**Research Note**

**Maturation and Survival of Cronobacter Biofilms on Silicone, Polycarbonate, and Stainless Steel after UV Light and Ethanol Immersion Treatments**

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**ABSTRACT**

*Cronobacter sakazakii* cells in biofilms formed on silicone, polycarbonate, and stainless steel coupons immersed in reconstituted powdered infant milk formula were treated with ethanol (10 to 70%) and UV light (12 to 2,160 mW·s/cm²) as antibacterial treatments. Biofilm maturation curves were determined after immersion at 25°C for up to 144 h. Populations increased after subsequent immersion at 25°C for 24 h in reconstituted powdered infant milk formula to the respective maximum levels of 7.96, 7.91, and 6.99 log CFU per coupon. Populations attached to silicone and polycarbonate surfaces to a greater extent than to stainless steel (P < 0.05). Treatment with 10% ethanol did not cause a significant decrease in the level of *C. sakazakii*, but treatment with 30, 40, and 50% ethanol reduced the levels to approximately 1.73, 3.02, and 4.17 log CFU per coupon, respectively. *C. sakazakii* was not detected on any coupon after treatment with 70% ethanol or 2,160 mW·s/cm² UV light. A synergistic effect of sequential ethanol and UV treatments was not observed.

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*Cronobacter* is a recently defined genus that consists of six species. Seventy-eight *Cronobacter* strains (60 *C. sakazakii* [Enterobacter sakazakii], 8 *C. malonaticus*, 5 *C. dublinensis*, 2 *C. muytjensii*, 1 *C. turicensis*, 1 *Cronobacter* genomospecies, and 1 *Cronobacter* sp.) representing clinical and environmental isolates from various geographical locations have been investigated (8). The change in classification was proposed based on the results of DNA-DNA hybridization experiments, biochemical reactions, pigment production, and antibiotic susceptibility (18). Updating the original taxonomy of *E. sakazakii* has resulted in the clear definition of at least five new species based on extensive genotypic and phenotypic evaluation. Iversen et al. (11) proposed that these species be moved to the novel genus *Cronobacter*. These pathogenic species are etiological agents in rare cases of meningitis, necrotizing enterocolitis, and bacteremia in neonates (18, 22, 24, 33). Powdered infant formula and milk powder have been implicated as vehicles in outbreaks of *C. sakazakii* infection (2, 9). *C. sakazakii* was detected in 20 (14.2%) of 141 powdered infant formulas originating from 35 different countries (23).

Attachment of bacterial cells to surfaces may be followed by bacterial growth, production of exopolysaccharides, and biofilm formation (15). Biofilms have been defined as sessile communities of cells attached to a surface or to each other, usually embedded in polymeric substances produced by the bacteria (21). *C. sakazakii* has been reported to attach and form biofilms on silicone, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride (10, 17). Contact of these and other foods containing the pathogen with abiotic or biotic surfaces can result in attachment and biofilm formation. Attachment of biofilms of *C. sakazakii* on equipment surfaces used in formula preparation and in feeding areas or in produce processing plants can increase the risk of infection for infants and immunocompromised adults. *C. sakazakii* colonization on the surfaces of spoons, brushes, and blenders used for infant formula preparation has been documented in a clinical setting where neonatal infections were reported (1, 24, 26, 32). Reuse of enteral feeding tubes and delivery bags after washing can increase the risk of microbial infection (28).

The objectives of the present study were to evaluate the efficacy of ethanol and UV treatments to reduce *C. sakazakii* in biofilms formed on silicone, polycarbonate, and stainless steel coupons immersed in reconstituted powdered infant milk formula (RIMF).

**MATERIALS AND METHODS**

*C. sakazakii* strain and culture conditions. *C. sakazakii* KCTC 2949 was used in this study. The biofilm formation ability of KCTC 2949 and 10 other wild *C. sakazakii* isolates from foods in Korea was tested. We selected this KCTC 2949 strain because of its superior ability to form biofilms and its higher resistance to interventions. A stock culture was maintained at −70°C in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) containing 50% glycerol. Cells were grown in TSB for 24 h at 37°C and subcultured weekly.
Preparation and cleaning treatments for three coupon types. Silicone, polycarbonate, and stainless steel coupons (2 by 2 cm²) were tested in this study. The coupons were sonicated in a 15% phosphoric acid solution for 20 min, rinsed with distilled water, scrubbed for 1 min with a neutral detergent, and rinsed again with distilled water. The washed coupons were dried at 55°C for 2 h and autoclaved for 15 min at 121°C in sealed tubes before use.

Attachment of C. sakazakii to coupons and biofilm formation. C. sakazakii grown in TSB at 37°C for 24 h was centrifuged at 4,000 × g for 15 min at 4°C, and the cells were then suspended in 0.1% peptone water. Sterile coupons were immersed in 100-ml test tubes containing an approximately 7 log CFU/ml cell suspension (10 ml) and incubated at 25°C for 4 h to facilitate attachment of cells to coupons. After incubation, coupons were removed from the test tubes with sterile forceps and washed twice in 200 ml of sterile distilled water. The washed coupons were immersed in 50-ml centrifuge tubes containing 15 ml of RIMF and incubated at 25°C to allow for the maturation of biofilms.

