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Novel wash aid T-128 enhances the efficacy of chlorine against Salmonella on tomatoes

Abstract

A novel wash aid, T-128, was evaluated for its capacity to enhance the efficacy of chlorine solution for tomato washing against *Salmonella enterica* in the presence of high organic load. Incremental amount of tomato juice was added to chlorinated wash solutions to simulate free chlorine depletion during tomato washing operations. Cells of *S. enterica* were either directly inoculated into the wash solutions or onto the tomatoes that are washed in chlorinated solutions with or without T-128. *Salmonella* cells survived in the wash solution, in the stem-scar areas, and in the internal tissues of tomatoes were enumerated. Application of T-128 significantly reduced the survival of *Salmonella* in chlorinated water when free chlorine approached depletion. It also resulted in a significantly higher reduction of *S. enterica* population on stem scars compared to the control. When inoculated and non-inoculated tomatoes were washed together, cross contamination by *Salmonella* cells to the non-inoculated tomatoes were reduced in the presence of T-128. The application of T-128 also significantly improved the chlorine efficacy against *Salmonella* internalization when chlorine concentration was near depletion. These results indicated that T-128 has the potential to improve chlorine efficacy against pathogen survival, cross-contamination and internalization in the wash solution when chlorine neared depletion, thus improve the safety margin of the process control.

Keywords: wash aid, free chlorine, tomato stem scar, infiltration, *S. enterica, Escherichia coli*

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Introduction

Salmonella enterica is one of the most common causing agents of food-borne illness in the United States. Based on an estimate by the Center for Disease Control, foodborne *Salmonella* causes 415 deaths, 1.4 million infections and about 2.65 billion dollars economic damages annually.[1] *Salmonella* outbreaks linked to consumption of fresh fruits and vegetables have been increasingly reported recently.[2] Tomatoes are one of the common vehicles of produce-associated salmonellosis and have been associated with several outbreaks in the United States.[3–6] Pre-harvest contamination has been linked to several recent produce associated outbreaks of *S. enterica* serovars and enterohemorrhagic *Escherichia coli*.[5–8] Implementation of good agriculture practices (GAP) can minimize but not eliminate the possibilities of in-field contamination. Outbreak investigations linked to tomatoes suggest that contaminated wash water in tomato packing facilities is a major contributing factor to microbial contamination.[6,9] Therefore, an effective post-harvest anti-microbial intervention is critical for ensuring fresh produce safety.

Traditionally, tomato packing house operators have maintained a 10°F temperature differential between the submerging water and tomato pulp in order to prevent plant pathogen infiltration and soft rot disease development. In the absence of scientific information pertaining to human pathogen infiltration, this procedure has been adopted as a food safety regulation. However, the effectiveness of this temperature differential in preventing pathogen infiltration has been increasingly questioned.[10]

Chlorinated water is one of the most widely used sanitizing solutions for tomato post- harvest processing. However, free chlorine is highly reactive with organic substances and can be readily depleted with high organic loads in the washing solution due to the accumulation of tomato juice, stems, leaves, and field debris.[11] This problem cannot be effectively solved by increasing the chlorine input because repeated addition of chlorine to wash water that is high in organic load also results in the increased formation of toxic chlorine by-products and off-gas.[12]

T-128 is a wash aid recently developed by scientists in the produce industry[13] for the purpose of stabilizing chlorine in wash water. This proprietary formula is composed of chemicals with GRAS (Generally Recognized as Safe) status,[14] including a common inorganic acid and organic polydiols. Previously, we have demonstrated that addition of T-128 to chlorinated water improved the efficacy of bacterial inactivation in wash solutions for lettuce processing and significantly reduced the likelihood of cross contamination, without negatively impacting the quality of lettuce.[15] T-128 was also found to increase the inactivation of *Salmonella* in biofilms formed on stainless steel surface by chlorine solutions.[16] In this study, we examined the potentials of T-128 for enhancing the efficacy of chlorinated water in reducing *Salmonella* populations and preventing cross contamination during tomato wash operations.

