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Effectiveness of Disinfectants in Killing *Enterobacter sakazakii* in Suspension, Dried on the Surface of Stainless Steel, and in a Biofilm ▽

Hoikyung Kim,[1] Jee-Hoon Ryu,[2]* and Larry R. Beuchat[1]*

Center for Food Safety and Department of Food Science and Technology, University of Georgia, 1109 Experiment Street, Griffin, Georgia,[1] and Graduate School of Life Sciences and Biotechnology, Korea University, Anam-dong, Sungbuk-ku, Seoul 136-791, Republic of Korea[2]

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The effectiveness of 13 disinfectants used in hospitals, day-care centers, and food service kitchens in killing *Enterobacter sakazakii* in suspension, dried on the surface of stainless steel, and in biofilm was determined. *E. sakazakii* exhibited various levels of resistance to the disinfectants, depending on the composition of the disinfectants, amount and type of organic matrix surrounding cells, and exposure time. Populations of planktonic cells suspended in water (7.22 to 7.40 log CFU/ml) decreased to undetectable levels (<0.30 log CFU/ml) within 1 to 5 min upon treatment with disinfectants, while numbers of cells in reconstituted infant formula were reduced by only 0.02 to 3.69 log CFU/ml after the treatment for 10 min. The presence of infant formula also enhanced the resistance to the disinfectants of cells dried on the surface of stainless steel. The resistance of cells to disinfectants in 6-day-old and 12-day-old biofilms on the surface of stainless steel was not significantly different. The overall order of efficacy of disinfectants in killing *E. sakazakii* was planktonic cells > cells inoculated and dried on stainless steel > cells in biofilms on stainless steel. Findings show that disinfectants routinely used in hospital, day-care, and food service kitchen settings are ineffective in killing some cells of *E. sakazakii* embedded in organic matrices.

Enterobacter sakazakii causes meningitis (7, 19), sepsis (47), bacteremia (40), and necrotizing enterocolitis (51) in preterm neonates and infants. Powdered infant formula has been implicated as a source of *E. sakazakii* in outbreaks of infections (2, 5, 6, 21, 39, 40, 47, 51). In surveys done to determine the presence of *E. sakazakii* in powdered infant formula, the organism was detected in 2.4 to 14.2% of the products tested (22, 38).

The presence of *E. sakazakii* on the surface of utensils and equipment used for infant formula preparation has been reported to occur in clinical settings where neonatal infections have been documented (2, 10, 40, 47). The ability of bacteria to form biofilms on abiotic surfaces (22, 27, 30) raises the possibility that infections may occur following cross-contamination of freshly prepared infant formulas upon contact with soiled surfaces in formula preparation areas in hospitals, day-care centers, food service kitchens, and the home. Food-borne pathogens, e.g., *Escherichia coli* O157:H7 and *Listeria monocytogenes*, and spoilage bacteria such as *Pseudomonas* spp. have enhanced resistance to antibiotics or sanitizers when cells are in biofilms (13, 17, 18, 43), thus increasing the potential for survival on these surfaces.

Surface disinfection is routinely carried out in formula preparation areas in hospitals, food service kitchens, and day-care centers by applying liquid chemical disinfectants to food contact and non-food contact surfaces. The microbicidal activity of commercial surface cleaners and disinfectants is largely based on quaternary ammonium compounds, phenolic compounds, organic acids, alcohols, chlorine, and iodophors. Various commercial hard-surface cleaners and disinfectants have been evaluated for their efficacy in killing bacteria capable of causing food-borne infections (15, 45, 49, 53). During infant formula preparation and feeding, reconstituted formula containing *E. sakazakii* may contaminate abiotic surfaces. These surfaces may be treated with disinfectants immediately after contamination occurs, after the formula remains on the surface and dries, or after growth of *E. sakazakii* and the formation of biofilm. The efficacy of commercial disinfectants used in formula preparation areas in hospitals and child day-care centers in killing *E. sakazakii* in dried infant formula and biofilm has not been described.

We undertook studies to determine the effectiveness of disinfectants in killing *E. sakazakii* in suspension, dried on the surface of stainless steel, and embedded in biofilm on stainless steel. Quaternary ammonium and phenolic disinfectants commonly used in infant formula preparation areas, laboratories, and hospital, food service, and child day-care settings were evaluated. The effects of time elapsed after drying cells on stainless steel as well as the age of biofilms on resistance of cells to disinfectants were determined.

MATERIALS AND METHODS

Bacterial strains and preparation of cells for treatment with disinfectants. *E. sakazakii* strains 3231, isolated from cerebral spinal fluid of an infant, and 3439, isolated from a commercially manufactured powdered infant formula, were grown in tryptic soy broth (BBL/Difco, Sparks, Md.) at 37°C for 24 h. Cultures were transferred by loop inoculum (ca. 10 μl) three times at 24-h intervals. Each culture was centrifuged at 4,000 × *g* for 15 min at 4°C. Cells resuspended in two

* Corresponding author. Mailing address for Larry R. Beuchat: Center for Food Safety, University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797. Phone: (770) 412-4740. Fax: (770) 229-3216. E-mail: lbeuchat@uga.edu. Mailing address for Jee-Hoon Ryu: Graduate School of Life Sciences and Biotechnology, Korea University, Anam-dong, Sungbuk-ku, Seoul 136-791, Republic of Korea. Phone: 82 2 3290 3409. Fax: 82 2 3290 3918. E-mail: escheri@korea.ac.kr.

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TABLE 1. Disinfectants evaluated for lethality to *E. sakazakii*

Disinfectant no.	Product name	Type of active ingredients	Active ingredient(s) (listed on label)	Concn of active ingredient (%)[a]	Application(s) recommended by manufacturers
1	ZEP FS Amine Z[b]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride	3.0	Dairies, restaurants, kitchens, food processing, dairy farms, bakeries, meat packaging plants, sanitary services, bottling plants, etc.
			Didecyl dimethyl ammonium chloride	1.5	
			Dioctyl dimethyl ammonium chloride	1.5	
			Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	4.0	
2	ZEP DZ-7[b]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride	0.814	Major hospital areas, including recovery rooms, patient rooms, nursery, maternity, pediatrics, animal research areas, office areas, nursing homes, child day care services, etc.
			Dioctyl dimethyl ammonium chloride	0.407	
			Didecyl dimethyl ammonium chloride	0.407	
			Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	1.085	
3	Lemonex[b]	Quaternary ammonium compounds	*n*-alkyl dimethyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) benzyl ammonium chlorides	0.8	Bathrooms, kennels, nursing homes, hospitals, hotels, child day care services, etc.
			n-alkyl dimethyl ethylbenzyl ammonium chlorides	0.8	
4	ZEP Micronex[b]	Quaternary ammonium compounds	Didecyl dimethyl ammonium chloride	10.14	Major hospital areas, including recovery rooms, patient rooms, nursery, maternity, pediatrics, etc.
			n-alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	6.76	
5	T.B.Q.[c]	Quaternary ammonium compounds	Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	8	Veterinary and research institutions, industrial plants, food preparation and service facilities, etc.
6	ZEP FS Formula 386 L[b]	Quaternary ammonium compounds/acid	Octyl decyl dimethyl ammonium chloride	2.295	Dairies, restaurants, kitchens, food processing, dairy farms, bakeries, meat packaging plants, nursing homes, hospitals, etc.
			Dioctyl dimethyl ammonium chloride	0.918	
			Didecyl dimethyl ammonium chloride	1.377	
			Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	3.060	
7	Perosan Liquid Sanitizer[b]	Peroxyacetic acid/hydrogen peroxide	Peroxyacetic acid	5.1	Eating and drinking establishments, bottling companies, food preparation (manufacturing), etc.
			Hydrogen peroxide	21.7	
8	LpH se[c]	Phenolic compounds	*o*-phenylphenol	7.7	Floors, walls, and other equipments in hospitals, nursing homes, clinics, etc.
			p-tertiary amylphenol	7.6	
9	Vesphene IIse[c]	Phenolic compounds	*o*-phenylphenol	9.09	Hospitals, nursing homes, medical and dental offices, pharmaceutical plants, etc.
			p-tertiary amylphenol	7.66	
10	Coverage Spray HB Plus[c]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride	0.025	Laboratories and veterinary clinics
			Dioctyl dimethyl ammonium chloride	0.010	
			Didecyl dimethyl ammonium chloride	0.015	

