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ORIGINAL ARTICLE

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Effect of micro-pattern topography on the attachment and survival of foodborne microorganisms on food contact surfaces

Jaesung Lee | Melvin A. Pascall

Department of Food Science and Technology, Ohio State University, Columbus, Ohio

Correspondence
Jaesung Lee, Department of Food Science and Technology, Ohio State University, 2015 Fyffe Road, Columbus, OH 43210.
Email: lee.1506@osu.edu

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Abstract

This study investigated the use of a nonchemical antimicrobial approach to limit the contamination and survival of foodborne bacteria on polymeric films. This was done by monitoring the difference between micropatterned topography films and regular (smooth) films for the attachment and survival of *Escherichia coli* and *Listeria innocua* during drying, biofilm formation, and sanitization. Initial bacteria attachments and numbers of survivors on the micro-pattern topography films were significantly lower in 3 hr short-term drying tests. Contaminated buffer solution and a milk-based medium at 10 and 23 °C, for 5 days, were used for the biofilm formation and sanitization tests. No significant differences in numbers of viable bacteria in the biofilms from the two test films were observed prior to cleaning and sanitizing. Viable cells were not detectable on the micro-pattern topography films after the application of sanitizing solutions, while evidence of cell viability was seen on the smooth films.

Practical applications

The overall results suggest that the attachment and survivability of foodborne bacteria on films can be inhibited by using physical modification of food contact surfaces. Development of the micro-pattern materials can also be helpful to reduce the number of viable bacteria on food contact surfaces. This will delay the reformation of biofilms after sanitization.

1 | INTRODUCTION

Cross-contamination from improperly cleaned surfaces continues to be a major source of bacterial transfer to humans and the ultimate cause of many infections. This has been reported by many studies which focused on bacterial adhesion to and transfer between biomaterial surfaces to humans at health institutions and in food processing establishments (Dancer, 2014; French et al., 2004; Otter, Yezli, & French, 2011). However, few published reports document cross-contamination between contaminated inanimate surfaces and resultant infections experienced by personnel in and around food manufacturing. At the same time, cross-contamination during food preparation was identified in several reports as a major factor associated with foodborne illness (Kusumaningrum, Van Asselt, Beumer, & Zwietering, 2004). These reports illustrate cross-contamination between inadequately sanitized food contact surfaces coming into contact with ready-to-eat foods. In many cases the problem originated with exposure to contaminated animal and dairy products (Keeratipibul & Lekroengsin, 2008). Although several approaches have been done to prevent this problem, the issue continues and it requires the continued quest for alternative methods to minimize the prevalence and spread of pathogenic microorganisms.

To minimize the growth, survival and formation of biofilms by harmful microorganisms, the incorporation of antimicrobial metals such as silver and copper (Lee, Lee, Jones, Sharek, & Pascall, 2011; Salgado et al., 2013), or chemical agents such as polyethylene glycol, alkanethiols, and quaternary surfactants (Cheng, Zhang, Chen, Bryers, & Jiang, 2007; Hou et al., 2007; Park et al., 1998), on the surface or in the matrix of food contact materials have been used. However, the concentration of residual chemicals on these materials could be a major concern if they are toxic and negatively impact the health of workers and or the consuming public (Gaulin, Le, Shum, & Fong, 2011).

As an alternative to chemically absorbed antimicrobial compounds in packaging materials, a physical approach has been applied to limit bacterial growth on inanimate surfaces. Initially, a surface energy model was developed to compare the interaction between fluid and solid phases with bioadhesion to characterize antifouling topographies. This model was designed using the concept of a biomimetic structure on the skin of sharks. As a result, an engineered roughness index was

