Evaluation of Chemical Disinfectants for the Elimination of *Salmonella* Biofilms from Poultry Transport Containers

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ABSTRACT Containers used in transporting live poultry between production and processing units are a primary source of contamination for processed poultry products. Because disinfection of transport containers (TC) has been difficult to accomplish, it is probable that the choice of appropriate disinfectant and its application are partially or wholly responsible for the failure to adequately eliminate pathogens from TC. Therefore, 13 commercial disinfectants were selected and evaluated for their capacities to destroy *Salmonella*. The disinfectants were applied in various concentrations on prescribed areas (10 cm diameter circle) of galvanized steel surfaces (representative of TC material) that were artificially contaminated with *Salmonella* (10^8 cfu/mL) with a mixture of organic material. Likewise, coupons (1.9 cm^2) made of the same metallic surfaces and covered with biofilms of *Salmonella* spp. were tested in the same manner for each disinfectant. Two of the disinfectants completely eliminated *Salmonella* on the artificially contaminated and biofilm-covered surfaces. These compounds produced logarithmic reductions in *Salmonella* populations as high as 7.18 within 2 min. One of the two effective disinfectants contained sodium hypochlorite and was effective at a concentration of 0.05% (vol/vol). The other disinfectant was an alkaline peroxide compound and was effective at a concentration of 1% (wt/vol). Evaluation of these two disinfectants under simulated conditions suggested that application under the prescribed regimen could result in effective elimination of *Salmonella* from TC within a limited period.

(*Key words*: cleaning, biofilm, contamination, *Salmonella*, disinfectant)

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INTRODUCTION

Concerns for food safety have stimulated increasing consumer awareness of potential pathogen contamination of food resulting in human diseases such as salmonellosis that may be caused by consuming poultry or other products contaminated with *Salmonella* spp. An average of 76 million cases of foodborne illnesses (Mead et al., 1999) and 40,000 cases of salmonellosis are reported in the United States each year, of which approximately 1,000 persons die each year with acute salmonellosis (National Center for Health Statistics, 2001). Poultry that enter the processing plant carrying *Salmonella*, internally or externally, are a major source of contamination in final poultry products (Morris and Wells, 1970). During transportation, the birds may shed *Salmonella* and thus contaminate transport containers (TC) (Bhatia and McNabb, 1980). If the containers are not properly cleaned and decontaminated after transport, the microorganisms deposited from the previous trip may contaminate subsequent flocks transported in the same, unclean TC. Therefore, an adequate cleaning and decontamination system for poultry TC is necessary to assure that poultry are not exposed to pathogens at this stage of processing.

Several chemical agents are commercially available for elimination of *Salmonella* in a suspension or nonbiofilm situation. However, decontamination of TC surfaces poses a different challenge in that bacterial cells are protected from the disinfectant by fecal material (wet or dry), if the TC is not properly precleaned. Carr et al. (1999) found that most disinfectants were ineffective against *Salmonella* in a field situation, because of persistently adherent and protected bacteria, probably in a biofilm state. Because organic material diminishes its efficiency, there is an increased demand for disinfectant during the decontamination process. In addition, bacteria present on metal or other surfaces may form a biofilm (Mafu et al., 1990), which is a slimy layer of organic polymer matrix, adhering to a surface, in which microbes are embedded. The most effective disinfectants against bacterial cells in suspension may not be as effective when treating bacterial cells embedded in a biofilm (Holah et al., 1990). Biofilm presence, as well as organic load, will increase the de-

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Abbreviation Key: TC = transport containers.