Continued on following page

TABLE 1—*Continued*

Disinfectant no.	Product name	Type of active ingredients	Active ingredient(s) (listed on label)	Concn of active ingredient (%)[a]	Application(s) recommended by manufacturers
			Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	0.034	
11	Coverage Spray TB[c]	Quaternary ammonium compounds	*n*-alkyl dimethyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) benzyl ammonium chlorides	0.105	Infant care equipment, laboratory equipment and surfaces, medical and dental equipment surfaces
			n-alkyl dimethyl ethylbenzyl ammonium chlorides	0.105	
12	ZEP Kitchen Surface Sanitizer[b]	Quaternary ammonium compounds	Octyl decyl dimethyl ammonium chloride	0.01050	Industrial and institutional kitchens, restaurants, delis, cafeterias, butcher shops, bakeries, supermarkets
			Didecyl dimethyl ammonium chloride	0.00525	
			Dioctyl dimethyl ammonium chloride	0.00525	
			Alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	0.01400	
13	ZEP FS RTU-D2[b]	Alcohol and quaternary ammonium compounds	Isopropyl alcohol	58.6000	Poultry, red meat, and dairy processing, restaurants, beverage plants, food service locations, and other food processing facilities
			Octyl decyl dimethyl ammonium chloride	0.0075	
			Didecyl dimethyl ammonium chloride	0.0045	
			Dioctyl dimethyl ammonium chloride	0.0030	

[a] Undiluted disinfectants.
[b] ZEP Manufacturing Co., Atlanta, Ga.
[c] Steris Co., St. Louis, Mo.

carriers, sterile synthetic hard water (400 μg of $CaCO_3$/ml) and reconstituted infant formula, were used in various experiments.

Media in which cells were suspended. Standard synthetic hard water was prepared according to the AOAC International (1) official methods of analysis. Phosphate-buffered saline (PBS) (pH 7.4) containing (per liter of distilled water) NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g) was used as a medium to suspend cells for attachment to stainless steel. Reconstituted infant formula was made by combining Similac Neosure Advance powdered infant formula (Ross Pediatrics, Abbott Laboratories, Columbus, Ohio) with distilled water at a ratio of 1:10 (wt/vol), dissolving by heating at 50 to 60°C, and autoclaving at 121°C for 15 min.

Preparation of stainless steel coupons for spot inoculation and biofilm studies. Stainless steel coupons (type 304; 5 cm by 2 cm) with no. 4 finish were used. The coupons were sonicated in 15% phosphoric acid solution at 80°C for 20 min, rinsed with distilled water, sonicated in alkali detergent solution (FS Pro-Chlor; Zep, Atlanta, Ga.) at 80°C for 20 min, and rinsed again with distilled water. The washed stainless steel coupons were dried and sterilized by autoclaving before use.

Preparation of disinfectant solutions. Descriptions of disinfectants evaluated in the study are shown in Table 1. All disinfectants were tested at minimum concentrations recommended by the manufacturers. Thirteen products were evaluated for their efficacy in killing planktonic cells of *E. sakazakii*. Disinfectants 1 to 9 were prepared at double the strength (2×) of the desired treatment concentrations. Equal volumes of cell suspensions and 2× disinfectant solutions were combined to form the treatment mixture. Disinfectants 10 to 13, intended to be used without dilution, were tested at concentrations received from the manufacturers. For experiments involving spot inoculation of cells on stainless steel and cells in biofilms, disinfectants 2, 5, 6, 7, and 9 were diluted in sterile hard water to obtain the minimum treatment concentrations recommended; undiluted disinfectant 11 was also tested.

Efficacy of disinfectants in killing planktonic cells. Cells in pellets obtained as described above were resuspended in 100 ml of sterile hard water or reconstituted infant formula to give populations of ca. 7 log CFU/ml. Ten milliliters of cell suspension was deposited in a sterile 25- by 150-mm test tube containing 10 ml of 2× disinfectants 1 to 9 or sterile hard water (control) at 22 ± 2°C and thoroughly mixed. For evaluation of disinfectants 10 to 13, cells from pellets were resuspended in 1 ml of sterile hard water or reconstituted infant formula to give a population of ca. 9 log CFU/ml. Cell suspensions (0.1 ml) were added to sterile 25- by 150-mm test tubes containing 20 ml of disinfectants 10 to 13 or sterile hard water (control) and mixed thoroughly.

At time zero (within 10 s after combining the cell suspension with sterile hard water) and after holding the treatment mixtures (suspension to which water [control] or disinfectants were added) for 1, 5, and 10 min at 22 ± 2°C, 2 ml of the suspension was withdrawn and combined with 2 ml of 2× Dey-Engley (DE) neutralizing broth (BBL/Difco). According to Sutton et al. (48), at concentrations of quaternary ammonium and phenolic disinfectants used in our study, DE broth neutralizes components otherwise lethal to *Enterobacteriaceae*, such as *Salmonella* and *Escherichia coli*. Preliminary experiments showed that viability of *E. sakazakii* is unaffected when cells are suspended in DE broth to which disinfectants evaluated in our study were added. Undiluted suspensions (0.25 ml in quadruplicate and 0.1 ml in duplicate) and suspensions (0.1 ml in duplicate) serially diluted in 0.1% peptone water were surface plated on tryptic soy agar (TSA; BBL/Difco). Plates were incubated at 37°C for 48 h before colonies were counted.

Efficacy of disinfectants in killing *E. sakazakii* spot inoculated and dried on stainless steel. Sterile stainless steel coupons were placed on a wire screen elevated 7 cm above the work surface in a laminar-flow biosafety cabinet. Suspensions (100 μl) of *E. sakazakii* in sterile hard water or reconstituted infant formula, prepared as described above, were deposited on each coupon to give ca. 8 log CFU/coupon. The inoculum was dried for 20 h (45% ± 7% relative humidity) at 22 ± 2°C in a laminar-flow biosafety cabinet. Inoculated coupons were immersed in sterile 25- by 150-mm test tubes containing 25 ml of disinfectant 2, 5, 6, 7, or 9 prepared at minimum treatment concentrations recommended by manufacturers, 25 ml of undiluted (full-strength) disinfectant 11, or sterile hard water (control) at 22 ± 2°C and thoroughly mixed. After treatment for 0 min (within 10 s after immersing coupons in sterile water) and after treatment for 1, 5, and 10 min in water or disinfectant solution, each coupon was transferred to a 50-ml tube containing 30 ml of DE broth and 3 g of sterile glass beads. The tube containing DE broth, coupon, and glass beads was vortexed at maximum speed for 1 min. Imme-

TABLE 2. Survival of planktonic cells of *E. sakazakii* strain 3231 as affected by treatment with disinfectants