Materials and methods

Plant materials

Mature red tomatoes (*Solanum lycopersicum* Mill.) were obtained from a local fresh produce wholesale establishment and used within 24 hours. Tomatoes were visually inspected to ensure consistence in size, shape, and integrity. Tomatoes of irregular shapes, sizes, ripeness, and those with visible defects or damages were excluded. Selected tomatoes were equilibrated to room temperature (22°C)

overnight before use to ensure uniform pulp temperature. In separate experiments, tomatoes (c.v. Sunbright) at the mature green stage were harvested from a local tomato farm and used to investigate *Salmonella* infiltration. Tomato extract was prepared using a vegetable juicer and filtered through a perforated polyethylene filter (330μm) in a WhirlPak bag (Nasco, Fort Atkinson, WI. US) to remove coarse particles before being used to modulate organic contents in wash solutions.

Bacterial strains

S. enterica sv. Thompson strain RM1987 transformed with plasmid pGT-KAN, which conferred green fluorescence and gentamicin resistance,[17] was kindly provided by Dr. Maria Brandl (USDA-ARS, WRRC., US). The parental strain RM1987 was a clinical isolate from a salmonellosis outbreak linked to a produce outbreak in California in 1999. *S. enterica* sv. Newport strain 2757 was isolated from tomatoes involved in a 2002 outbreak in Pennsylvania and was kindly provided by Dr. Jie Zheng (FDA, CFSAN, US). This strain was adapted for resistance to nalidixic acid by repeated subculturing in tryptic soy agar (TSA) (BD, Sparks, MD., US) supplemented with an increasing concentration of nalidixic acid (Sigma, St Luis, MO., US). The selected nalidixic acid resistant mutant did not display any detectable change in growth pattern in TSB and on XLT-4 (Neogen, Lansing, MI., US) media. The resistance was stable after five consecutive subcultures in TSB without selective pressure. No cross inhibition,[18] was observed when strains 2757 and RM1987 were cross-streaked on tryptic soy agar plates or mix-cultured in tryptic soy broth (TSB) (BD, Sparks, MD., US) without antibiotics.

Inoculum preparation and inoculation

Bacterial inocula were prepared using overnight cultures grown at 37°C with shaking in TSB containing appropriate antibiotics. Cultures were harvested by centrifugation, washed in phosphate buffered saline (PBS, pH7.2) and resuspended in equal volumes of PBS. Inocula, composed of cells from RM1987or a cocktail of both strains, were diluted directly in distilled water or a wash solution to achieve a concentration of 10^6 CFU/ml. For inoculation of tomatoes, five tomatoes per replicate were submerged in 4.5L of the *Salmonella* suspension (10^6 CFU/ml) in sterile distilled water. The inoculated tomatoes were removed from the suspension after 1 min and were air-dried for 30min in a biosafety cabinet. All inoculated tomatoes were used for treatment within 2hrs after inoculation.

Inactivation of Salmonella in wash solution containing high organic loads

Chlorine solutions (25mg/L) were prepared using 6% sodium hypochlorite (NaOCl) (Clorox, Oakland, CA. US) and distilled water that had been equilibrated to room temperature (22-25°C). The pH of treatment solutions was adjusted to 3.0 or 5.0 using T-128, while the pH of the control was adjusted to 6.5 (industry standard practice) using citric acid. Aliquots of tomato juice were added to the chlorine solutions to decrease the chlorine concentration to desired levels. Inocula were added to the wash solution to achieve approximately 10^6 CFU/ml initial cell concentration. Following exposure for 30 seconds, the reaction was stopped by neutralizing free chlorine with a sodium thiosulfate based dechlorinating reagent (Hach, Loveland, CO. US). *Salmonella* survival in the wash solution was determined based on the most probable number (MPN) method detailed in the Microbial Analysis section.