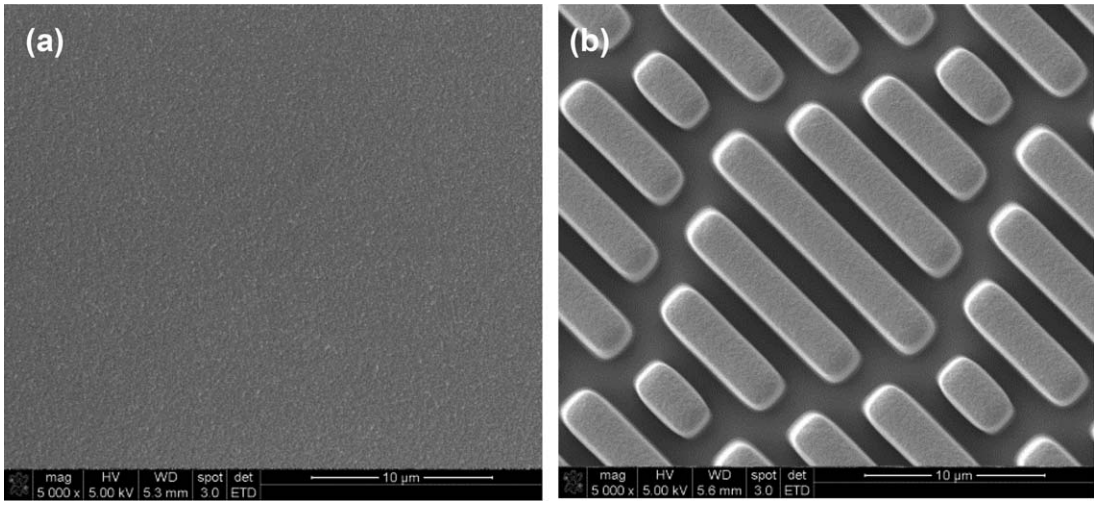

FIGURE 1 Scanning electron microscopic images of the surfaces of (a) smooth and (b) micro-pattern topography films

developed as an extension of this model and polymeric materials with unique topographic variants were designed as seen in Figure 1. Recent studies have shown that micro-patterns imprinted on polymer surfaces can indeed retard the colonization of microorganisms in clinical settings (Chung et al., 2007; May et al., 2015; Reddy et al., 2011).

The purpose of this study was to evaluate the effect of micro-pattern topography on attachment, survivability, and biofilm formation of foodborne microorganisms on polymeric food contact surface materials. The effect of food sanitization processes on the biofilms was also investigated. In addition, different cell enumeration methods on numbers of survivors from the surface of films were compared for each assay. *Escherichia coli* K12 (gram-negative bacteria) and *Listeria innocua* (gram-positive bacteria) were tested as surrogates for *E. coli* O157:H7 and *Listeria monocytogenes*. The use of surrogates has been proven to be a practical alternative for foodborne pathogen testing (Moce-Livina, Muniesa, Pimenta-Vale, Lucena, & Jofre, 2003).

2 | MATERIALS AND METHODS

2.1 | Sample fabrication

Polymer film materials with and without (smooth) the selected micro-patterns were provided by Sharklet Technologies Inc. for testing. These samples were secured to the bottom of 100 mm Petri dishes. All samples were sprayed with 70% ethyl alcohol, rinsed with sterile de-ionized water then allowed to completely dry before use.

2.2 | Bacteria preparation

E. coli K12 (ATCC 29181) and *L. innocua* Seeliger (ATCC 33090) cells were grown on Tryptic soy agar (TSA) slants and transferred to Tryptic soy broth, then incubated at 37 °C for 21 hr. The final cell concentrations in the broth were 9–10 log CFU/ml. The cells were then harvested by centrifugation (Sovall RC5C Plus, Newtown, CT) at 10,000 g for 10 min at 4 °C. The supernatant was decanted, and the pelleted cells were re-suspended in 20 ml of sterile phosphate buffer solution (PBS, pH 7.2) to obtain viable cell populations of approximately 9 log CFU/ml. The cell suspensions were serially inoculated into sterile PBS and mixed to give desired initial numbers.

2.3 | Attachment and survivability assay

A 25 ml volume of each cell suspension (10^5 CFU/ml) was added to Petri dishes containing test films (smooth or micro-pattern topography) and incubated at 23 ± 1 °C for 1 hr to allow for bacterial attachment. The bacterial cells were removed by pouring off the suspension. Each film in a dish was washed three times by gently pouring 20 ml PBS onto them, waiting 5 s, swirling for 10 s, and pouring off the PBS. After the final rinse, any remaining large PBS wash droplets were removed by firmly shaking the dish three times. The cell counts at time zero were obtained when cells on the inoculated films were cultured immediately after rinsing to quantify the extent of bacterial adhesion. Subsequent time-points were defined by the duration of exposure in a laminar air flow hood at 23 ± 1 °C. All Petri dishes for these time-points were left in the hood with the lids ajar before sampling at 1 and 3 hr. This dry period is recommended to simulate the normal practice in restaurants and food service institutions (NSF/ANSI, 2012).