TABLE 1. Efficiencies of disinfectants in reducing *Salmonella* from artificially contaminated surfaces in the presence of an organic load

Disinfectant[1]	Active ingredient	*Salmonella* population (cfu/cm^2)			*Salmonella* reduction	
		Initial inoculation A	Before treatment B	After treatment C	$Log_{10}B - log_{10}C$	$[(B-C)/B] \times 100$ (%)
A	Sodium hypochlorite	7.97×10^8	7.20×10^7	5.34×10^6	1.13	92.58
B	Enzymes	1.66×10^9	5.54×10^5	5.12×10^5	0.03	7.51
C	Sodium chlorite, potassium	1.66×10^9	5.54×10^5	4.42×10^2	3.10	99.92
D	Sodium chlorite	7.97×10^8	7.20×10^7	8.19×10^4	2.95	99.89
E	Sodium chlorite	7.97×10^8	7.20×10^7	6.72×10^3	4.03	99.99
F	Quaternary ammonium	7.97×10^8	7.20×10^7	1.84×10^7	0.59	74.44
G	Quaternary ammonium	7.97×10^8	7.20×10^7	5.12×10^6	1.15	92.89
H	Iodine	7.97×10^8	7.20×10^7	5.18×10^5	2.14	99.28
I	Quaternary ammonium	6.11×10^8	1.01×10^8	2.15×10^6	1.67	97.88
J	Quaternary ammonium	6.11×10^8	1.01×10^8	3.94×10^5	2.41	99.61
K	Quaternary ammonium	6.11×10^8	1.01×10^8	1.24×10^7	0.91	87.80
L	Phenol, cresol	6.11×10^8	1.01×10^8	2.03×10^4	3.70	99.98

[1]The product names are encoded to maintain company confidentiality. Disinfectant M listed in Table 4 was not tested in this experiment.

mand for the disinfecting compound (Characklis, 1980; Wright et al., 1991). The objective of this study was to identify a disinfectant that was effective in reducing or, optimistically, eliminating *Salmonella* populations, embedded in organic materials, or in biofilms, or both, from poultry TC.

MATERIALS AND METHODS

Thirteen commercially available disinfectants against *Salmonella* species were identified, based on their use in poultry house disinfection, or their suggested use by the manufacturer, or both (Table 1). The target surface for the disinfectants was galvanized steel, the material used for the construction of many of the TC commonly used in the poultry industry. In this study, the disinfectants were evaluated on the surfaces of galvanized steel samples.

Artificial Contamination Tests

Five serotypes of *Salmonella enterica* that had been isolated previously from poultry TC, i.e., Typhimurium, Thompson, Berta, Hadar, and Johannesburg (El-Assaad, 1992) were used in this study. Each *Salmonella* serotype was grown separately in 25 mL brain-heart infusion broth at 37 C for 24 h. The turbidities of the broth cultures were compared with a calibration curve (optical density vs. microbial population) to obtain approximately 10^9 cfu/mL. A chicken fecal slurry was prepared by dissolving one part chicken manure in five parts of distilled water to produce an organic load, based on previously determined biological oxygen demand, that would resemble the load during the actual cleaning of TC (E. T. Mallinson, 1997, Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, MD, personal communication). One milliliter each of the five *Salmonella* broth cultures, containing about 10^9 organisms and 5 mL of the sterilized chicken fecal slurry, were mixed to yield an organic suspension containing approximately 5×10^8 cfu/mL. The solutions of chemical disinfectants were prepared according to the manufacturer's recommendations.

Galvanized steel plates ($12.7 \times 12.7 \times 0.3$ cm, with a 10 cm diameter circle scribed in the center) were prepared and autoclaved. One-half milliliter of the *Salmonella*-contaminated chicken fecal slurry was seeded on the sterile galvanized steel surfaces and spread over the 10 cm diameter circle. Using a sterile metal template with a 2.5×1.25 cm excised area, the galvanized steel surface was swabbed within the scribed area with a cotton swab dipped in 0.85% saline and then wrung out. Three galvanized steel test surfaces were swabbed immediately after seeding to establish the average initial *Salmonella* population. All other galvanized steel surfaces were allowed to dry at ambient conditions for 45 min to form a dry coating of organic material and bacteria. These surfaces then simulated conditions on the TC. Again, three galvanized steel surfaces were swabbed using sterile templates to determine if there was a reduction in the population of *Salmonella* spp. after drying.