Disinfectant[b]	Carrier for cells	pH[c]	Population (log CFU/ml) after exposure for[a]:			
			0 min	1 min	5 min	10 min
Water (control)	Water	7.39	A 7.33 A	a A 7.01 A	a A 7.20 A	a A 7.19 A
	Infant formula	7.01	A 7.17 A	a A 7.26 A	ab A 7.33 A	a A 7.27 A
1	Water	7.60	A 7.22 A	c B 1.76 B	b B <0.30[d] C	b B <0.30 C
	Infant formula	6.96	A 7.06 AB	b A 6.78 B	a A 7.38 A	a A 7.39 A
2	Water	6.94	A 7.22 A	e B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	6.82	A 7.06 A	a A 7.29 A	ab A 6.99 A	a A 7.04 A
3	Water	10.71	A 7.22 A	e B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	9.69	A 7.06 A	b A 6.70 A	c A 5.26 B	c A 4.01 C
4	Water	8.33	A 7.22 A	e B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	6.90	A 7.06 A	a A 7.21 A	ab A 7.13 A	a A 7.25 A
5	Water	9.30	A 7.31 A	d B 0.91 B	b B <0.30 B	b B <0.30 B
	Infant formula	7.61	A 7.19 A	a A 7.16 A	b A 6.79 A	a A 6.67 A
6	Water	2.72	A 7.22 A	b B 3.00 B	b B <0.30 C	b B <0.30 C
	Infant formula	5.64	A 7.06 A	a A 7.26 A	ab A 7.15 A	a A 7.18 A
7	Water	5.31	A 7.22 A	e B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	6.02	A 7.06 A	c A 6.12 B	c A 5.76 BC	b A 5.18 C
8	Water	2.76	A 7.31 A	e B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	5.77	A 7.19 A	a A 7.06 A	ab A 7.11 A	a A 7.32 A
9	Water	9.91	A 7.31 A	e B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	8.75	A 7.19 A	a A 7.29 A	ab A 7.10 A	a A 7.11 A
Water (control)	Water	7.39	A 7.33 A	a A 7.01 A	a A 7.20 A	a A 7.19 A
	Infant formula	7.01	A 7.17 A	a A 7.26 A	a A 7.33 A	a A 7.27 A
10	Water	10.31	A 7.31 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	10.31	A 7.19 A	b A 1.02 B	b A <0.30 B	b A <0.30 B
11	Water	12.14	A 7.31 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	12.17	A 7.19 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
12	Water	8.33	A 7.31 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	8.57	A 7.19 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
13	Water	9.56	A 7.31 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	9.03	A 7.19 A	b A <0.30 B	b A <0.30 B	b A <0.30 B

[a] For comparison of the effect of treatment time, mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$). For comparison of the effect of carrier (water versus infant formula), within treatment time and within water (control) or each disinfectant treatment, mean values that are not preceded by the same uppercase letter are significantly different ($P \leq 0.05$). For comparison of the effect of disinfectants 1 to 9, within water (control) and all disinfectant treatments 1 to 9, type of carrier, and treatment time, mean values that are not preceded by the same lowercase letter are significantly different ($P \leq 0.05$). For comparison of the effect of disinfectants 10 to 13, within water (control) and all disinfectant treatments 10 to 13, type of carrier, and treatment time, mean values that are not preceded by the same lowercase letter are significantly different ($P \leq 0.05$).

[b] See Table 1 for a description of disinfectants. Disinfectants 1 to 9 were prepared by diluting with water to minimum concentrations per the manufacturers' recommendations. Disinfectants 10 to 13 were applied without dilution (full strength) per the manufacturers' recommendations.

[c] pH of treatment mixture (water or infant formula plus disinfectant).

[d] Detection limit was 2 CFU/ml (0.30 log CFU/ml).

diately after vortexing, undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) and samples (0.1 ml in duplicate) serially diluted in 0.1% peptone water were surface plated on TSA and incubated at 37°C for 48 h. Colonies were counted, and populations (log CFU/coupon) of *E. sakazakii* remaining on stainless steel coupons before and after treatment with disinfectants were calculated.

Efficacy of disinfectants in killing *E. sakazakii* in biofilm on stainless steel. Each sterile stainless steel coupon was immersed in a sterile 25- by 150-mm test tube containing 25 ml of a suspension of *E. sakazakii* in PBS (ca. 7 log CFU/ml) and incubated at 4°C for 24 h to facilitate attachment of cells. The coupons were transferred to 50-ml tubes containing 30 ml of sterile reconstituted infant formula prepared as described above and incubated at 25°C for 6 or 12 days. Coupons were removed from the formula and washed in 400 ml of sterile water (22 ± 2°C) with agitation for 15 s to remove most of the cells not present in or firmly attached to the biofilm matrix. The washed coupons were transferred to sterile 25- by 150-mm test tubes containing 25 ml of disinfectants 2, 5, 6, 7, and 9 prepared at minimum treatment concentrations recommended by manufacturers, undiluted disinfectant 11, or sterile hard water (control). After treatment for 0 min (within 10 s after immersing coupons in sterile water) and after treatment for 1, 5, and 10 min in water or disinfectant solution, each coupon was transferred to a 50-ml tube containing 30 ml of DE broth and 3 g of sterile glass beads and vortexed at maximum speed for 1 min to dislodge cells from the biofilms. The undiluted suspensions (0.25 ml in quadruplicate and 0.1 ml in duplicate) and suspensions (0.1 ml in duplicate) serially diluted in 0.1% peptone water were surface plated on TSA. Plates were incubated at 37°C for 48 h before the colonies

TABLE 3. Survival of planktonic cells of *E. sakazakii* strain 3439 as affected by treatment with disinfectants

Disinfectant[b]	Carrier for cells	pH[c]	Population (log CFU/ml) after exposure for[a]: 0 min	1 min	5 min	10 min
Water (control)	Water	7.39	A 7.42 A	a A 7.60 A	a A 7.32 A	a A 7.12 A
	Infant formula	7.01	A 7.44 A	a A 7.41 A	a A 7.26 A	a A 7.25 A
1	Water	7.60	A 7.40 A	d B <0.30[d] B	b B <0.30 B	b B <0.30 B
	Infant formula	6.96	A 6.91 B	a A 7.28 AB	a A 7.32 AB	a A 7.40 A
2	Water	6.94	A 7.40 A	d B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	6.82	A 6.91 A	a A 7.10 A	a A 6.99 A	a A 7.15 A
3	Water	10.71	A 7.40 A	d B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	9.69	A 6.91 A	b A 6.41 A	c A 4.04 B	c A 3.22 B
4	Water	8.33	A 7.40 A	d B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	6.90	A 6.91 A	a A 7.24 A	a A 7.04 A	a A 7.02 A
5	Water	9.30	A 7.37 A	c B 3.15 B	b B <0.30 C	b B <0.30 C
	Infant formula	7.61	A 7.46 A	a A 7.17 A	a A 6.85 A	a A 6.81 A
6	Water	2.72	A 7.40 A	b B 3.71 B	b B <0.30 C	b B <0.30 C
	Infant formula	5.64	A 6.91 A	a A 7.24 A	a A 7.22 A	a A 7.38 A
7	Water	5.31	A 7.40 A	d B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	6.02	A 6.91 A	b A 6.12 B	b A 5.63 BC	b A 5.16 C
8	Water	2.76	A 7.37 A	d B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	5.77	A 7.46 A	a A 7.49 A	a A 7.15 A	a A 7.37 A
9	Water	9.91	A 7.37 A	d B <0.30 B	b B <0.30 B	b B <0.30 B
	Infant formula	8.75	A 7.46 A	a A 7.50 A	a A 7.33 A	a A 7.31 A
Water (control)	Water	7.39	A 7.42 A	a A 7.60 A	a A 7.32 A	a A 7.12 A
	Infant formula	7.01	A 7.44 A	a A 7.41 A	a A 7.26 A	a A 7.25 A
10	Water	10.31	A 7.37 A	b A 0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	10.31	A 7.46 A	b A 0.56 B	b A <0.30 B	b A <0.30 B
11	Water	12.14	A 7.37 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	12.17	A 7.46 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
12	Water	8.33	A 7.37 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	8.57	A 7.46 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
13	Water	9.56	A 7.37 A	b A <0.30 B	b A <0.30 B	b A <0.30 B
	Infant formula	9.03	A 7.46 A	b A <0.30 B	b A <0.30 B	b A <0.30 B

[a] For comparison of the effect of treatment time, mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$). For comparison of the effect of carrier (water versus infant formula), within treatment time and within water (control) or each disinfectant treatment, mean values that are not preceded by the same uppercase letter are significantly different ($P \leq 0.05$). For comparison of the effect of disinfectants 1 to 9, within water (control) and all disinfectant treatments 1 to 9, type of carrier, and treatment time, mean values that are not preceded by the same lowercase letter are significantly different ($P \leq 0.05$). For comparison of the effect of disinfectants 10 to 13, within water (control) and all disinfectant treatments 10 to 13, type of carrier, and treatment time, mean values that are not preceded by the same lowercase letter are significantly different ($P \leq 0.05$).