In the first part of this sampling, a Replicate Organism Direct Agar Contact (RODAC) plate method was used to collect bacterial cells from the surface of the films. Three smooth and three micro-pattern topography films were touch-inoculated onto RODAC plates (TSA with 0.07% Lecithin and 0.5% Polysorbate 80, 60-mm diameter) for 3–5 s to recover attached bacterial cells from the surface of the films. Viable cell counts were determined after 36 hr incubation at 37 °C. In the second part of the sampling, hygiene swabs were used to collect the organisms from the same sized surface circle area (60 mm diameter) of three smooth and three micro-pattern topography films. These swabs were made with sterile calcium alginate fiber tips on wood applicators (Fisher Scientific, Pittsburgh, PA). They were moistened before use with sterile maximum recovery diluent (MRD) supplied by Oxoid Limited (Basingstoke, UK). These swabs were used to transfer bacterial cells to test-tubes containing 2 ml of the MRD. These tubes were then

vigorously vortexed to release the cells from the fiber tip of the applicators. The cell suspensions were serially diluted and plated onto TSA to determine their viable counts after 24 hr incubation at 37 °C.

2.4 | Biofilm formation and sanitization

Two contaminating solutions (PBS and a milk-based medium), for 5 days, were tested for their possible influences on cell adherence and biofilm formation. A 2% reduced fat ultra-high temperature milk was used as the milk-based medium. An initial amount (10^7 CFU/ml) of bacterial cells in the contaminating solutions (20 ml) were poured into the Petri dishes containing the attached films. The Petri dishes were sealed and incubated at pre-established temperatures. After 5 days of incubation at 23 °C, the contaminating solutions were withdrawn and the films rinsed (10 s) twice with sterile phosphate buffer to release the nonadherent cells. The films containing biofilms were reduced to 8-mm round pieces using Miltex disposable biopsy punches (Cardinal Health, OH) and detached from the Petri dishes using a sterile stainless steel tweezer. These film pieces were placed in test-tubes containing peptone water. Cells in the biofilm that adhered to the polymeric (silicone) films were collected by duplicate processes of vortexing (30 s) and sonication (30 s at 42 kHz) in a Kendal Commercial Ultrasonic Cleaner. This process has been recommended to remove biofilm cells from silicone material (Hamilton, 2003). The samples were serially diluted, and plated onto TSA plates to establish the bacterial cells survival after incubation.

One detergent and two sanitizers (quaternary ammonium compounds [QAC] and sodium hypochlorite [chlorine]) commonly used to clean food contact surfaces in food processing plants were used in this part of the study. After 5 days of incubation the contaminating solutions (milk and PBS) were withdrawn and the films rinsed (10 s) twice with the phosphate buffer to release the nonadherent cells. Bacteria on the unwashed (control) films were detached by the 8 mm biopsy punch. Other pieces of films were also detached and used for the cleaning process (washing for 30 s in a Petri dish with 20 ml of washing solution [1% alkaline detergent] and rinsing for 5 s by spraying water on to it). For the sanitization process, two of the films that were rinsed were placed in a Petri dish with 20 ml of a sanitizing solution (200 ppm of QAC or 100 ppm of Chlorine) and immersed for 30 s. After each step, the film samples were withdrawn and immersed in Difco neutralizing buffer (#236210, Difco laboratories, Sparks, MD) solution to inactivate QAC and Chlorine. The bacterial cells on the film surfaces were collected by duplicate processes of vortexing (30 s) and sonication for 30 s at 120 W. The samples were serially diluted, and plated onto TSA to establish their survival after incubation.

