Survival of *Salmonella* Transformed To Express Green Fluorescent Protein on Italian Parsley as Affected by Processing and Storage

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ABSTRACT

To study the effect of processing and storage parameters on the survival of *Salmonella* on fresh Italian parsley, parsley bunches were dipped for 3 or 15 min in suspensions that were preequilibrated to 5, 25, or 35°C and inoculated with *Salmonella* transformed to express enhanced green fluorescent protein. Loosely attached and/or associated, strongly attached and/or associated, and internalized and/or entrapped *Salmonella* cells were enumerated over 0, 1, and 7 days of storage at 25°C and over 0, 1, 7, 14, and 30 days of storage at 4°C using surface-plating procedures. Leaf sections obtained from samples after 0, 1, and 7 days of storage were examined using confocal scanning laser microscopy. Temperature of the dip suspension had little effect on the attachment and survival of *Salmonella* cells on parsley. Regardless of the temperature or duration of dip, *Salmonella* was internalized. Immersion for longer times resulted in higher numbers of attached and internalized cells. Microscopic observations supported these results and revealed *Salmonella* cells near the stomata and within cracks in the cuticle. Storage temperature had the greatest impact on the survival of *Salmonella* cells on parsley. When stored at 25°C, parsley had a shelf life of 7 days, and *Salmonella* populations significantly increased over the 7 days of storage. For parsley stored at 4°C, numbers of *Salmonella* cells decreased over days 0, 1, and 7. After 7 days of storage, there were no viable internalized *Salmonella* cells detected. Storage temperature represents an important control point for the safety of fresh parsley.

Fresh produce is an important source of enteric pathogens in foodborne illness outbreaks. Fruits and vegetables, which are commonly consumed without any cooking treatment, may potentially harbor many foodborne pathogens (12, 21). Ingestion of fresh parsley has been associated with *Shigella sonnei* and enterotoxigenic *Escherichia coli* infections (23). Salmonellosis outbreaks have been epidemiologically associated with the consumption of cantaloupes, watermelon, tomatoes, lettuce, celery, alfalfa sprouts, parsley, unpasteurized orange juice, and other raw salad vegetables (10, 11, 15, 17, 20). To assess the risk factors involved in the contamination of fresh produce with human pathogens, it is important to understand the microbial ecology of the various products and possible vectors during harvesting and processing that may contribute to product contamination.

Cultivation, harvesting, and retail preparation of fruits and vegetables provide numerous opportunities for human and equipment contact (5, 18). Typical manual labor operations including picking, sorting, trimming, tying, bunching, and removal of visible soil in rinse water, all of which subject the product to extensive handling and exposure to a variety of microorganisms. Rinse water can be of questionable bacteriological quality (18). Reina et al. (28) found that recycled water developed *Enterobacteriaceae* populations of up to 10⁶ CFU/g during a typical day's operation. Human pathogenic bacteria can become internalized in fruit

through immersion when there is a negative temperature differential (7, 9, 37). Zhuang et al. (37) demonstrated the internalization of *Salmonella* in tomatoes. Handling by workers and improperly sanitized equipment can further increase risk for contaminated products (5).

Following harvesting, parsley and other leafy greens are commonly submerged in a rinse tank to remove both field heat and visible soil contamination. Following this step, there may or may not be a spray rinse. If pathogenic bacteria were introduced into the rinse tank, there would be ample opportunity for product to be inoculated during rinsing. The purpose of this study was to examine attachment, growth, and survival of *Salmonella* inoculated onto parsley through rinsing in a contaminated rinse tank. In this study, parsley bunches were dipped in a suspension inoculated with *Salmonella* transformed to express enhanced green fluorescent protein (EGFP) to simulate a contaminated rinse tank. To examine the effect of a temperature differential and also the effect of length of immersion on attachment, parsley bunches were dipped in suspensions preequilibrated to 5, 25, and 35°C for 3 or 15 min. The presence of GFP-transformed salmonellae on parsley was evaluated using surface-plating procedures and confocal scanning laser microscopy.

MATERIALS AND METHODS

Bacterial cultures. Three *Salmonella* strains obtained from a previous study were transformed to express the plasmid vector EGFP (pEGFP, 3.4 kb; Clontech Laboratories, Inc., Palo Alto,

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Calif.). Transformed strains were *Salmonella* Javiana isolated from the surface of a cantaloupe and *Salmonella* Rubislaw and *Salmonella* Anatum isolated from irrigation water. The pEGFP, which carries a red-shifted variant of wild-type GFP, encodes a variant of the *Aequorea victoria* GFP that has been optimized for brighter fluorescence and higher expression. The EGFP gene was inserted in the frame with the *lacZ* initiation codon from pUC19, an *E. coli* cloning vector, so that the protein is expressed from the *lac* promoter. The donor (pUC) backbone also contained an ampicillin resistance gene for propagation and selection.