[b] See Table 1 for a description of disinfectants. Disinfectants 1 to 9 were prepared by diluting with water to minimum concentrations per the manufacturers' recommendations. Disinfectants 10 to 13 were applied without dilution (full strength) per the manufacturers' recommendations.

[c] pH of treatment mixture (water or infant formula plus disinfectant).

[d] Detection limit was 2 CFU/ml (0.30 log CFU/ml).

were counted, and the number of cells (log CFU/coupon) surviving treatment was calculated.

Statistical analysis. All experiments were replicated three times. In experiments involving stainless steel coupons, two coupons were examined at each sampling time. Data were analyzed using the general linear model of the Statistical Analysis Systems procedure (SAS; SAS Institute, Cary, N.C.). Statistically significant differences in populations of planktonic cells, spot-inoculated cells, dried cells, and cells of *E. sakazakii* in biofilms caused by treatment with disinfectants were determined. The influence of the presence of infant formula on efficacy of disinfectants in reducing populations of planktonic cells and spot-inoculated dried cells and the efficacy of disinfectants in killing cells in biofilms as affected by the maturation period (age) were determined using Fisher's least significant difference test. Significant differences are presented at a 95% confidence level ($P \leq 0.05$).

RESULTS AND DISCUSSION

Resistance of planktonic cells to disinfectants. Numbers of *E. sakazakii* strain 3231 (Table 2) and strain 3439 (Table 3) suspended in hard water and reconstituted infant formula not containing disinfectants did not change significantly ($P > 0.05$) within 10 min at 22°C. Treatment of both strains suspended in water containing disinfectants for 1 min resulted in significant reductions ($P \leq 0.05$) in populations compared to the number of cells recovered from water not containing disinfectants. For disinfectants 1 to 9, with the exceptions of planktonic cells in water treated with disinfectants 1, 5, and 6, populations of *E. sakazakii* strain 3231 (Table 2) decreased to <0.30 log CFU/ml within 1 min; with the exceptions of disinfectants 5 and 6, populations of strain 3439 (Table 3) were reduced to <0.30 log CFU/ml within 1 min. This indicates that disinfectants 1, 5, and 6, at the concentrations tested, have the lowest lethality among disinfectants 1 to 9 to *E. sakazakii* suspended in water. Disinfectants 1, 5, and 6 contain alkyl (50% C_{14}, 40% C_{12}, and 10% C_{16}) dimethyl benzyl ammonium chloride as a major active ingredient (3 to 8%). After treatment of cells in water with disinfectants 1 to 9 for 5 min, populations of strains 3231 and 3439 were reduced from initial populations of 7.01 and 7.60 log CFU/ml, respectively, to <0.30 log CFU/ml, indicating that with sufficient exposure time, disinfectants 1 to 9 are equivalent in lethality to *E. sakazakii*. Populations of strains 3231 and 3439 suspended in infant formula were decreased significantly ($P \leq 0.05$) by treatment with disinfectants 3 and 7 for 1 to 5 min, while treatment with the other disinfectants for 10 min did not decrease the populations.

Disinfectants 10 to 13 are spray products which are ready to use without dilution or additional preparation. Treatment of cells of *E. sakazakii* strains 3231 (Table 2) and 3439 (Table 3) suspended in water containing disinfectants 10 to 13 reduced initial populations of 7.01 and 7.60 log CFU/ml, respectively, to ≤0.30 log CFU/ml within 1 min. Of the ready-to-use products tested, disinfectant 10 had the lowest initial lethality to *E. sakazakii*, regardless of carrier composition.

Disinfectants 1 to 6 and 10 to 12 are quaternary ammonium-based disinfectants. At the concentrations tested, all contain alkyl dimethyl benzyl ammonium chloride (benzalkonium) at concentrations of 0.006 to 0.105%. The time required to achieve the same level of lethality to *E. sakazakii* in water, however, differed among these disinfectants, indicating that pH and constituents other than benzalkonium chloride contribute to bactericidal activity. Disinfectants 3 and 11, containing alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, and 5% C_{18}) dimethyl benzyl ammonium chloride, showed the greatest lethality, whereas disinfectants 1, 5, 6, and 10, which had lowest lethality, contain alkyl (50% C_{14}, 40% C_{12}, and 10% C_{16}) dimethyl benzyl ammonium chloride. These differences may in part be responsible for differences in efficacy of these two groups of quaternary ammonium compounds in killing *E. sakazakii* suspended in water. Benzalkonium chloride is known to have different bactericidal activities depending on the length of its hydrophobic chain (24). The general order of antibacterial activity, which depends on the length of the alkyl chain, has been reported to be $C_{14} > C_{16} > C_{12} \geq C_{18} > C_{10} > C_8$ (24, 29, 46). Merianos (35) reported that C_{12}, C_{14}, and C_{16} homologues are most effective in inactivating yeasts and molds, gram-positive bacteria, and gram-negative bacteria, respectively. Observations in our study that quaternary ammonium compounds with the highest alkyl C_{14} and C_{16} content cause higher lethality to *E. sakazakii* are in agreement with these findings.

The type of cell carrier, i.e., water or infant formula, in which *E. sakazakii* was suspended significantly ($P \leq 0.05$) affected the efficacy of disinfectants 1 to 9 in killing both test strains (Tables 2 and 3). The lethality of all disinfectants was markedly decreased in the presence of infant formula. The microbicidal activity of chlorine, quaternary ammonium compounds, peroxyacetic acid, hydrogen peroxide, and phenolic compounds is known to be reduced upon contact with organic materials (4, 25, 28, 33, 41, 50). In addition, upon combining infant formula (pH 6.6) with disinfectant solutions, the pH shifted toward 7.0. Disinfectants 6 and 8, for example, had the lowest pH (2.72 and 2.76, respectively) when combined with cells suspended in water, while respective pH values were 5.64 and 5.77 when combined with infant formula. The pHs of disinfectants 3, 5, and 9 ranged from 9.30 to 10.71 when combined with water containing cells but was reduced to 7.61 to 9.69 when combined with infant formula. Exposure of *E. sakazakii* to low pH (<4.0) is known to cause reductions in populations (14, 26). Exposure of *E. sakazakii* to environments at pHs 9.30 to 10.71, however, may not cause immediate death. Gurtler and Beuchat (20) reported that populations of *E. sakazakii* decreased by 0.5 log CFU/ml when cells were exposed to an environment at pH 11.25 for 5 min. Considering disinfectants 1 to 9, only in inoculated formulas treated with disinfectants 3 and 7 did significant ($P \leq 0.05$) reductions in populations of both strains occur within 10 min. Disinfectant 3 contains *n*-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, and 5% C_{18}) dimethyl benzyl ammonium chloride and *n*-alkyl dimethyl ethylbenzyl ammonium chloride, whereas disinfectant 7 contains peroxyacetic acid and hydrogen peroxide as active ingredients.