The chemical treatments were applied to the dried surfaces for 2 min, which corresponded to the minimal time available between unloading the chickens from the poultry TC and reloading the containers on the truck (El-Assaad, 1992). Each disinfectant was tested on two dried surfaces. One-half milliliter of the disinfectant solution was applied to the dried surface by using a pipette and was spread over the entire 10 cm area with a sterile, glass, spreading rod. After a 2-min contact, the test surfaces were swabbed with sterile swabs. The swabs were placed in 10 mL of 0.85% saline solution and vortexed for 1.0 min; the microbial population was quantified by standard bacteriological serial dilution and spread plate methods on XLT-4 media incubated at 37 C for 24 h (Ramesh, 1999). The number of *Salmonella* present on the surface before application of disinfectant and after disinfection treatment indicated the effectiveness of each disinfectant.

Biofilm Tests

Preparation of Biofilms. Smaller sized galvanized steel coupons (1.9×1.9 cm^2) were preferred because they could be used in 15.0-mL culture wells for biofilm formation, and more samples could be tested with greater efficiency.

The galvanized steel coupons were autoclaved to remove any contamination and then were placed in each well of the culture plates containing six 5.0 cm diameter wells to which 5.0 mL of tryptic soy broth with glucose (10 g/L) was added as the growth medium (Krysinski et al., 1992). Ten microliters of each of the five *Salmonella* cultures grown separately in brain-heart infusion broth were then dispensed into each well. The culture plates were incubated at 37 C and observed each day for growth (by increasing turbidity and by random, microscopic examination of the coupons). Tryptic soy broth media were replaced every 2 d up to 16 d to encourage biofilm formation.

Within a day, *Salmonella* attached to the coupon surfaces. There were 32 companion coupons used to follow biofilm formation over 16 d. The coupons were removed from culture media and gently washed with PBS to remove the planktonic (floating) cells. The coupons were then swabbed and streaked on a glass slide for Gram staining and microscopic examination. Microscopic observation of the slide showed the presence of Gram-negative rods resembling *Salmonella*. Presence of intact bacteria in the biofilm was confirmed with a scanning electron microscope.[2] Growth of *Salmonella* in the biofilm was monitored for companion samples using scanning electron microscopy every day for 16 d of biofilm development. Based on our observation of the densities of the biofilms, we decided to use coupons with 3- or 4-d-old biofilms for the evaluation of the disinfectants.

Evaluation Against 3- and 4-d-old Biofilms. Disinfectants that performed best against bacteria in suspension may not be similarly efficient in the elimination of biofilms, in which the same types of bacteria are embedded. Thus, the disinfectants were tested against biofilms containing all five *Salmonella* serotypes used in the previous experiment. Those disinfectants that were previously able to reduce the *Salmonella* population by at least 85% (A, C, D, E, G, H, I, J, K, and L) plus one more product (M), an enzymatic compound that later came to our attention as a potential biofilm remover, were tested against *Salmonella* 3-d-old biofilms.

To more stringently test the disinfectants, those that killed more than 99% of the *Salmonella* population in the study on 3-d-old biofilms (A, C, G, H, and M) were tested on 4-d-old biofilms (Table 2).

Measurement of the Effect on Biofilms. Culture plates containing six 5.0 cm diameter wells each with 15-mL capacity were used as treatment chambers. Three of the six wells were filled with 5 mL of disinfectant solution each. A galvanized steel coupon with biofilm was removed from the 3- or 4-d growth chamber, was gently washed with PBS three times using a squirt bottle, and then was placed into a well containing disinfectant solution. After 2 min of treatment, the coupon was removed from the solution and the surface was swabbed with a sterile cotton swab, which was then analyzed for *Salmonella* population size by serial dilution and spread plating methods. The swabs were immediately placed in 10 mL of 0.85% saline solution for serial dilution, which essentially inactivated the killing effect of the disinfectant. Therefore, a separate inactivation step was not performed. In order to enumerate the *Salmonella* populations in the biofilm, the galvanized steel coupon with biofilm grown on the surface was removed from its growth chamber and was washed gently with sterile PBS three times to remove the planktonic cells. The coupon was then swabbed on its entire surface with a sterile dry cotton swab, which was vortexed for 1.0 min and then the size of the *Salmonella* population was determined by using standard serial dilution and spread plating methods. Three control coupons were swabbed with dry swabs to determine the initial bacterial count constituting the biofilm population for a particular group of samples. Three galvanized steel coupons with biofilm were subjected to a disinfection treatment with each chemical tested and then were quantified as described above.