For the scanning electronic microscopy (SEM), film pieces containing *L. innocua* were taken out before and after the cleaning process and fixed with 10 ml 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The fixatives were left in contact with the samples overnight at 4 °C. The samples were then washed with buffer and post-fixed for 1 hr in 1% osmium tetroxide in phosphate buffer. Following this, the samples were rinsed with the buffer, dehydrated with serial concentrations of ethanol, and then dried on a critical point drier. The dried cells were coated with gold-palladium and examined by a Philips XL-30 scanning electron microscope at 30 kV (FEI, Inc., Hillsboro, OR).

2.5 | Quantitative microbial analysis

All experimental trials were repeated at least twice. No less than three sample replicates were used for each trial. The significances between the mean values of the microbial viability were analyzed by multifactor analysis of variance (ANOVA). The data analyses were performed by the General Linear Model function and Tukey's comparison testing with Minitab 16 (State College, PA) to determine the level of significance between film types and the viability of the tested microorganisms. The level of significance was set at $p < .05$.

3 | RESULTS AND DISCUSSION

3.1 | Bacterial attachment and survival on micro-pattern and smooth films at short-term drying condition

The film samples tested in this part of the study were smooth and micro-pattern topography acrylic polymeric films. The bacterial attachment (at time 0) and their survivability (after drying for 1 and 3 hr) for *E. coli* and *L. innocua* on the smooth and micro-pattern topography films are shown in Table 1. The results show that the initial viable counts of attached cells of both bacterial strains on the micro-pattern topography films were not significantly less ($p > .05$) than those on the smooth films (Table 1). After air drying in the laminar air flow hood for 3 hr at 23 °C, the bacterial survivors on the micro-pattern topography films were at least 0.5 log lower than those on the smooth films. No viable *L. innocua* cells were found on the micro-pattern topography films after 3 hr air drying. The number of viable cells on the micro-pattern topography films was lower than that of cells on the smooth surface after short-term air drying indicating the micro-pattern have interfered cell attachment. It has been observed that unattached foodborne bacterial cells are very sensitive to air drying, with one log drop in viability occurring during the 90 min on glass surface (Katz & Hammel, 1987). In a previous survival study at clinical setting, Reddy et al. (2011) reported that the micro-patterns of the sample films caused a reduction (76%) in the viable bacterial cell numbers attached to polymer surfaces after 24 hr of storage. Our result indicated that the effect of micro-pattern on the bacterial survival occurred much earlier when storage was in an air flow condition. The reduction in viable *E. coli* numbers on the surface of the films during the air drying step indicated that the desiccation stability of *E. coli* was higher than that of the *L. innocua*. This result was similar to results obtained from our earlier study where those bacteria were contaminated and dried on the surface of table utensils before ware-washing (Lee, Cartwright, Grueser, & Pascall, 2007).

Two sample collection methods were also evaluated in this attachment study. These were: (1) hygiene swabbing; and (2) RODAC plate testing. It has been known that the maximum recovery (~80%) of microorganisms with nonporous surfaces is similar for both methods

TABLE 1 Bacterial attachment and their survivability for *L. innocua* and *E. coli* on the smooth and micro-pattern topography films at short-term drying conditions

	Drying time (hr)					
	0		1		3	
	Smooth	Micro-pattern	Smooth	Micro-pattern	Smooth	Micro-pattern
L. innocua						
Rodac plate	[a] 2.5 ± 0.5^{A}	1.5 ± 0.4^{AB}	0.7 ± 0.4^{A}	0.4 ± 0.4^{AB}	0.7 ± 0.4^{A}	0.1 ± 0.1^{B}
Cotton Swab	1.3 ± 0.5^{B}	1.0 ± 0.5^{B}	0.1 ± 0.2^{B}	[b]ND	ND	ND
E. coli						
Rodac plate	1.8 ± 0.3^{A}	1.7 ± 0.3^{A}	0.8 ± 0.4^{A}	0.6 ± 0.4^{A}	0.6 ± 0.5^{A}	0.2 ± 0.2^{B}
Cotton Swab	2.0 ± 0.4^{A}	1.4 ± 0.4^{AB}	0.7 ± 0.2^{A}	0.4 ± 0.3^{B}	0.4 ± 0.4^{AB}	0.2 ± 0.2^{B}

[a]Viable counts (log_{10} CFU) of microbial strains on collecting area (28.3 cm^2).
[b]No colony was detected on plates from sample replicates.
Means that do not share a letter are significantly different in the rows at a same drying time.