In contrast to the decreased effect infant formula has on lethality of disinfectants 1 to 9 to planktonic *E. sakazakii*, the type of carrier had no effect on lethality of disinfectants 10 to 13. This is attributed to the low concentration of organic material in the reaction mixture, which was 100-fold less than the amount introduced by the infant formula in experiments involving the evaluation of disinfectants 1 to 9.

Resistance of spot-inoculated, dried cells to disinfectants. Table 4 shows populations of *E. sakazakii* strains 3231 and 3439 recovered from the surface of stainless steel coupons on which cells in water and infant formula were dried and treated with disinfectants 2, 5, 6, 7, 9, and 11. These disinfectants were selected for evaluation because they had different levels of lethality to *E. sakazakii* cells in suspension, represent a wide range of recommended applications, and are based on various types of microbicides, viz., quaternary ammonium compounds, phenolic compounds, and a combination of peroxyacetic acid and hydrogen peroxide. Treatment of coupons that had been inoculated with cells suspended in water with disinfectant 7, which contains peroxyacetic acid and hydrogen peroxide, and disinfectant 9, which contains phenolic compounds, reduced initial populations of 7.98 and 8.01 log CFU/coupon for strains 3231 and 3439, respectively, to <1.48 log CFU/coupon within 5 min. Both strains were reduced to populations <1.48 log CFU/coupon by treating coupons for 1 min with disinfectant 11, a quaternary ammonium compound product. Studies have shown that hydrogen peroxide (42), peroxyacetic acid (16), and phenolic compounds (52) are effective in reducing microbial populations on abiotic surfaces. We have observed a peroxyacetic acid-based sanitizer to be effective in killing *E. sakazakii* on produce (28). Cells of strains 3231 and 3439 applied to stainless steel using water as a carrier and initially at popula-

TABLE 4. Survival of *E. sakazakii* strains 3231 and 3439 spot inoculated and dried on the surface of stainless steel coupons as affected by the type of carrier (water or infant formula) in which cells were suspended

Disinfectant[a]	pH[b]	Treatment time (min)	Population recovered (log CFU/coupon) by strain and carrier							
			Strain 3231				Strain 3439			
			Water		Infant formula		Water		Infant formula	
			Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]
Water (control)	7.20	0	7.98 a		8.74 a		8.01 a		8.65 a	
		1	a 8.01 a		a 8.44 a		a 8.35 a		bc 8.58 a	
		5	a 8.22 a		a 8.51 a		a 8.31 a		a 8.44 a	
		10	a 8.29 a		a 8.45 a		a 8.29 a		ab 8.43 a	
2	6.68	0	7.98 a		8.74 a		8.01 a		8.65 b	
		1	c 6.43 ab	1.58 a	a 8.46 a	+0.02 b	b 7.62 ab	0.73 a	a 9.01 a	+0.43 b
		5	c 5.21 b	3.01 a	a 8.04 b	0.47 b	b 7.14 ab	1.17 a	a 8.38 b	0.06 b
		10	d 1.08 c	7.21 a	d 7.40 c	1.05 b	b 6.78 b	1.51 a	a 8.53 b	+0.1 b
5	8.99	0	7.98 a		8.74 a		8.01 a		8.65 a	
		1	b 7.54 a	0.47 a	a 8.22 a	0.22 a	b 7.87 a	0.48 a	bc 8.63 a	+0.05 b
		5	b 6.86 b	1.36 a	a 8.44 a	0.07 b	b 7.34 ab	0.97 a	a 8.63 a	+0.19 b
		10	c 4.75 c	3.54 a	ab 8.36 a	0.09 b	b 6.42 b	1.87 a	a 8.55 a	+0.12 b
6	2.72	0	7.98 a		8.74 a		8.01 a		8.65 a	
		1	c 6.64 b	1.37 a	a 8.61 ab	+0.17 b	b 7.49 ab	0.86 a	ab 8.75 a	+0.17 a
		5	b 6.35 b	1.87 a	a 8.16 ab	0.35 b	b 7.07 ab	1.24 a	a 8.60 a	+0.16 a
		10	b 6.21 b	2.08 a	bc 8.02 b	0.43 b	b 6.76 b	1.53 a	ab 8.28 a	0.15 a
7	5.24	0	7.98 a		8.74 a		8.01 a		8.65 a	
		1	c 6.40 b	1.61 a	a 8.16 a	0.28 b	b 7.29 a	1.06 a	b 8.70 a	+0.12 b
		5	d <1.47[e] c	≥6.74 a	b 4.96 b	3.55 b	c <1.48 b	≥6.83 a	b 6.48 b	1.96 b
		10	d <1.48 c	≥6.81	e <1.48 c	≥6.97	c <1.48 b	≥6.81	c <1.48 c	≥6.95
9	9.98	0	7.98 a		8.74 a		8.01 a		8.65 ab	
		1	d 4.37 b	3.64 a	a 8.67 a	+0.23 b	c 5.79 b	2.56 a	ab 8.87 a	+0.29 b
		5	d <1.48 c	≥6.74 a	a 8.59 a	+0.08 b	c <1.48 c	≥6.83 a	a 8.28 bc	0.16 b
		10	d <1.48 c	≥6.81 a	c 7.95 b	0.50 b	c <1.48 c	≥6.81 a	b 8.04 c	0.39 b
11	12.04	0	7.98 a		8.74 a		8.01 a		8.65 a	
		1	e <1.48 b	≥6.53 a	a 8.35 b	0.10 b	d <1.48 b	≥6.87 a	c 8.36 a	0.07 b
		5	d <1.48 b	≥6.74	c <1.48 c	≥7.03	c <1.48 b	≥6.83	c <1.48 b	≥6.96
		10	d <1.48 b	≥6.81	e <1.48 c	≥6.97	c <1.48 b	≥6.81	c <1.48 b	≥6.95

[a] See Table 1 for a description of the disinfectants.
[b] pH of water or treatment solution (disinfectant).
[c] For comparison of the effect of disinfectants, within the same strain and the same treatment time, mean values in the same column that are not preceded by the same letter are significantly different ($P \leq 0.05$). For comparison of the effect of treatment time, within the same strain and within water or each disinfectant treatment, mean values in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$).
[d] Reduction or increase in population compared to the number of *E. sakazakii* recovered from stainless steel coupons treated with water (control) for the same length of time. For comparison of the effect of carrier (water versus infant formula), within the same strain, mean values for R in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$).
[e] The detection limit was 30 CFU/coupon (1.48 log CFU/coupon).

tions of 7.98 and 8.01 log CFU/coupon, respectively, survived 10-min treatments with disinfectants 2, 5, and 6, all quaternary ammonium products containing alkyl (50% C_{14}, 40% C_{12}, and 10% C_{16}) dimethyl benzyl ammonium chloride as the major microbicide. Disinfectant 11, the most effective among the six disinfectants tested, contains alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, and 5% C_{18}) dimethyl benzyl ammonium chloride and *n*-alkyl dimethyl ethylbenzyl ammonium chloride.

As with planktonic cells treated with disinfectants 1 to 9 (Tables 2 and 3), the composition of the carrier used to suspend cells significantly ($P \leq 0.05$) affected the efficacy of disinfectants in killing cells of *E. sakazakii* strains 3231 and 3439 dried on the surface of stainless steel (Table 4). Cells of both strains of *E. sakazakii* in infant formula dried on stainless steel showed significantly ($P \leq 0.05$) higher resistance to all disinfectants compared to the resistance of cells applied to stainless steel using water as a carrier (Tables 4). With the exceptions of disinfectants 7 and 11, ≥7.40 and ≥8.04 log CFU/coupon inoculated with strains 3231 and 3439, respectively, survived in infant formula initially containing 8.74 and 8.65 log CFU/coupon, respectively, after treatment with disinfectants for 10 min. Treatment with disinfectant 7 for 10 min or disinfectant 11 for 5 min reduced the number of *E. sakazakii* cells in dried infant formula to <1.48 log CFU/coupon.