Analysis for the Optimal, and Most Effective, and Economic Concentrations

Those disinfectants that reduced *Salmonella* by 100% on 4-d-old biofilms (A, C, and H) were selected as disinfectants that could potentially be used in decontaminating poultry TC. The optimal effectiveness of those selected disinfectants was tested at different concentrations and contact times. The concentrations used included higher and lower levels than used in the previous tests. The incremental levels of the various disinfectants differed because of the manufacturers' original recommendations. Because decontamination is a concentration and time dependent treatment process, those compounds (A, C, and H) that produced 100% efficiency were tested at various concentrations and contact times. The efficiencies of the decontaminants were compared with a control in which no chemical was used. Compound A was tested at 0, 0.025, 0.05, 0.075, and 0.1%; C was tested at 0, 1, 1.5, 2, and 2.5%; and H was tested at 0, 0.5, 1, 1.5, and 2%. The concentrations for each decontaminant were selected to bracket the concentration that produced 100% reduction in the previous tests. Each level was tested at 1, 2, and 3 min of contact. Each treatment was repeated three times, and the *Salmonella* populations before and after each treatment were recorded. The logarithmic reduction in *Salmonella* populations was examined to select the most effective disinfectants (Table 3).

Statistical Design

The time-concentration experiment was conducted as a randomized complete block design with test day as

[2]Model 1000A, Amray, Bedord, MA.

TABLE 2. Efficiencies of disinfectants in reducing *Salmonella* from 4-d-old biofilms grown on galvanized steel surfaces

Disinfectant[1]	*Salmonella* population (cfu/cm^2)		*Salmonella* reduction	
	Before treatment B	After treatment C	Log $Log_{10}(B+1) - log_{10}(C+1)$	$[(B - C)/B] \times 100$ (%)
A	4.28×10^7	0	7.63	100
C	4.28×10^7	0	7.63	100
G	4.28×10^7	1.78×10^7	0.38	58.30
H	1.98×10^7	0	7.30	100
M	1.98×10^7	3.17×10^5	1.80	98.40

[1]The product names are encoded to maintain company confidentiality.

TABLE 3. Effect of the three selected disinfectants (A, C, and H) at varying concentrations and times on 4-d-old biofilms of *Salmonella* grown on galvanized steel surfaces

Disinfectant	Concentration	Time (min)	*Salmonella* reduction log (BT) – log (AT)[1]
A	0 ppm	1	0.97 ± 0.27
		2	0.71 ± 0.30
		3	0.67 ± 0.20
	250 ppm	1	6.26 ± 0.98
		2	7.18 ± 0.25
		3	6.51 ± 0.74
	500 ppm	1	5.76 ± 1.47
		2	7.18 ± 0.25[a]
		3	7.18 ± 0.25
	750 ppm	1	7.18 ± 0.25
		2	7.18 ± 0.25
		3	7.18 ± 0.25
	1,000 ppm	1	7.18 ± 0.25
		2	7.18 ± 0.25
		3	7.18 ± 0.25
C	0%	1	0.68 ± 0.28
		2	0.56 ± 0.09
		3	0.43 ± 0.12
	1%	1	6.35 ± 0.82
		2	7.12 ± 0.30[a]
		3	7.12 ± 0.30
	1.5%	1	7.12 ± 0.30
		2	7.12 ± 0.30
		3	7.12 ± 0.30
	2%	1	7.12 ± 0.30
		2	7.12 ± 0.30
		3	7.12 ± 0.30
	2.5%	1	7.12 ± 0.30
		2	7.12 ± 0.30
		3	7.12 ± 0.30
H	0%	1	0.53 ± 0.16
		2	0.58 ± 0.14
		3	0.69 ± 0.13
	0.5%	1	0.49 ± 0.28
		2	0.62 ± 0.08
		3	0.70 ± 0.06
	1%	1	1.10 ± 0.27
		2	0.83 ± 0.26
		3	0.85 ± 0.32
	1.5%	1	0.50 ± 0.32
		2	0.92 ± 0.21
		3	1.12 ± 0.47
	2%	1	1.31 ± 0.48[a]
		2	0.73 ± 0.15
		3	0.94 ± 0.02

[a]Level of significance at $P \leq 0.05$.