Salmonella reduction on tomato surface and stem-scar areas

Wash solutions containing free chlorine levels (0, 0.2, 0.5, 2, 5, 50 and 100mg/L) were prepared using 6% NaOCl, and pH was adjusted to 6.5 using citric acid as control, or to pH 5.0 using T-128. Five inoculated tomatoes were washed in each wash solution for 30 s or 2 min with gentle manual agitation. Dechlorinating reagent was added to the spent wash water to stop the reaction. After removal from washing solution and a brief rinse with sterile water containing dechlorinating agent, the smooth surface of the tomato was repeatedly wiped using a moistened sterile paper tissue for 1 min, avoiding the stem scar. The paper tissue was then stomached with TSB supplemented with 0.1% of sodium pyruvate (Sigma, St. Luis, MO. US) (TSBP) in a filtered stomacher bag. Then the stem scar of the tomato was removed carefully using two sterile knives and a sterile cork borer, weighed in a filtered stomacher bag, mechanically crushed, and macerated for 2 min with 10 volumes (tomato weight basis) of TSBP. Spent wash water, smooth surface samples, and stem scar samples were subjected to microbial analyses to evaluate survival of *Salmonella* in wash solutions and on fruit surfaces.

Salmonella cross contamination during tomato wash

The effect of T-128 on preventing cross contamination was examined by washing inoculated and non-inoculated tomatoes (3 each) in chlorinated water containing 0, 0.2, 5, 10, 50mg/L free chlorine, with pH adjusted to 6.5 using citric acid as control, or to pH 5.0 using T-128. *Salmonella* survival in spent wash water and on the surfaces of the non-inoculated tomato was determined using modified MPN method as described above.

Salmonella infiltration of tomato internal tissues

Wash solutions with an initial free chlorine concentration of 10mg/L were prepared and pH adjusted to 6.5 with citric acid or to 5.0 using T128. Tomato juice extract, as organic load simulator, was gradually added to the chlorine solution to modulate the residual free chlorine concentration. Two *Salmonella* strains were inoculated into wash solutions to achieve a targeted initial concentration of approximately 10^6 CFU/ml. Meanwhile, five tomatoes were simultaneously submerged into the wash solution. After immersion for 2min, the tomatoes were retrieved and a cylindrical plug of internal tissue was removed as described previously.[10] The cylindrical plug of tomato core tissues was weighed, crushed, and macerated for 2min with 10 volumes of TSBP.

Microbial analysis

The presence of viable *Salmonella* cells in wash solutions, spent wash water, on smooth surface and stem scars, and in internal tissues were enumerated using a microplate-based 8-well 6-dilution most probable number (MPN) method as previously described with modifications.[19] In brief, eight 0.3 ml aliquots (3.0ml for the infiltration experiments) were serially 10-fold diluted in TSBP in a deep-well microplate and incubated at 37°C overnight. The following day, 3μl droplets of enriched cultures for all dilutions were arrayed on TSA plates supplemented with 50mg/L gentamicin (for strain RM1987) using an 8-channel pipet. After 6h incubation, growth of *Salmonell*a was confirmed by fluorescence and growth patterns were recorded and analyzed using MPN calculator software.[20] For *S. enterica* sv. Newport strain 2757, overnight enrichment cultures were

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similarly arrayed and incubated overnight on XLT-4 agar containing 50mg/L nalidixic acid to confirm the growth of *Salmonella*; results were calculated for MPN as described above. Unless specified, data are the average of three replicate experiments.

Experimental design and statistical analysis

The MPN data were log-transformed to meet the requirement for normality and homogenous variance. For the different experiments, statistical analyses were performed using the general linear model (GLM) of SAS (SAS Institute, Cary, NC. US). The Fisher's LSD test was used to determine differences among means at α=0.05.

Results and discussion

Inactivation of Salmonella in wash solution containing high organic loads

The effect of wash aid T-128 on the survival of *Salmonella* strain in chlorinated water with increasing concentrations of freshly prepared tomato juice was determined. In comparison to the control, application of the T-128 significantly enhanced the bactericidal activity of the wash water when increasing concentrations of tomato juice were added as a source of organic load (Table 1). No *Salmonella* survival was observed when the wash solutions contained 0.5mg/L or higher free chlorine, irrespective of T-128 application. However, when the free chlorine concentrations decreased to 0.24 and 0.12 mg/L, the survival of *Salmonella* was observed in the control solution, but not in the solutions containing T-128, either at pH 3.0 or 5.0. This agrees with our previous report that the presence of T-128 significantly enhanced the efficacy of free chlorine against *E. coli* O157: H7 in the presence of high organic loads for leafy green processing.[15,21]