The greatest reductions in populations of spot-inoculated, dried cells were achieved by treatment with disinfectant 11, regardless of type of cell carrier and strain (Table 4). In addition to its unique alkyl ammonium chloride composition, among the disinfectants tested, disinfectant 11 has the highest pH (12.04). The alkaline pH of disinfectant 11 may in part contribute additively to

TABLE 5. Survival of *E. sakazakii* strains 3231 and 3439 in biofilm formed on the surface of stainless steel coupons immersed in infant formula at 25°C for 6 or 12 days as affected by treatment with disinfectants

Disinfectants[a]	pH[b]	Treatment time (min)	Population recovered (log CFU/coupon) by strain and biofilm type							
			Strain 3231				Strain 3439			
			6-day biofilm		12-day biofilm		6-day biofilm		12-day biofilm	
			Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]	Recovered[c]	R[d]
Water (control)	7.20	0	7.68 a		7.80 a		7.74 a	8.23 a		
		1	a 7.74 a		a 7.91 a		a 7.67 a	8.07 a		
		5	a 7.69 a		a 7.85 a		a 7.82 a	7.99 a		
		10	a 7.71 a		a 7.81 a		a 7.55 a	8.11 a		
2	6.68	0	7.68 a		7.80 a		7.74 a	8.23 a		
		1	ab 7.45 a	0.29 a	a 7.24 ab	0.67 a	a 7.71 a	+0.04 a	a 7.79 ab	0.28 a
		5	a 7.12 a	0.57 a	ab 6.79 b	1.06 a	b 7.06 b	0.76 a	ab 7.34 bc	0.65 a
		10	a 7.03 a	0.68 a	ab 6.43 b	1.38 a	a 6.67 b	0.88 a	a 6.86 c	1.25 a
5	8.99	0	7.68 a		7.80 a		7.74 a		8.23 a	
		1	a 7.67 a	0.07 a	a 7.35 ab	0.56 a	a 7.80 a	+0.13 a	a 7.87 ab	0.20 a
		5	a 7.08 a	0.61 a	ab 6.84 b	1.01 a	a 7.80 a	0.02 a	a 7.89 ab	0.10 a
		10	a 7.12 a	0.59 a	a 6.95 b	0.86 a	a 7.16 b	0.39 a	a 7.30 b	0.81 a
6	2.72	0	7.68 a		7.80 a		7.74 a		8.23 a	
		1	a 7.69 a	0.05 a	a 7.30 ab	0.61 a	a 7.63 a	0.04 a	a 7.96 ab	0.11 a
		5	a 7.09 a	0.60 a	b 6.45 b	1.40 a	b 7.21 a	0.61 a	a 7.41 bc	0.58 a
		10	a 7.02 a	0.69 a	a 6.98 ab	0.83 a	a 6.35 b	1.20 a	a 6.87 c	1.24 a
7	5.24	0	7.68 a		7.80 a		7.74 a		8.23 a	
		1	ab 7.46 ab	0.28 a	ab 7.01 ab	0.90 a	b 6.71 a	0.96 a	a 7.19 ab	0.88 a
		5	ab 6.61 b	1.08 a	b 6.47 bc	1.38 a	c 5.70 a	2.12 a	b 6.73 b	1.26 a
		10	b 5.26 c	2.45 a	b 5.70 c	2.11 a	b 2.72 b	4.83 a	b 5.22 c	2.89 a
9	9.98	0	7.68 a		7.80 a		7.74 a		8.23 a	
		1	a 7.64 a	0.10 a	a 7.84 a	0.07 a	a 7.63 ab	0.04 a	a 7.96 ab	0.11 a
		5	a 7.04 ab	0.65 a	a 7.39 ab	0.46 a	b 7.09 bc	0.73 a	a 7.57 b	0.42 a
		10	a 6.90 b	0.81 a	a 7.19 b	0.62 a	a 6.60 c	0.95 a	a 6.75 c	1.36 a
11	12.04	0	7.68 a		7.80 a		7.74 a		8.23 a	
		1	b 6.97 b	0.77 b	b 6.37 b	1.54 a	c 3.68 b	3.99 a	b 5.01 b	3.06 a
		5	b <1.48[e] c	≥6.21	c <1.48 c	≥6.37	d <1.48[e] c	≥6.34	c <1.48 c	≥6.51
		10	c <1.48 c	≥6.23	c <1.48 c	≥6.33	c <1.48 c	≥6.07	c <1.48 c	≥6.63

[a] See Table 1 for a description of the disinfectants.
[b] pH of water or treatment solution (disinfectant).
[c] For comparison of the effect of disinfectants, within the same strain and the same treatment time, mean values in the same column that are not preceded by the same letter are significantly different ($P \leq 0.05$). For comparison of the effect of treatment time, within the same strain and within water or each disinfectant treatment, mean values in the same column that are not followed by the same letter are significantly different ($P \leq 0.05$).
[d] Reduction or increase in population compared to the number of *E. sakazakii* cells recovered from stainless steel coupons treated with water (control) for the same length of time. For comparison of the effect of carrier (water versus infant formula), within the same strain, mean values for R in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$).
[e] The detection limit was 30 CFU/coupon (1.48 log CFU/coupon).

reductions in populations. Highly alkaline pH environments can cause disruption of the cell membrane of gram-negative bacteria, resulting in leakage of cytoplasm and death (34).

Compared to planktonic cells, a lower percentage of cells dried on stainless steel coupons were killed upon treatment with disinfectants. Although a portion of the dried cells would be expected to be injured by desiccation, thereby potentially increasing sensitivity to disinfectants, organic material (in the case of the infant formula carrier) and cells at or near the surface of the dried inoculum would provide protective barriers against contact with disinfectants. Some of the cells dried on the surface of stainless steel may have undergone starvation during drying for 20 h, a condition also known to increase the resistance of bacteria to sanitizers (3, 23, 54). Mosley et al. (36) reported that bacteria inoculated on stainless-steel strips were more resistant than planktonic cells to sanitizers. Treatment with various sanitizers, including quaternary ammonium compounds and peroxyacetic acid, was reported to be effective in killing *Pseudomonas fluorescens* and *Yersinia enterocolitica* in liquid suspension but relatively ineffective in killing cells attached to surfaces (37). Our observations on the behavior of *E. sakazakii* are in agreement with these reports.

Efficacy of disinfectants in killing *E. sakazakii* in biofilm. Biofilms formed by *E. sakazakii* on stainless steel immersed in reconstituted infant formula for 6 or 12 days were treated with water and disinfectants 2, 5, 6, 7, 9, and 11 for 1, 5, and 10 min. Populations of strains 3231 and 3439 surviving treatments are shown in Table 5. Treatment with disinfectants 2, 5, and 6 did not significantly ($P > 0.05$) reduce the population of strain 3231 (7.68 log CFU/coupon) in 6-day-old biofilm compared to treatment with water (control). Treatment with disinfectants 7 and 9 for 10 min significantly ($P \leq 0.05$) reduced populations but only by 2.45 and 0.81 log CFU/coupon, respectively. Treatment with disinfectant 11 decreased the population of strain 3231 in 6-day-old biofilm by 0.77 log CFU/coupon within 1 min and subsequently caused decreases to an undetectable level (<1.48 log CFU/coupon) at 5 min. As with strain 3231, treatment with disinfectant 11 for 5 min resulted in significant reductions in populations of strain 3439 in 6-day-old biofilms to an undetectable level (<1.48 log CFU/coupon). Exposure of biofilms to disinfectants 2, 5, 6, and 9 for 5 to 10 min caused significant reductions in populations of strain 3439 in 6-day-old biofilms, but ≥6.35 log CFU/coupon survived after treatment for 10 min.