Determination of biofilm maturation curves. After biofilms developed on coupons immersed in RIMF at 25°C for 4 h, the population of C. sakazakii was recorded for up to 144 h. At each sampling time, the coupons were washed in sterile water (200 ml) with gentle agitation for 5 s and then transferred to 50-ml centrifuge tubes containing 15 ml of 0.1% peptone water and 3 g of sterile glass beads. These centrifuge tubes were vortexed for 1 min at maximum speed. Samples were serially diluted in 0.1% peptone water and pour plated on tryptic soy agar (TSA; Difco, Becton Dickinson). Inoculated plates were incubated at 25°C for 36 to 48 h before colonies were counted (12).

Design of disinfection experiments. Disinfection experiments for three treatments (ethanol alone, UV alone, and a sequential ethanol-UV treatment) were performed at room temperature. Coupons immersed in RIMF at 25°C for 24 h for biofilm maturation were washed in 200 ml of sterile water with agitation for 5 s to remove most of the cells not present in or firmly attached to the biofilm matrix. For the ethanol alone treatment, the washed coupons were separately immersed for 5 min in ethanol (10, 30, 40, 50, or 70%) and then immersed in a neutralizing medium for 5 min. The neutralizing medium consisted of 3 g of lecithin (Fluka, Buchs, Switzerland), 30 g of polysorbate 80 (Fluka), 5 g of sodium thiosulfate (Sigma-Aldrich, St. Louis, MO), 1 g of L-histidine (Sigma-Aldrich), and 30 g of saponin (Fluka) per 1,000 ml of sterile distilled water. Neutralized coupons were then autoclaved for 15 min at 121°C. The coupons were subsequently transferred to tubes containing 15 ml of 0.1% peptone water with 3 g of sterile glass beads and vortexed at maximum speed for 1 min to dislodge cells from the biofilms. Samples were then serially diluted in 0.1% peptone water, surface plated on TSA, and incubated at 25°C for 36 to 48 h before colonies were counted. For the UV alone treatment, both sides of the washed coupons were irradiated for various lengths of time (10, 30, 60, 300, 600, 1,200, or 1,800 s). UV treatment was performed in a batch disinfection device. UV lamps of 10, 15, and 20 W (200- to 260-nm low-pressure UV lamp, Sankyo Denki, Yamanashi-ken, Japan) were horizontally suspended 20 cm above the coupons on a prop. The light intensity was measured using a photoradiometer (HD 2102.2, Delta OHM, Padua, Italy) and set at 1.2 mW/cm². Procedures after irradiation were the same as after ethanol treatment except for neutralization. For the sequential ethanol-UV treatment, washed coupons were treated with ethanol (10, 30, 40, or 50%), and then irradiated for 60, 300, 600, or 1,200 s to obtain levels of 72, 360, 720, or 1,440 mW·s/cm², respectively.

Statistical analysis. Coupons were examined at each sampling time in duplicate in all experiments. Data were analyzed using the analysis of variance (ANOVA) procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC). The ANOVA was performed to determine significant differences (P < 0.05) in mean values for silicone, polycarbonate, and stainless steel coupons.

RESULTS AND DISCUSSION

Biofilm maturation curves. Biofilm maturation curves for C. sakazakii biofilms on silicone, polycarbonate, and stainless steel are shown in Figure 1. Populations of 6.20, 6.10, and 5.74 log CFU per coupon, respectively, were attached to the coupon surfaces during immersion in the cell suspension for 4 h at 25°C (at 0 h in Fig. 1). Populations increased after subsequent immersion for 24 h in RIMF to maximum levels of 7.96, 7.91, and 6.99 log CFU per coupon. Cells attached more readily to the silicone and polycarbonate than to the stainless steel (P < 0.05). Everaert et al. (6) reported that a silicone rubber surface attracts huge quantities of adhering yeasts and bacteria cells. Yokota (34), Nakamura (25), and Quirbes et al. (29) found that precipitation of the ε-Cu phase on stainless steel allows Cu ions to be dissolved and thus come into contact with bacteria on the stainless steel coupons. These Cu ions can remove bacteria by destroying the cell wall and membrane because Cu ions extract electrons from the bacteria and induce strong reduction, causing leakage of the cytoplasm and oxidization of the nucleus. Lee (16) studied biofilm formation for 71 strains of C. sakazakii. Eleven of these strains (4, 55, 60, 501B, 800DS, FSM 30, FSM 298, FSM 321, FSM 324, 2.43, and 2.84) readily formed biofilms within 24 h at 22°C. These results are similar to those of the present study in which C. sakazakii formed biofilms fully on all three coupon types after 24 h with no additional biofilm growth during the subsequent 120 h.
Bactericidal efficacy of ethanol and UV treatments against *C. sakazakii* in biofilms. The survival of *C. sakazakii* in biofilms formed on three types of coupons in RIMF after treatment with various concentrations of ethanol (10, 30, 40, 50, and 70%) are shown in Figure 2. Treatment with 10% ethanol did not cause a significant decrease in the level of *C. sakazakii*, but treatment with 30, 40, and 50% ethanol reduced the levels by approximately 1.73, 3.02, and 4.17 log CFU per coupon, respectively. Treatment with 70% ethanol resulted in a reduction in the level of *C. sakazakii* to the limit of detection (<1.18 log CFU per coupon). The bactericidal efficacy of ethanol was similar among all three types of surfaces.