Table 1 Survival of *S. enterica* sv. Thompson RM1987 in chlorinated wash solution in the presence or absence of T-128

Residual Free Chlorine (mg/L)[1]	*Salmonella* Survival (log MPN/ml)		
	Citric acid, pH6.5	T-128, pH3.0	T-128, pH 5.0
4.96	ND[2]	ND	ND
0.99	ND	ND	ND
0.49	ND	ND	ND
0.24	1.44±0.06	ND	ND
0.12	2.02±0.05	ND	ND
0	5.51±0.15 A[3]	5.47±0.10 A	5.48±0.32 A

[1]Free chlorine level was achieved by adding tomato juice to wash solution containing 25mg/L of free chlorine. Data was not shown for higher chlorine concentrations.
[2]Not detected at the detection limit of -0.42 log MPN/ml.
[3]Treatment means within each row with different capital letters are significantly different at α=0.05.

Effect of T-128 on Salmonella reduction on tomatoes

Chlorinated water is widely used to reduce potential pathogen contamination in commercial processing. In this study, we evaluated the potential of T-128 increasing chlorine efficacy for pathogen reduction on tomatoes. *Salmonella* cells attached to the smooth surface were readily removed or inactivated by chlorine wash, probably because the smooth skin of tomatoes was relatively resistant to pathogen attachment. *Salmonella* cells were not recovered from the smooth surface after exposure for 30 sec to wash solutions containing as low as 0.2 mg/L free chlorine, irrespective of T-128 presence (data not shown). To ensure that failure to recover viable *Salmonella* cells from the smooth surface was not due to the sampling procedure, pieces of the smooth skin of washed tomatoes were removed and enriched in TSBP overnight. No viable *Salmonella* cells were recovered from these enriched smooth tomato skin samples. However, significant numbers of *Salmonella* cells were recovered in the stem scar areas, both in the absence and presence of T-128 (Table 2). Increasing chlorine concentration significantly reduced *Salmonella* survival on tomato stem-scars. Compared to tomatoes, washed in the absence and presence of T-128 (pH5.0) for 30 sec and 2 min, there was a significant correlation ($P<0.05$) between *Salmonella* survival and T-128 application and treatment time. For 30-second wash, no significant difference ($P>0.05$) in *Salmonella* survival was observed between chlorine solutions containing T-128 (pH 5.0) and citric acid (pH 6.5). However, when the treatment time was increased to 2 min, the application of T-128 significantly reduced the pathogen population recovered from the stem scars. For washing solutions with residue free chlorine ranging from 0.2 to 100mg/L, *Salmonella* survival on the stem scars was consistently lower by approximately 1 log10 unit with T-128 application than without this wash aid. This time-dependent effect of T-128 on *Salmonella* reduction suggests that T-128 might facilitate the penetration of chlorine in the stem scars. The observed difference for *Salmonella* survival between the stem scars and the smooth surface of tomatoes are likely due to the differences in the surface characteristics. Tomato stem scars have a rough surface and are more vulnerable to pathogen attachment and colonization.[22] Therefore, tomato stem scars should be closely examined when assessing the effectiveness of antimicrobial interventions.

Table 2 Survival of *S. enterica* sv. Thompson RM 1987 on tomato stem scars after washing in chlorinated water with or without T-128

Free Chlorine (mg/L)	*Salmonella* recovered from stem scars (log MPN/g)[1]			
	Wash Time 30 s		Wash Time 2 min	
	Control	T-128	Control	T-128
0	4.39±0.01 A[2]	4.38±0.01 A	4.33±0.05 A	4.34±0.04 A
0.2	4.29±0.02 A	4.39±0.01 A	4.18±0.06 B	3.200±0.14 C
0.5	3.97±0.03 B	4.26±0.02 A	3.92±0.03 B	2.94±0.06 C
2	3.87±0.02 A	3.96±0.02 A	3.72±0.03 B	2.75±0.06 C
5	3.66±0.05 A	3.72±0.03 A	3.57±0.05 B	2.61±0.08 C
50	3.31±0.10 A	3.57±0.05 A	3.20±0.06 B	2.24±0.13 C
100	2.93±0.05 A	2.81±0.10 B	2.81±0.10 B	1.85±0.08 C

[1]Data represents the average of three replicates of 5 tomatoes each.
[2]Treatment means within each row with different capital letters are significantly different at α=0.05.