The behavior of *E. sakazakii* in 12-day-old biofilms was similar to that in 6-day-old biofilms, indicating that the age of the biofilms did not have a major effect on the resistance of cells to disinfectants. Significant ($P \leq 0.05$) reductions in numbers of both strains of *E. sakazakii* in 12-day-old biofilms to <1.48 log CFU/coupon occurred only upon treatment with disinfectant 11 for 5 min. Treatment with all other disinfectants significantly reduced populations of strains 3231 and 3439 in 12-day-old biofilms; however, reductions in populations were ≤2.89 log CFU/coupon upon treatment for 10 min. Regardless of strain or age of biofilms, disinfectant 11 had the greatest lethality to *E. sakazakii*; disinfectant 7 had the second greatest lethality.

At the concentrations tested, the lethality of all test disinfectants to *E. sakazakii* in biofilms was lower than that observed for planktonic cells or spot-inoculated, dried cells on the surface of stainless steel. The overall order of efficacy of all disinfectants in killing *E. sakazakii* was planktonic cells > spot-inoculated, dried cells > cells in biofilms. *E. sakazakii* has been observed to form biofilms on the surfaces of stainless steel, silicon, latex, polycarbonate, glass, and polyvinyl chloride (22, 27, 30); however, inactivation of the bacterium in biofilms by the disinfectants examined in our study was not reported.

Mechanisms that enhance the resistance of bacteria in biofilms to environmental stresses have been proposed. Extracellular polymeric substances produced by microorganisms during biofilm formation behave as protective barriers against exposure to environmental stresses (11, 31, 32). Ryu and Beuchat (43) reported that exopolysaccharide is a major factor enhancing resistance of *E. coli* O157:H7 in biofilms to sanitizers. Oxidizing sanitizers, including hydrogen peroxide, can be neutralized and lose bactericidal activity upon contact with the surface of biofilms (9, 12, 32, 55). Some strains of *E. sakazakii* are reported to produce extracellular polysaccharide (30, 44), the amount produced being affected by nutrient availability and temperature. The production of exopolysaccharide by *E. sakazakii* during biofilm formation would likely provide a protective barrier against disinfectants.

While *E. sakazakii* cells at or near the surface of biofilms would utilize nutrients and oxygen from the surrounding environment, cells deeply within the biofilm matrix may have undergone starvation, which may increase their resistance to stress. *Pseudomonas aeruginosa* in biofilms showed higher resistance than non-biofilm formers to several antibiotics (13). Resistance of *E. coli* O157:H7 (43) and *L. monocytogenes* (17, 18) to sanitizers is significantly greater in biofilms on abiotic surfaces compared to resistance of planktonic cells. Peracetic acid, mercuric chloride, and formaldehyde at otherwise lethal concentrations were shown to be ineffective in killing microorganisms in biofilms (8).

In summary, the disinfectants evaluated in this study exhibited various levels of lethality to *E. sakazakii*, depending on the composition of the carrier used to suspend cells and treatment time. The two test strains, one isolated from a clinical specimen and the other from a food source, behaved similarly upon exposure to experimental test parameters. The presence of infant formula enhanced the resistance of planktonic cells and cells spot inoculated and dried on the surface of stainless steel to the disinfectants. The overall order of resistance of *E. sakazakii* to disinfectants was planktonic cells < cells spot inoculated and dried on stainless steel < cells in biofilms on stainless steel.

The results emphasize the importance of proper cleaning of surfaces soiled by rehydrated infant formula and other foods. Otherwise, infant formula remaining on these surfaces protects *E. sakazakii* against the lethality of disinfectants or may serve as a source of nutrients, resulting in growth and production of biofilm. The results provide information useful in assessing the efficacy of disinfectants in killing *E. sakazakii* embedded in organic matrices on surfaces in formula preparation and feeding areas in hospitals, day-care centers, food service kitchens, and the home.

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REFERENCES

1. **AOAC International.** 2000. Official methods of analysis, 17th ed., method 960.09. AOAC International, Gaithersburg, MD.
2. **Bar-Oz, B., A. Preminger, O. Peleg, C. Block, and I. Arad.** 2001. *Enterobacter sakazakii* infection in the newborn. Acta Pediatr. **90:**356–358.
3. **Berg, J. D., A. Matin, and P. V. Roberts.** 1982. Effect of antecedent growth-conditions on sensitivity of *Escherichia coli* to chlorine dioxide. Appl. Environ. Microbiol. **44:**814–818.
4. **Beuchat, L. R., B. B. Adler, and M. M. Lang.** 2004. Efficacy of chlorine and peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. J. Food Prot. **67:**1238–1242.
5. **Biering, G., S. Karlsson, N. V. C. Clark, K. E. Jonsdottir, P. Ludvigsson, and O. Steingrimsson.** 1989. Three cases of neonatal meningitis caused by *Enterobacter sakazakii* in powdered milk. J. Clin. Microbiol. **27:**2054–2056.
6. **Block, C., O. Peleg, N. Minster, B. Bar-Oz, A. Simhon, I. Arad, and M. Shapiro.** 2002. Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. Eur. J. Clin. Microbiol. Infect. Dis. **21:**613–616.
7. **Burdette, J. H., and C. Santos.** 2000. *Enterobacter sakazakii* brain abscess in the neonate: the importance of neuroradiologic imaging. Pediatr. Radiol. **30:**33–34.
8. **Carpentier, B., and O. Cerf.** 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. J. Appl. Bacteriol. **75:**499–511.
9. **Chen, X., and P. S. Stewart.** 1996. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. Environ. Sci. Technol. **30:**2078–2083.
10. **Clark, N. C., B. C. Hill, C. M. O'Hara, O. Steingrimsson, and R. C. Cooksey.** 1990. Epidemiological typing of *Enterobacter sakazakii* in two neonatal nosocomial outbreaks. Diagn. Microbiol. Infect. Dis. **13:**467–472.
11. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711–745.
12. **de Beer, D., R. Srinivasan, and P. S. Stewart.** 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. **60:**4339–4344.
13. **Delissalde, F., and C. F. Amábile-Cuevas.** 2004. Comparison of antibiotic susceptibility and plasmid content, between biofilm producing and non-

producing clinical isolates of *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents **24:**405–408.

