Mei HC, Subbiahdoss G, Jutte PC, van den Dungen JJ, Zaat SA, *et al.* Biomaterial-associated infection: locating the finish line in the race for the surface. Science Translational Medicine. 2012; 4: 153rv10-153rv10.
8. Rosmaninho R, Santos O, Nylander T, Paulsson M, Beuf M, Benezech T, *et al.* Modified stainless steel surfaces targeted to reduce fouling-Evaluation of fouling by milk components. Journal of Food Engineering. 2007; 80: 1176-1187.
9. Zhao Q, Su X, Wang S, Zhang X, Navabpour P, Teer D. Bacterial attachment and removal properties of silicon- and nitrogen-doped diamond-like carbon coatings. Biofouling. 2009; 25: 377-385.
10. Boxler C, Augustin W, Scholl S. Influence of surface modification on the composition of a calcium phosphate-rich whey protein deposit in a plate heat exchanger. Dairy Science Technology. 2014; 94: 17-31.
11. Moreira JMR, Fulgêncio R, Alves P, Machado I, Bialuch I, Melo LF, *et al.* Evaluation of SICAN performance for biofouling mitigation in the food industry. Food Control. 2016; 62: 201-207.
12. Moreira JMR, Fulgêncio R, Oliveira F, Machado I, Bialuch I, Melo LF, *et al.* Evaluation of SICON® surfaces for biofouling mitigation in critical process areas. Food and Bioproducts Processing. 2016; 98: 173-180.
13. Gomes LC, Piard JC, Briandet R, Mergulhão FJ. *Pseudomonas grimontii* biofilm protects food contact surfaces from *Escherichia coli* colonization. LWT - Food Science and Technology. 2017; 85: 309-315.
14. Hirt CW, Nichols BD. Volume of fluid (VOF) method for the dynamics of free boundaries. Journal of Computational Physics. 1981; 39: 201-225.
15. Youngs DL. Time-dependent multi-material flow with large fluid distortion. Numerical Methods for Fluid Dynamics. 1982.
16. Gomes LC, Moreira JMR, Teodósio JS, Araújo JDP, Miranda JM, Simões M, *et al.* 96-well microtiter plates for biofouling simulation in biomedical settings. Biofouling. 2014; 30: 535-546.
17. Meireles A, Fulgêncio R, Machado I, Mergulhão F, Melo L, Simões M. Characterization of the heterotrophic bacteria from a minimally processed vegetables plant. LWT - Food Science and Technology. 2017; 85: 293-300.
18. Teodósio JS, Simões M, Melo LF, Mergulhão FJ. Flow cell hydrodynamics and their effects on *E. coli* biofilm formation under different nutrient conditions and turbulent flow. Biofouling. 2011; 27: 1-11.
19. Grischke M, Hieke A, Morgenweck F, Dimigen H. Variation of the wettability of DLC-coatings by network modification using silicon and oxygen. Diamond and Related Materials. 1998; 7: 454-458.
20. Santos O, Nylander T, Rosmaninho R, Rizzo G, Yiantsios S, Andritsos N, *et al.* Modified stainless steel surfaces targeted to reduce fouling–surface characterization. Journal of Food Engineering. 2004; 64: 63-79.
21. Lelièvre C, Legentilhomme P, Gaucher C, Legrand J, Faille C, Bénézech T. Cleaning in place: effect of local wall shear stress variation on bacterial removal from stainless steel equipment. Chemical Engineering Science. 2002; 57: 1287-1297.
22. Jensen BBB, Friis A. Predicting the cleanability of mix-proof valves by use of wall shear stress. Journal of Food Process Engineering. 2005; 28: 89-106.
23. Liu Z, Lin YE, Stout JE, Hwang CC, Vidic RD, Yu VL. Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. Journal of Applied Microbiology. 2006; 101: 437-442.
24. Cunault C, Faille C, Bouvier L, Föste H, Augustin W, Scholl S, *et al.* A novel set-up and a CFD approach to study the biofilm dynamics as a function of local flow conditions encountered in fresh-cut food processing equipments. Food Bioproducts Processing. 2015; 93: 217-223.
25. Cunault C, Faille C, Briandet R, Postollec F, Desriac N, Benezech T. *Pseudomonas* sp. biofilm development on fresh-cut food equipment surfaces–a growth curve–fitting approach to building a comprehensive tool for studying surface contamination dynamics. Food Bioproducts Processing. 2018; 107: 70-87.
26. Dunne WM. Bacterial Adhesion: Seen Any Good Biofilms Lately? Clinical Microbiology Reviews. 2002; 15: 155-166.
27. Boks NP, Norde W, van der Mei HC, Busscher HJ. Forces involved in bacterial adhesion to hydrophilic and hydrophobic surfaces. Microbiology. 2008; 154: 3122-3133.
28. Li J, Busscher HJ, van der Mei HC, Norde W, Krom BP, Sjollema J. Analysis of the contribution of sedimentation to bacterial mass transport in a parallel plate flow chamber: Part II: Use of fluorescence imaging. Colloids Surf B Biointerfaces. 2011; 87: 427-432.
29. Gomes da Cruz L, Ishiyama EM, Boxler C, Augustin W, Scholl S, Wilson DI. Value pricing of surface coatings for mitigating heat exchanger fouling. Food and Bioproducts Processing. 2015; 93: 343-363.