(Ismaïl et al., 2013; Knechtges, 2012). The results showed that the cell counts for the collected bacterial survivors from the film surfaces were significantly lower for the hygiene swab when compared with the RODAC plate method (Table 1). These results suggest that the RODAC plate method was better suited for quantification of surviving organisms on the test film surfaces. Previous studies showed that the microbial recovery performance of hygiene swab is greatly reduced on dry surface condition when compared to other sampling methods (Foschino, Picozzi, Civardi, Bandini, & Faraday, 2003; Vorst, Todd, & Ryser, 2004). However, the RODAC plate method has been designed for enumerating less than 200 cells on each target surface, and it is not suitable for counting cell numbers in a biofilm where higher than 10^3 cells are normally found (Hart, French, Eitzen, & Ritter, 1973). Also, viable cells still remained on the test films surface after application of the RODAC plate test method. In recent bacterial attachment studies, fluorescence microscopic images were used to enumerate live bacterial cells on a nano-porous membrane surfaces (Feng et al., 2014; Hsu, Fang, Borca-Tasciuc, Worobo, & Moraru, 2013). However, the microscopic method also has obvious detection limits for sizing. Therefore, we modified the arrangement of the films in the Petri dishes by inserting a silicone layer between the film and dish interface. Later, we applied the micro-pattern topography film directly onto the surface of the silicone and used it for the analysis. Silicone can be more easily detached from Petri dishes when compared with double sided adhesive tape. As a result, the silicone films with the imprinted micro-patterns were removed from the Petri dishes using the punch biopsy procedure as a bacteria collection method. Bacteria survivors on the silicone film surface were then enumerated with much better accuracy after a series of vortex and sonicating processes. This modified method was then used for all future biofilm formation and sanitizing tests.

3.2 | Biofilm formation on micro-pattern and smooth films

The abilities of *E. coli* and *L. innocua* to form biofilms were tested on the surface of smooth-silicone and micro-pattern topography-silicone films. The results show that the viable numbers in the milk-based biofilm were higher than 5 log CFU for *L. innocua* after 5 days storage at 23 °C (control numbers in Table 2), while less numbers were observed for *E. coli* at the same time and condition. The literature reports that surface attachment and growth of gram-positive bacteria is less affected by charges from insoluble powders in carrier liquids as well as substratum charges (Speier & Malek, 1982). Since the silicone film surface basically has no net charge (neutral), the charged milk particles could have negatively affected the biofilm formation of *E. coli* cells in this study. As we expected, the viable cell numbers in the biofilm formed at 10 °C was greater for *L. innocua*, which is known as a psychrotrophic bacterium. For the viable counts on the PBS-based biofilm

TABLE 2 Milk-based biofilm formation and efficacy of cleaning and sanitizing on biofilms developed on the two film surfaces after 5 days storage at different temperatures

	Storage temperature (°C)	Smooth				Micro-pattern			
		Control	Washing/ rinsing	Sanitizing A[a]	Sanitizing B[b]	Control	Washing/ rinsing	Sanitizing A	Sanitizing B
L. innocua	23	[c] 6.0 ± 1.0	4.0 ± 0.8	0.7 ± 0.6	0.2 ± 0.2	6.0 ± 0.6	3.3 ± 0.8	[d]ND	0.2 ± 0.1
	10	5.7 ± 0.8	3.8 ± 0.6	0.2 ± 0.2	0.2 ± 0.1	5.8 ± 0.3	3.8 ± 0.7	0.2 ± 0.2	ND
E. coli	23	5.1 ± 0.4	3.2 ± 0.3	0.2 ± 0.2	ND	5.2 ± 0.5	2.8 ± 0.3	ND	ND
	10	4.1 ± 0.2	0.7 ± 0.6	ND	ND	4.2 ± 0.3	0.7 ± 0.7	ND	ND

[a]200 ppm of QAC.
[b]100 ppm of Chlorine.
[c]Viable counts (log_{10} CFU) of microbial strains on detached film piece (0.5 cm^2).
[d]No colony was detected on plates from sample replicates.