14. **Edelson-Mammel, S. G., and R. L. Buchanan.** 2004. Acid resistance of twelve strains of *Enterobacter sakazakii*, Abstr. P170, p. 107. Program and abstract book, 91st Annu. Mtg. Int. Assn. Food Prot., 8 to 11 August 2004, Phoenix, AZ
15. **Exner, M., V. Vacata, B. Hornei, E. Dietlein, and J. Gebel.** 2004. Household cleaning and surface disinfection: new insights and strategies. J. Hosp. Infect. **56:**S70–S75.
16. **Farrell, B. L., A. B. Bonner, and A. C. L. Wong.** 1998. Attachment of *Escherichia coli* O157:H7 in ground beef to meat grinders and survival after sanitation with chlorine and peroxyacetic acid. J. Food Prot. **61:**817–822.
17. **Folsom, J. P., and J. F. Frank.** 2006. Chlorine resistance of *Listeria monocytogenes* biofilms and relationship to subtype, cell density, and planktonic cell chlorine resistance. J. Food Prot. **69:**1292–1296.
18. **Frank, J. F., and R. A. Koffi.** 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J. Food Prot. **53:**550–554.
19. **Gallagher, P. G., and W. S. Ball.** 1991. Cerebral infarction due to CNS infection with *Enterobacter sakazakii*. Pediatr. Radiol. **21:**135–136.
20. **Gurtler, J. B., and L. R. Beuchat.** 2005. Performance of media for recovering stressed cells of *Enterobacter sakazakii* as determined using spiral plating and ecometric techniques. Appl. Environ. Microbiol. **71:**7661–7669.
21. **Himelright, I., E. Harris, V. Lorch, and M. Anderson.** 2002. *Enterobacter sakazakii* infections associated with the use of powdered infant formula—Tennessee, 2001. JAMA **287:**2204–2205.
22. **Iversen, C., M. Lane, and S. J. Forsythe.** 2004. The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Lett. Appl. Microbiol. **38:**378–382.
23. **Jones, J. G., and R. W. Pickup.** 1989. The effect of organic carbon supply in water on the antibiotic resistance of bacteria. Aqua **33:**131–135.
24. **Jono, K., T. Takayama, M. Kuno, and E. Higashide.** 1986. Effect of alkyl chain length of benzalkonium chloride on the bactericidal activity and binding to organic materials. Chem. Pharm. Bull. (Tokyo) **34:**4215–4224.
25. **Kennedy, J., J. Bek, and D. Griffin.** 2006. Selection and use of disinfectant. NebGuide. http://www.ianrpubs.unl.edu/epublic/pages/publicationD.jsp?publicationId=431.
26. **Kim, H., and L. R. Beuchat.** 2005. Survival and growth of *Enterobacter sakazakii* on fresh-cut fruits and vegetables and in unpasteurized juice as affected by storage temperature. J. Food Prot. **68:**2541–2552.
27. **Kim, H., J.-H. Ryu, and L. R. Beuchat.** 2006. Attachment and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. Appl. Environ. Microbiol. **72:**5846–5856.
28. **Kim, H., J.-H. Ryu, and L. R. Beuchat.** 2006. Survival of *Enterobacter sakazakii* on fresh produce as affected by temperature and effectiveness of sanitizers for its elimination. Int. J. Food Microbiol. **111:**134–143.
29. **Kitahara, T., N. Koyama, J. Matsuda, Y. Aoyama, Y. Hirakata, S. Kamihira, S. Kohno, M. Nakashima, and H. Sasaki.** 2004. Antimicrobial activity of saturated fatty acids and fatty amines against methicillin-resistant *Staphylococcus aureus*. Biol. Pharm. Bull. **27:**1321–1326.
30. **Lehner, A., K. Riedel, L. Eberl, P. Breeuwer, B. Diep, and R. Stephan.** 2005. Biofilm formation, extracellular polysaccharide production, and cell-to-cell signaling in various *Enterobacter sakazakii* strains: aspects promoting environmental persistence. J. Food Prot. **68:**2287–2294.
31. **Lewis, K.** 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. **45:**999–1007.
32. **Mah, T. F., and G. A. O'Toole.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. **9:**34–39.
33. **Marriott, N. G., and R. B. Gravani.** 2006. Principles of food sanitation, 5th ed. Springer, New York, NY.
34. **Mendonca, A. F., T. L. Amoroso, and S. J. Knabel.** 1994. Destruction of gram-negative food-borne pathogens by high pH involves disruption of the cytoplasmic membrane. Appl. Environ. Microbiol. **60:**4009–4014.
35. **Merianos, J. J.** 1991. Quaternary ammonium antimicrobial compounds, p. 225–262. *In* S. S. Block (ed.), Disinfection, sterilization and preservation, 4th ed. Lea and Febiger, Philadelphia, PA.
36. **Mosley, E. B., P. R. Elliker, and H. Hays.** 1976. Destruction of food spoilage indicator and pathogenic organisms by various germicides in solution and on a stainless steel surface. J. Milk Food Technol. **39:**830–836.
37. **Mosteller, T. M., and J. R. Bishop.** 1993. Sanitizer efficiency against attached bacteria in milk biofilm. J. Food Prot. **56:**34–41.
38. **Muytjens, H. L., W. H. Roelofs, and G. H. J. Jasper.** 1988. Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteraceae*. J. Clin. Microbiol. **26:**743–746.
39. **Muytjens, H. L., H. C. Zanen, H. J. Sonderkamp, L. A. Kolee, I. K. Wachsmuth, and J. J. Farmer.** 1983. Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. J. Clin. Microbiol. **18:**115–120.
40. **Noriega, F. R., K. L. Kotloff, M. A. Martin, and R. S. Schwalbe.** 1990. Nosocomial bacteremia caused by *Enterobacter sakazakii* and *Leuconostoc mesenteroides* resulting from extrinsic contamination of infant formula. Pediatr. Infect. Dis. **9:**447–449.
41. **Ostrander, W. E., and L. J. Griffith.** 1964. Evaluation of disinfectants for hospital housekeeping use. Appl. Microbiol. **12:**460–463.
42. **Robbins, J. B., C. W. Fisher, A. G. Moltz, and S. E. Martin.** 2005. Elimination of *Listeria monocytogenes* biofilms by ozone, chlorine, and hydrogen peroxide. J. Food Prot. **68:**494–498.
43. **Ryu, J.-H., and L. R. Beuchat.** 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. Appl. Environ. Microbiol. **71:**247–254.
44. **Scheepe-Leberkühne, M., and F. Wagner.** 1986. Optimization and preliminary characterization of an ecopolysaccharide synthesized by *Enterobacter sakazakii*. Biotechnol. Lett. **8:**695–700.
45. **Sharma, M., and L. R. Beuchat.** 2004. Sensitivity of *Escherichia coli* O157:H7 to commercially available alkaline cleaners and subsequent resistance to heat and sanitizers. Appl. Environ. Microbiol. **70:**1795–1803.
46. **Shelton, R. S., M. G. Van Campen, C. H. Tilford, H. C. Lang, L. Nisonger, F. J. Bandelin, and H. L. Rubenkoenig.** 1946. Quaternary ammonium salts as germicides. I. Non-acylated quarternary ammonium salts derived from aliphatic amines. J. Am. Med. Chem. Soc. **68:**753–755.
47. **Simmons, B. P., M. S. Gelfand, M. Haas, L. Metts, and J. Ferguson.** 1989. *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered infant formula. Infect. Control Hosp. Epidemiol. **10:**398–401.
48. **Sutton, S. V., D. W. Proud, S. Rachui, and D. K. Brannan.** 2002. Validation of microbial recovery from disinfectants. J. Pharm. Sci. Technol. **56:**255–266.
49. **Taormina, P. J., and L. R. Beuchat.** 2002. Survival of *Listeria monocytogenes* in commercial food-processing equipment cleaning solutions and subsequent sensitivity to sanitizers and heat. J. Appl. Microbiol. **92:**71–80.
50. **Troller, J. A.** 1993. Sanitation in food processing, 2nd ed. Academic Press, San Diego, CA.
51. **Van Acker, V., F. de Smet, G. Muyldermans, A. Bougatef, A. Naessens, and S. Lauwers.** 2001. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J. Clin. Microbiol. **39:**293–297.
52. **Vesley, D., and G. S. Michaelsen.** 1964. Application of a surface sampling technique to the evaluation of bacteriological effectiveness of certain hospital housekeeping procedures. Health Lab. Sci. **1:**107–113.
53. **Vijayakumar, C., and C. E. Wolf-Hall.** 2002. Minimum bacteriostatic and bactericidal concentrations of household sanitizers for *Escherichia coli* strains in tryptic soy broth. Food Microbiol. **19:**383–388.
54. **Walters, M. C., F. Roe, A. Bugnicourt, M. J. Franklin, and P. S. Stewart.** 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob. Agents Chemother. **47:**317–323.
55. **Xu, X., P. S. Stewart, and X. Chen.** 1996. Transport limitation of chlorine disinfection of *Pseudomonas aeruginosa* entrapped in alginate beads. Biotechnol. Bioeng. **49:**93–100.