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Effect of T-128 against Salmonella cross contamination during tomato wash process

One of the main objectives of sanitizer application for fresh produce processing is to maintain the microbial quality of the wash water and thus preventing cross contamination by bacterial pathogens.[11] Since tomatoes are often washed in a dump tank system, the accumulation of debris, leaves, soils, and tomato juice can lead to depletion of free chlorine, thus increasing the potential for cross contamination. To assess the effect of T-128 on preventing cross contamination, inoculated and non-inoculated tomatoes were washed in various chlorine solutions with or without T-128, and samples from both the smooth skins and stem-scars were examined. Cross-contamination of the tomato skin (smooth surface) was not observed except when inoculated and non-inoculated tomatoes were washed together in distilled water (data not shown). In the stem-scars, however, the frequency of *Salmonella* recovered and the associated population was significantly affected by the chlorine concentrations and T-128 applications (Table 3). *Salmonella* cells were recovered from the stem scars of the non-inoculated tomatoes when washed in solutions containing no or low levels of residue free chlorine. With no chlorine treatment, *Salmonella* cross-contamination were detected on all of the non-inoculated tomatoes following washing with inoculated tomatoes in distilled water for 2min with or without T-128, with average contamination level of 2.6 log MPN/g of the stem scar tissues. Cross contamination occurred at 100% rate when tomatoes were washed in 0.2mg/L or lower free chlorine solution irrespective of T-128 application; but the *Salmonella* populations recovered on the tomatoes stem scars were significantly lower when washed at the presence of T-128 (1.25 log MPN/g) than at the absence of T-128 (2.3 log MPN/g). When increasing the chlorine concentration to 5mg/L, no cross contamination was observed with T-128 treatment, while 67% of the tomatoes were cross-contaminated when washed at the absence of T-128. Increasing chlorine concentration to 10mg/L or above prevented the recovery of viable *Salmonella* cells in all of the treatments.

The observation that T-128 enhanced the efficacy of chlorine against *Salmonella* cross contamination of tomatoes was comparable to our earlier findings that T-128 increased chlorine efficacy against *E. coli* O157:H7 cross contamination during fresh-cut lettuce washing.[15] Cross contamination can occur either by direct contact between tomato fruits or mediated by the wash solution. Direct contact between the concaved stem scars seems unlikely to occur at high frequency. Therefore, wash solution mediated indirect contact at the stem scar is likely the major mechanism for pathogen cross contamination. This underlines the importance of ensuring wash water quality to prevent the potential survival by bacterial pathogens.

Table 3 *Salmonella* enterica sv. Thompson RM 1987 recovered in the stem-scars of non-inoculated tomatoes

Free Chlorine (mg/L)	No. of Tomatoes Washed[1]	Control		T128	
		% Positive	log MPN/g	% Positive	log MPN/g
50	15	0	ND[2]	0	ND
10	15	0	ND	0	ND
5	15	67	0.76±0.16	0	ND
0.2	15	100	2.32±0.05 A[3]	100	1.25±0.11 B
0	15	100	2.64±0.03 A	100	2.66±0.01 A

[1]Number represents total of five replicates of 3 tomatoes.
[2]Cross contamination not detected.
[3]Treatment means within each row with different capital letters are significantly different at α=0.05.