TABLE 3 PBS-based biofilm formation and efficacy of cleaning and sanitizing on biofilms developed on the two film surfaces after 5 days storage at different temperatures

	Storage temperature (°C)	Smooth				Micro-pattern			
		Control	Washing/ rinsing	Sanitizing A[a]	Sanitizing B[b]	Control	Washing/ rinsing	Sanitizing A	Sanitizing B
L. innocua	23	4.6 ± 0.7[c]	3.8 ± 0.7	0.4 ± 0.3	0.4 ± 0.4	4.7 ± 0.5	3.2 ± 0.3	ND[d]	ND
	10	4.6 ± 0.5	4.1 ± 0.6	0.3 ± 0.3	0.2 ± 0.1	4.5 ± 0.3	4.0 ± 0.2	ND	ND
E. coli	23	3.5 ± 0.8	3.0 ± 0.6	0.2 ± 0.2	0.2 ± 0.1	3.3 ± 0.4	1.4 ± 0.3	ND	ND
	10	2.2 ± 0.6	0.6 ± 0.4	ND	ND	2.3 ± 0.1	0.4 ± 0.5	ND	ND

[a]200 ppm of QAC.
[b]100 ppm of Chlorine.
[c]Viable counts (log_{10} CFU) of microbial strains on detached film piece (0.5 cm^2).
[d]No colony was detected on plates from sample replicates.