[1]BT = before treatment. AT = after treatment.

TABLE 4. Efficiencies of disinfectants in reducing *Salmonella* from 3-d-old biofilms grown on galvanized steel surfaces

Disinfectant	*Salmonella* population (cfu/cm^2)		*Salmonella* reduction	
	Before treatment B	After treatment C	Log $Log_{10}(B+1) - log_{10}(C+1)$	$[(B–C)/B] \times 100$ (%)
A	1.19×10^6	0	6.08	100
C	1.19×10^6	0	6.08	100
D	1.19×10^6	8.97×10^4	2.12	92.46
E	1.19×10^6	2.88×10^4	1.62	97.58
G	2.49×10^7	7.47×10^4	2.52	99.70
H	2.49×10^7	1.25×10^5	2.30	99.50
I	2.49×10^7	1.34×10^6	1.27	94.61
J	2.49×10^7	1.99×10^6	1.10	92.00
K	5.80×10^6	4.23×10^3	3.14	92.70
L	5.80×10^6	7.83×10^3	2.87	98.65
M	5.80×10^6	1.91×10^3	3.48	99.67

block. The *Salmonella* reduction [log (count before) – log (count after)] data were analyzed with the mixed model procedure.[3] The fixed portions of the model included the effects of disinfectant, concentration and time, and all two- and three-factor interactions. The random sources included the effect of day and the residual variance. Mean comparisons were based on the distribution probabilities.

RESULTS AND DISCUSSION

The efficiencies of the disinfectants in reducing the numbers of *Salmonella*, applied to artificially contaminated surfaces, are given in Table 1. Compounds B and F showed very low efficiencies, and therefore they were not tested on *Salmonella* biofilms. In general, halogen compounds were effective in reducing *Salmonella* under all conditions measured. The bactericidal action of the chlorine-releasing disinfectants is due to their oxidative reaction with cellular proteins that interfere with cellular function. Although it is believed that the antibacterial activity of iodine is through its reaction with cellular enzymes, the exact method of reaction is yet unknown (Ascenzi, 1996). Of the five quaternary ammonium compounds (F, G, I, J, and K), two of them (F and G) had the same listed active ingredients in identical proportions. Compound G, however, reduced *Salmonella* populations by 92% whereas compound F reduced them only by 74%. This disparity might be attributed to the composition of the inert ingredients—information that was not available for any of the tested chemical disinfectants. Varying effects of synergism or antagonism of the active ingredient with the inert ingredients might be responsible for the various levels of disinfection among the different disinfectants, but no definitive conclusions can be made, as these effects were not tested.

The existence of a biofilm was checked through Gram staining and was confirmed by scanning electron microscopy (Figure 1). Attachment of *Salmonella* and production of extracellular substances can be observed in these micrographs. The presence of *Salmonella* on galvanized steel surfaces after removing the planktonic cells indicated that they were attached to the metal surface (Figure 1A). Multiplying bacterial cells indicated that attached *Salmonella* actively proliferate to eventually form microcolonies. The microbes tended to populate densely within cracked and peeled surfaces of the sample (not shown). *Salmonella* started developing fibrils within 2 d of attachment, perhaps in response to the culture environment at the time. Figure 1B shows a relatively dense population of *Salmonella* on a galvanized steel surface with a network of fibrils apparently attaching the bacteria to the surface and to each other. Some cells might have been older than others because bacterial multiplication was observed throughout the growth period. The appearance of the bacterial cells (Figure 1C) indicates that they were present in different layers and suggests that the biofilm can grow in thickness, probably due to an increase in the number of bacterial cells and to secretion of extracellular material. The *Salmonella* also showed an obvious rough surface that can be attributed to the extracellular material secreted by the bacteria in the biofilm. The evolution of the biofilms shown here after prolonged culture demonstrates to some extent the development of natural biofilms on contaminated TC over several usages, as described by Carr et al. (1999). Similar polymer secretions by biofilm bacteria have been documented (Fletcher and Floodgate, 1973). It is the polymeric matrix that offers resistance to the penetration of chemical cleanser and disinfectants. The partial formation of a bacterial matrix is shown in Figure 1D with dividing bacterial cells indicating active growth.