Efficacy of T-128 against Salmonella infiltration into tomato internal tissues

It has been demonstrated that the stem scars are the principal route of pathogen infiltration into the internal tissues.[10] Since T-128 significantly reduced *Salmonella* survival on the stem scars, the application of T-128 could significantly reduce or eliminate the likelihood of *Salmonella* infiltration. To test this hypothesis, the internal tissues of tomatoes exposed to contaminated wash solutions with or without T-128 were examined for the presence of *Salmonella* cells. Of the total 30 tomatoes washed in each solution, infiltration by *Salmonella* cells was detected in 67% of tomatoes following immersion for 2 min in inoculated distilled water, averaging 1.7 log10 MPN/g for the two *Salmonella* strains, regardless the presence of T-128. For tomatoes immersed in wash solutions with varying levels of tomato juice and residual free chlorine, infiltration was not observed when free chlorine was maintained at 1mg/L or higher, irrespective of T-128 application. Without T-128, infiltration occurred in 13%, 33%, and 53% of washed tomatoes, averaging -0.1, 0.8 and 1.2 log10 MPN/g, respectively, when free chlorine level decreased to 0.5, 0.1 and 0.02mg/L as a result of incremental addition of tomato juice. In contrast, no pathogen infiltration was detected when washed in solution containing 0.5 and 0.1mg/L free chlorine in the presence of T128. When free chlorine was further reduced to 0.02 mg/L, infiltration was detected in 20% of tomatoes, averaging 0.4 log10 MPN/g (vs. 53% of tomatoes had infiltration, with an average cell count of 1.2 log10 MPN/g in the absence T-128) (Table 4).

Human salmonellosis outbreaks associated with fresh produce consumption continued to occur over the past years in the United States, in spite of heightened awareness and increased emphasis on good agriculture practices.[23] These outbreaks underline the needs for more effective interventions and the adoption of new technologies to further reduce the risk of *Salmonella* contamination at all stages of produce production, processing, and distribution. Electron beam, X-ray and microwave irradiations have been used to reduce *Salmonella* on tomatoes with various degrees of success.[24–27] However, issues such as throughputs, costs, effects on produce quality, and consumer perceptions have prevented wide adoption of these technologies in commercial processing. Several chemical sanitizers, including chlorine dioxide, acidified sodium chlorite, ozone, and electrolyzed acidic water can reduce *Salmonella* on

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tomato surface at levels comparable to that of chlorinated water.[28–30] Recently several essential oils have been shown to significantly reduce *Salmonella* on tomatoes.[31–33] The economics and practicability of such intervention strategies in commercial processing remain to be demonstrated. In this study, we demonstrated that the application of a novel wash aid in conjunction with the most traditional sanitizer used in the fresh produce industry significantly increased the efficacy of the sanitizer for inactivating *Salmonella* in the stem scars, by preventing cross contamination and pathogen infiltration into the internal tissues. Although additional reduction of approximately 1 log10 unit of *Salmonella* compared to the chlorine wash solution for 2 min is modest, the improved efficacy against pathogen cross-contamination and international can significantly increase the safety margin for washing process control. The potential of T-128 improving the efficacy of other common sanitizers remains to be investigated.

Our observations also re-emphasize the importance of the stem scars as the principal gateway for the attachment, colonization, and infiltration by bacterial pathogens into tomatoes. Bacterial cells attached to the stem scars are much less susceptible to sanitizer treatment. Studies failing to examine the stem scars as the focal site of bacterial contamination therefore often risk over-estimating the efficacy of treatments. Several factors, including healing time and plant variety, affect stem-scar mediated infiltration by *Salmonella*.[10] Practices that minimize the contamination of stem scars and treatments that more effectively inactivate bacterial cells protected by the stem scars could lead to significant improvement of safety for commercially processed tomatoes.

Table 4 The Frequency and population of *Salmonella* enterica recovered from the internal tissues of tomatoes washed in chlorine solution with and without T-128

Free Chlorine (mg/L)	No. of Tomatoes Washed[1]	Control		T128	
		% Positive	log CFU/g[§]	% Positive	log CFU/g[§]
5	30	0	ND[2]	0	ND
1	30	0	ND	0	ND
0.5	30	13	-0.11±0.21	0	ND
0.1	30	33	0.80±0.19	0	ND
0.02	30	53	1.21±0.34 A[3]	20	0.40±0.36 B
0	30	67	1.72±0.21 A	67	1.73±0.16 A

[1]Number represents total of three replicates of 10 tomatoes
[2]ND: Infiltration not detected.
[3]Treatment means within each row with different capital letters are significantly different at α=0.05.

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Conflicts of interest

Authors declare that there is no conflict of interest.

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