Salmonella Javiana cells were made competent and transformed using the calcium chloride method as described by Sambrook and Russell (29). *Salmonella* Anatum and *Salmonella* Rubislaw were transformed by electroporation to express EGFP. Electrocompetent *Salmonella* cells were prepared following procedures in The MicroPulser Electroporation Apparatus Operating Instructions and Applications Guide (Bio-Rad Laboratories, Hercules, Calif.) and methods described by O'Callaghan and Charbit (24) and were electroporated using a MicroPulser electroporator (Bio-Rad). Transformants produced by both the calcium chloride method and electroporation were selected by plating onto Luria-Bertani agar plates (Fisher Scientific, Pittsburgh, Pa.) supplemented with 100 µg/ml ampicillin (LBA-amp; Sigma-Aldrich Corp., St. Louis, Mo.). Transformed colonies were inoculated onto Protect Bacterial Preservers (Key Scientific Products, Round Rock, Tex.) and stored at -80°C.

Determination of stability of *Salmonella*/pEGFP strains.

Transformed *Salmonella* strains maintained at -80°C were transferred to 10 ml of Luria-Bertani broth supplemented with 100 µg/ml ampicillin (LBB-amp) and incubated in a Classic C76 Water Bath Shaker (New Brunswick Scientific Co., Edison, N.J.) at 37°C for 12 to 16 h with shaking (200 rpm). Cultures were then streaked for isolation using a sterile loop. Inoculated LBA-amp plates were incubated at 37°C and examined for fluorescing colonies using UV light (365 nm; model UVGL-58, UVP, Inc., San Gabriel, Calif.). A fluorescent colony was picked from each plate and used to inoculate a fresh LBA-amp plate. This procedure was repeated for 10 days to observe whether the *Salmonella* colonies maintained and/or continued to express the pEGFP.

LBA-amp plates inoculated with each of the transformed *Salmonella* strains were incubated at 37°C for 12 to 16 h and then storage at 4°C and observed biweekly over a 2-month period to determine whether the recombinant *Salmonella* strains continued to express green fluorescence.

Growth comparison of transformed *Salmonella* versus parent strains. The growth kinetics of the parent and recombinant strains of *Salmonella* were studied by constructing growth curves to ensure that GFP expression did not affect the growth of the transformed *Salmonella* cultures. For each of the three parent strains and their respective transformed strains, 10-ml aliquots of LBB and LBB-amp were inoculated with a 1% (vol/vol) overnight culture (18 to 24 h at 37°C) and allowed to incubate for 18 to 24 h at 37°C. Parent and recombinant strains were inoculated into 100-ml aliquots of LBB or LBB-amp and transferred into 500-ml Erlenmeyer flasks to achieve a final concentration of 10³ CFU/ml. The flasks were incubated at 37°C in a Classic C76 Water Bath Shaker (200 rpm), and at 0, 1, 2, 4, 6, 8, 12, and 24 h, a 5-ml sample was removed and plated onto LBA for parent *Salmonella* strains and LBA-amp for transformed *Salmonella* strains. Following incubation at 37°C for 24 h, colonies were counted.

Preparation of parsley and inoculum. Three cases of Italian (flat leaf) parsley (*Petroselinum crispum* var. *neapolitanum*)

were obtained from a packing shed. Parsley had been harvested within 24 h and had been subjected to a rinse in chlorinated water (city water supply, which contained 5 ppm chlorine). Upon arrival at the laboratory, bunches were placed onto aluminum foil and retied into bunches weighing 10 ± 0.1 g. Five centimeters of the stem end was removed using scissors sterilized by flaming with 95% alcohol. Parsley bunches were stored at 4°C in sterile plastic tubs prior to treatments (<10 h).

A three-strain inoculum was prepared from cultures of *Salmonella* Anatum, *Salmonella* Rubislaw, and *Salmonella* Javiana that were previously transformed to express EGFP. When preparing the inoculum, each strain was grown individually in 10 ml of LBB-amp and incubated overnight with shaking (200 rpm) at 37°C in a water bath shaker. Ten milliliters of each *Salmonella* culture was transferred to a 15-ml centrifuge tube and centrifuged three times (2,000 × *g* for 10 min at 21°C) with sterile 0.1% peptone water. Following the final wash, the cells were resuspended in 10 ml of sterile 0.1% peptone water to yield a final concentration of 10⁹ CFU/ml with an optical density at 600 nm of approximately 0.7. Equal volumes of each of the three *Salmonella*/pEGFP strains were combined. Eight milliliters of the three-strain suspension was transferred to 8 liters of sterile 0.1% peptone water preequilibrated to 5, 25, or 35°C in a Nalgene polypropylene sterilizing pan (54 by 43.5 by 13 cm; VWR Scientific, Suwanee, Ga.) to create an 8-liter dip suspension (inoculum) with a cell density of approximately 10⁶ CFU/ml. The aqueous peptone solution was used as a carrier for the inoculum to imitate practical conditions (4). As product is submerged in the dump tank, soil, leaves, and other organic material are also introduced into the water and it is common for more than one commodity to be processed in the same dump tank on the same day of processing. The cell density of the dip suspension was confirmed by surface plating appropriate decimal dilutions onto LBA-amp plates.