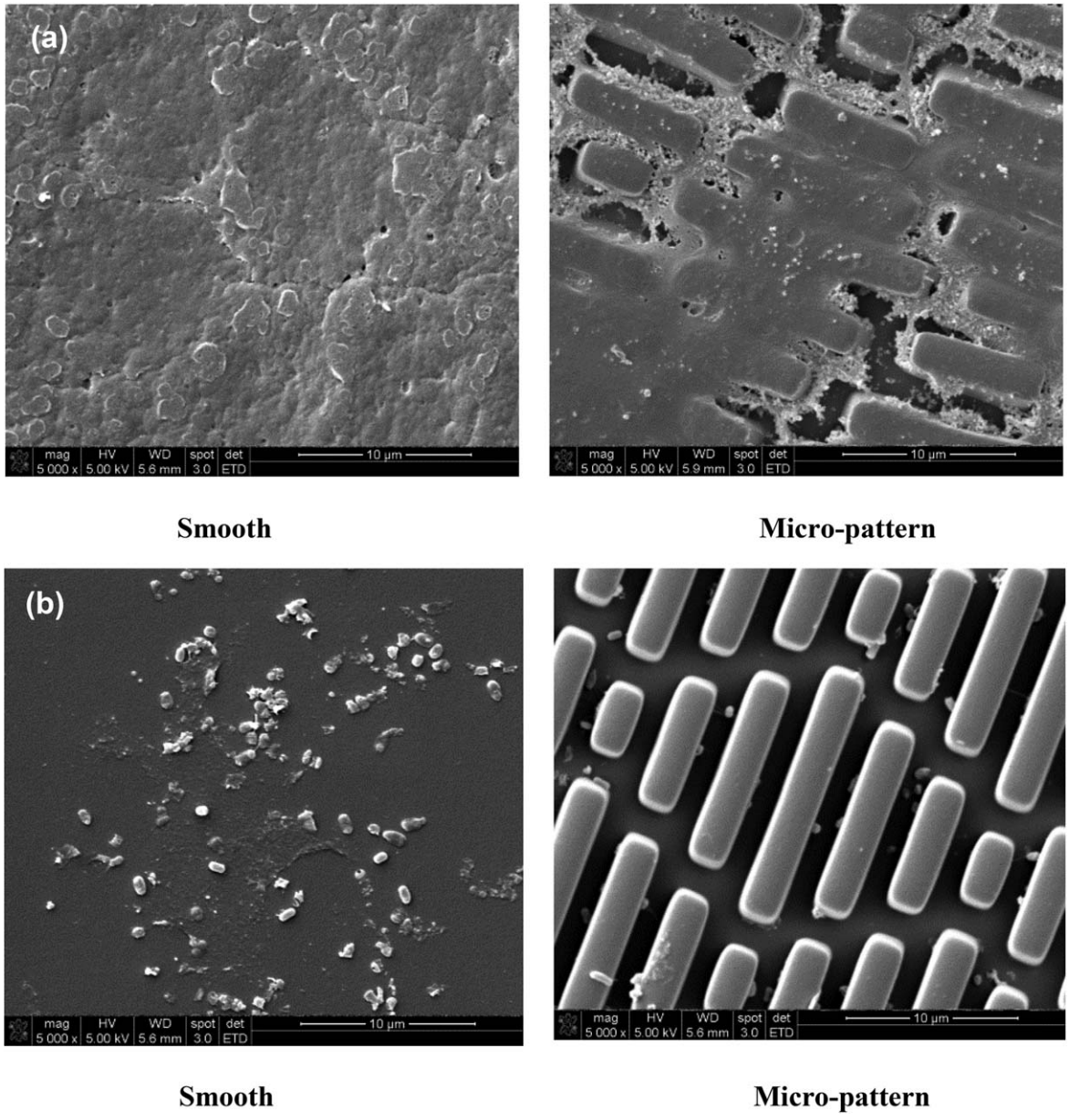

FIGURE 2 *Listeria innocua* cells in milk-based biofilm on the two film surfaces before (a) and after (b) washing and rinsing