The results of UV treatment (10, 30, 60, 300, 600, 1,200, and 1,800 s) against *C. sakazakii* are shown in Figure 3. UV treatment for up to 60 s resulted in a significant reduction (approximately 2 log CFU per coupon), and treatment for 1,800 s (2,160 mW·s/cm²) reduced the bacterial level to the limit of detection (<1.18 log CFU per coupon). The UV treatment had similar antibacterial effects on all three types of surfaces. Kim et al. (14) reported that UV doses of 60, 30, and 30 mW·s/cm² were required for a 4-log reduction of *Listeria monocytogenes*, *Salmonella Typhimurium*, and *Escherichia coli* O157:H7, respectively, on stainless steel chips (2 by 2 cm²) that had been immersed in a TSB culture of 10⁷ CFU/ml for 10 min, dried for 30 min, and swabbed with sterile cotton for enumeration. However, our results indicated that the UV dose required to achieve a 4-log reduction of *C. sakazakii* in a biofilm on stainless steel coupons (2 by 2 cm², sampled by vortexing) was 720 mW·s/cm². Although the pathogens used in these two studies were different, a higher UV dose was required to kill the pathogen in a biofilm than in a planktonic state on a surface. The population levels of *C. sakazakii* in all biofilms formed on all three coupon types decreased similarly.

The bactericidal efficacy of the sequential ethanol-UV treatment is shown in Table 1. A synergistic effect was not observed under these treatment conditions. Rather, the dual treatment produced the opposite effect. Costerton et al. (4), Lewis (19), and Mah and O’Toole (20) all reported the existence of mechanisms that enhance the resistance of bacteria in biofilms to environmental stresses. Extracellular polymeric substances produced by microorganisms during biofilm formation behave as protective barriers to environmental stresses. The attachment of microorganisms to biotic or abiotic surfaces can be followed by biofilm formation, and this biofilm is strongly resistant to sanitizers (7, 8, 15, 27, 30). Some strains of *C. sakazakii* produce extracellular polysaccharides (17, 31), and the amount produced is thought to be affected by nutrient availability and temperature. The exopolysaccharide produced by *C. sakazakii* in biofilms likely provides a protective barrier to disinfectants. Although *C. sakazakii* cells at or near the biofilm surface can use surrounding nutrients, cells deep within the biofilm matrix probably suffer starvation, which may increase their resistance to stress (13). *Pseudomonas aeruginosa* cells in biofilms were more resistant than nonbiofilm cells to several antibiotics (5). Exposure of coupons to ethanol in our experiments likely increased cell resistance to subsequent disinfection by UV irradiation. Cho et al. (3) found no synergistic effect after a sequential UV-chlorine treatment to kill *Bacillus subtilis* spores in water. These researchers hypothesized that the lack of a synergistic UV plus sanitizer effect was due to the fact that the cell wall of *B. subtilis* was not disrupted, but the pyrimidine of *B. subtilis* DNA formed a double bond to inhibit cell growth.

The maturation and survival characteristics of *C. sakazakii* KCTC 2949 in biofilms on silicone, polycarbonate, and stainless steel coupons immersed in RIMF at room temperature were studied. These results can be useful for developing treatment protocols to control the growth of *C. sakazakii* in biofilms formed on instrument surfaces in...
TABLE 1. Effect of sequential ethanol and UV treatments on the survival of C. sakazakii in biofilms formed on silicone, polycarbonate, and stainless steel coupons in reconstituted powdered infant milk formula.

<table>
<thead>
<tr>
<th>Material</th>
<th>Ethanol conc. (%)</th>
<th>0 s Survival</th>
<th>Effect&lt;sup&gt;a&lt;/sup&gt;</th>
<th>60 s (72 mW·s/cm²)</th>
<th>300 s (360 mW·s/cm²)</th>
<th>600 s (720 mW·s/cm²)</th>
<th>1,200 s (1,440 mW·s/cm²)</th>
<th>Survival</th>
<th>Effect</th>
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<td>10</td>
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<td>5.23</td>
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<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>6.19</td>
<td>5.31</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<tr>
<td></td>
<td>50</td>
<td>3.97</td>
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<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>5.41</td>
<td>4.53</td>
<td>3.21</td>
<td>2.06</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> Synergistic effect of ethanol and UV = log reduction by sequential ethanol and UV disinfection – (log reduction by ethanol disinfection + log reduction by UV disinfection).

<sup>b</sup> ND, not detected (<1.18 log CFU per coupon).

<sup>c</sup> NA, not applicable.

processing plants, hospitals, restaurants, and group food services. These results may contribute to reducing the risk of C. sakazakii infection for neonates and children.

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REFERENCES