The *Salmonella* population in biofilms was determined by swabbing the coupon surface after removing planktonic cells and analyzing the swabs through the serial dilution and spread plating methods. The *Salmonella* population in a biofilm could be quantified reasonably accurately only up to 4 d of growth because of increasing

[3]SAS software, Version 6.12, SAS Institute Inc., Cary, NC.

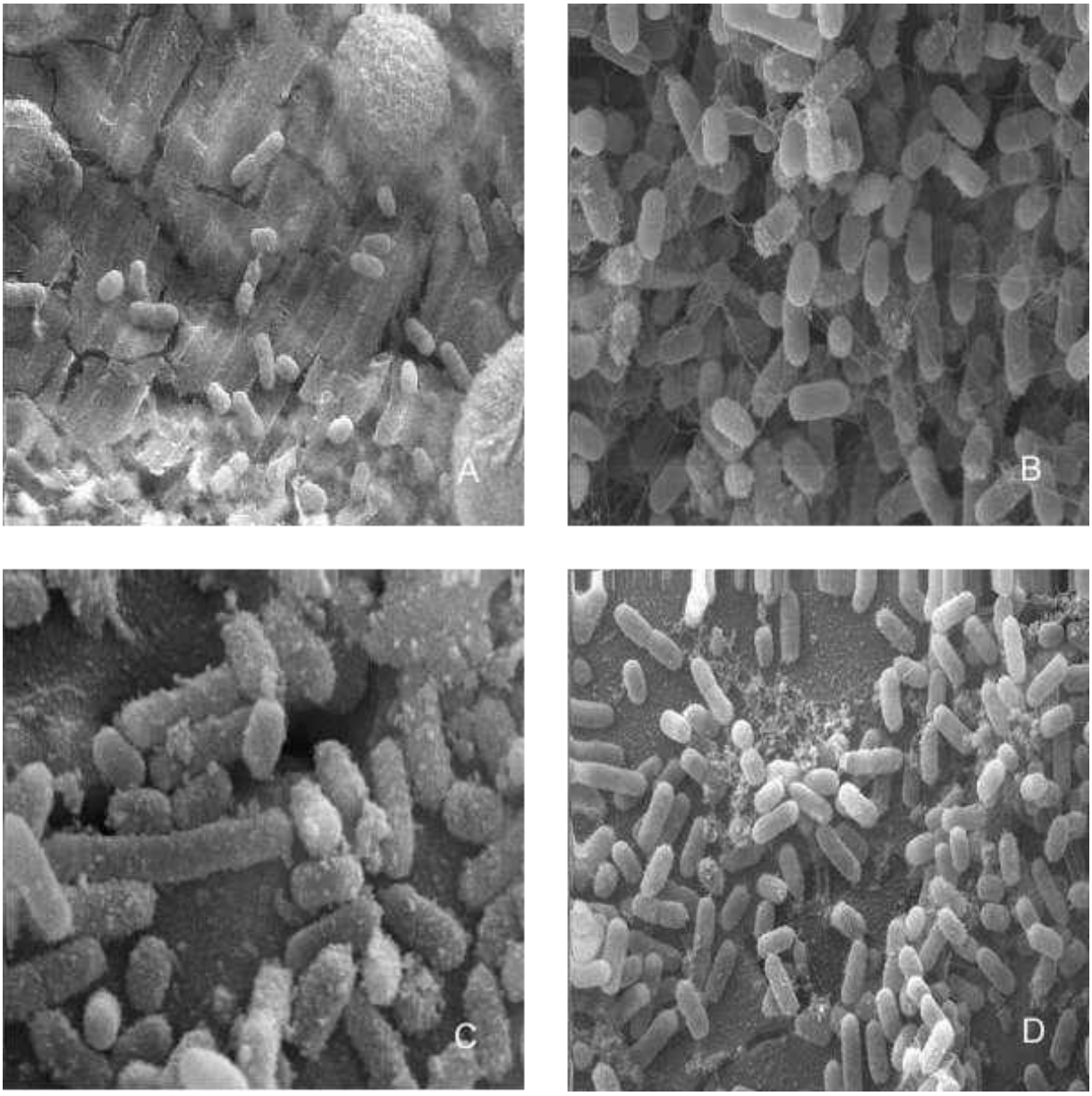

FIGURE 1. Electron scanning micrographs of various biofilms. A) Scanning electron micrograph of *Salmonella* on a 2-d-old biofilm grown on galvanized steel surface; original magnification: 2,600×. B) Scanning electron micrograph of *Salmonella* on an 8-d-old biofilm grown on a galvanized steel surface showing a network of fimbriae; original magnification: 4,100×. C) Scanning electron micrograph of *Salmonella* on an 8-d-old biofilm grown on a galvanized steel surface demonstrating a rough surface on the bacteria; original magnification: 5,800×. D) Scanning electron micrograph of *Salmonella* on a 16-d-old biofilm grown on a galvanized steel surface showing extracellular material formation; original magnification: 3,000×.

polymer thickness. The *Salmonella* count on a 1-d-old biofilm was similar to that observed in the artificial contamination tests. Scanning electron micrographs revealed that the spread of attached bacteria on the surface after 1 and 2 d of incubation was limited. Therefore, the disinfectants were tested on 3- and 4-d-old biofilms.

Except for compounds B and F, all disinfectants were tested on 3-d-old biofilms of *Salmonella*. The disinfectant (M) claimed by the manufacturer to be a biofilm remover was also tested on *Salmonella* biofilms. Thus, 11 disinfectants in total were tested on 3-d-old biofilms. Not all 11 biocides were effective in significantly reducing *Salmonella* in biofilms (Table 4). The nature of bacteria grown in a biofilm is different from those grown in broth culture. The slimy