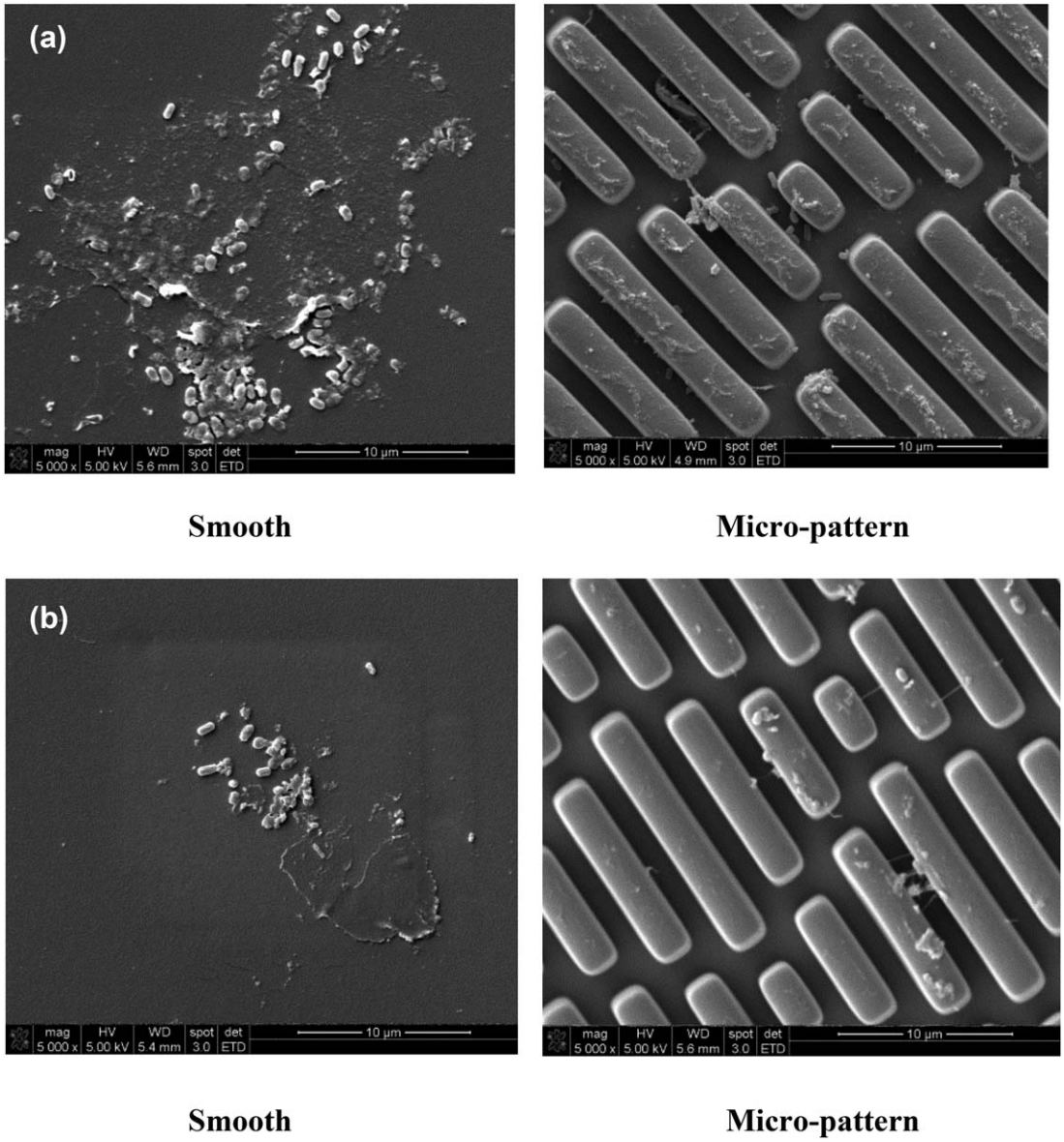

FIGURE 3 *Listeria innocua* cells in phosphate buffer-based biofilm on the two film surfaces before (a) and after (b) washing and rinsing

(control values in Table 3), the numbers were significantly lower ($p < .05$) for both bacteria, especially for *E. coli* cells in the PBS-based biofilm. It is known that biofilms are not able to grow well and mature without nutrients (Percival, Walker, & Hunter, 2000).

The SEM results show that most of the bacterial cells were attached to milk particulate substances that formed sediment on the surface of the films (Figure 2a). This could be why there was no significant difference in viable numbers in biofilms between the micro-pattern topography and the smooth film surfaces at each nutrient condition. A recent biofilm study on foodborne bacteria showed that the test cells were associated with food particulates attached to the abiotic surface rather than the base material surface itself (Brown et al., 2014). This suggests that food matrices could contribute to bacterial biofilm formation by covering and conditioning an abiotic surface and also become a source of nutrients.

3.3 | Biofilm sanitization

The ability of bacteria in the biofilm to survive cleaning (washing and rinsing) and sanitization was investigated. Washing of these samples with a detergent and rinsing with sterile water significantly reduced ($p < .05$) the biofilm associated cells on both film surfaces for *E. coli* and *L. innocua* (Tables 2 and 3). The cleaning efficacy was not significantly different ($p > .05$) on for biofilm associated cells on the micro-pattern topography films than on the cells associated with the smooth films in most of the storage conditions. A further reduction in bacterial numbers was found after the sanitizing process. On most of the micro-pattern topography surfaces, bacteria were not detectable after the application of QAC or chlorine solutions, while the evidence of cell viability was still shown on the smooth film after the sanitization. The presence of milk residue on the film surfaces resulted in greater

numbers of both bacteria when compared with the use of contaminated PBS.

SEM images of the attachment patterns for *L. innocua* cells on both milk and PBS-based biofilms are shown in Figures 2 and 3. After the washing and rinsing steps, bacterial colonies on the micropattern topography films were clearly isolated from each other due to the undulations in the surface of the material. At the same time, cell aggregation patterns are shown in the SEM images of the smooth films. The SEM results suggest that there is relationship between the cell attachment patterns on the films and the effectiveness of the sanitization process. The literature reports that cell aggregation can provide bacteria with a protective mode of colonization in a harsh environment (Alhede et al., 2011). Therefore, we expect that the survivability of the bacterial cells on the micropattern films will be lower than that of cells on the smooth films after sanitization.

We also believe that the cell aggregation patterns could have affected the accuracy of counting the bacterial cells. This is so because one cell could have showed up as one colony during the plate counting. At the same time, an aggregate of cells could have also showed up as one colony. The SEM images clearly showed that most cells on the micro-pattern surface were single units. However, on the smooth film samples, they showed up as clumps of cells. Therefore, although the data show similar numbers of bacteria survival on both surfaces, in reality the numbers on the micro-patterned films appear to be lower. Future research should focus on the use of an imaging technique that would more accurately quantify the actual bacterial cell numbers on the test films.

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