layer of polymer matrix offers resistance to the penetration of disinfectant solution and protects the bacteria embedded in the matrix. Only five of the 11 disinfectants (A, C, G, H, and M) produced more than 99% reduction in the *Salmonella* count. These five disinfectants were tested on 4-d-old biofilms. Three of the five compounds (A, C, and H) were able to completely kill *Salmonella* producing a 100% reduction (Table 2). All three of these effective compounds were from the halogen group. Two of them (A and C) were chlorine compounds, and the other (H) was an iodine compound.

The disinfectants were significantly more effective than the controls in reducing *Salmonella* ($P \leq 0.05$). Effectiveness of disinfectant H was significantly lower ($P \leq 0.05$) than

that of A or C. With a *Salmonella* population in the range of 7 log cycles present initially on the sample surfaces, compound H only reduced the population by 1.31 ± 0.48 log cycles within 2 min. Disinfectants A and C eliminated all of the *Salmonella* present on the galvanized steel surfaces. Disinfectant A, at 0.05% and above, reduced the *Salmonella* population by 7.18 ± 0.25 log cycles within 2 min. Disinfectant C at 1% solution (wt/vol) or above reduced the *Salmonella* population by 7.12 ± 0.30 log cycles within 2 min (Table 3).

In conclusion, there were two disinfectants, A and C, that were effective in reducing *Salmonella* in the presence of organic load and in eliminating *Salmonella* biofilms. Compound A, which had sodium hypochlorite as its active ingredient, was effective at a concentration of 500 ppm of sodium hypochlorite. Compound C, which had sodium chlorite and alkaline peroxide as its active ingredients, was effective at a concentration of 1% product solution. Both disinfectants eliminated *Salmonella* completely within 2 min. Either of the 2 compounds or both could be used for the decontamination of TC.

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