Processing and storage. Twelve 10-g parsley bunches (preequilibrated to 25°C) were placed in a sterile basket made from 19-gauge galvanized wire and then submerged in the 8-liter three-strain dip suspension preequilibrated to 5, 25, or 35°C for 3 or 15 min and rotated approximately every 1.5 min using sterile forceps. After dipping, the parsley was shaken gently to remove excess inoculum and allowed to stand for 1 h (at 25°C) to allow the excess inoculum to drain off and the leaves to dry before sampling or storing at 4 or 25°C. Parsley samples were stored in oxygen-permeable clear plastic, single-compartment hinged containers (21.1 by 21.1 by 7.3 cm; Sysco, Bryan, Tex.). Because parsley has a high respiration rate, six additional holes were created in the containers to allow ample oxygen transmission. Samples were analyzed on the day of inoculation (day 0) and after 1, 7, 14, and 30 days of storage at 4°C and after 1 and 7 days of storage at 25°C. For 4°C storage, containers with parsley were placed in a low-temperature incubator (Fisher Scientific) in a manner that allowed uniform air flow between containers. For 25°C storage, containers of parsley were placed in a closed metal storage cabinet. The 10-g parsley bunches not exposed to inoculum (controls) were sampled on day 0, after 1, 7, 14, and 30 days of storage at 4°C, and after 1 and 7 days of storage at 25°C.

Microbiological analyses. Loosely attached and/or associated, strongly attached and/or associated, and internalized and/or entrapped *Salmonella* cells were enumerated using the following procedures. To enumerate loosely attached and/or associated *Salmonella* cells, a 10-g bunch of parsley was added to 90 ml of sterile 0.1% peptone water in a sterile Whirl-Pak bag (17.8 by 27.9 cm; Nasco, Modesto, Calif.) and inverted 25 times. Bacteria in the peptone rinse were enumerated by surface plating appro-

TABLE 1. Mean counts of loosely attached, strongly attached, and internalized and/or entrapped *Salmonella*/pEGFP on parsley stored at 4°C stratified by dip temperature, duration of dip, and days of storage^a

Effect	<i>n</i>	Loosely attached ^b	Strongly attached ^c	Internalized/entrapped ^d
Temperature of dip (°C)	60	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001
5		2.1 B	1.9 B	1.4 A
25		1.7 C	1.7 C	1.2 B
35		2.6 A	2.4 A	1.5 A
Duration of dip (min)	270	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001
3		1.9 B	1.8 B	1.2 B
15		2.3 A	2.1 A	1.6 A
Storage (days)	36	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001
0		4.5 A	4.0 A	2.5 A
1		3.2 B	3.1 B	2.2 B
7		1.3 C	1.2 C	<1.0 C
14		<1.0 D	<1.0 D	<1.0 C
30		<1.0 D	<1.0 D	<1.0 C
Dip time × storage	18	<i>P</i> = 0.0002	<i>P</i> = 0.0001	<i>P</i> = 0.0001
3 min × 0 days		4.0 B	3.6 B	1.9 C
3 min × 1 day		2.8 C	2.8 C	1.9 C
3 min × 7 days		1.2 DE	1.2 DE	<1.0 D
3 min × 14 days		<1.0 E	<1.0 EF	<1.0 D
3 min × 30 days		<1.0 E	<1.0 F	<1.0 D
15 min × 0 days		4.9 A	4.4 A	3.0 A
15 min × 1 day		3.5 B	3.4 B	2.6 B
15 min × 7 days		1.5 D	1.3 D	<1.0 D
15 min × 14 days		<1.0 E	<1.0 F	<1.0 D
15 min × 30 days		<1.0 E	<1.0 F	<1.0 D

^a Counts are given as log CFU per gram. Within a column and for each effect, means with different letters are significantly different (*P* < 0.05).

^b Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml of 0.1% peptone water.

^c Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (prerinsed with 90 ml of 0.1% peptone water) with 90 ml of 0.1% peptone water.

^d Internalized/entrapped *Salmonella* were enumerated on parsley that was surface disinfected with a 2,000 mg/liter sodium hypochlorite solution.

priate decimal dilutions onto LBA-amp plates. To enumerate the strongly attached and/or associated bacteria, the peptone rinse was decanted and an additional 90 ml of sterile 0.1% peptone water was added to the 10 g of parsley and pummeled in a Stomacher Lab-Blender 400 (Tekmar Co., Cincinnati, Ohio) for 2 min. The resulting peptone rinse was surface plated onto LBA-amp plates. Plates were incubated at 37°C for 16 to 24 h and examined under UV light (365 nm), and green fluorescent colonies were counted.

A procedure employed by Buchanan et al. (7) to study the infiltration of *E. coli* O157:H7 into apples was used in this study to enumerate the internalized and/or entrapped *Salmonella* cells. A 10-g bunch of parsley was surface sanitized by immersion in 2.0 liters of a 2,000-mg/liter sodium hypochlorite solution at 25°C for 3 min, drained, and then immersed in sterile distilled water for two successive 1-min rinses. The 2,000-mg/liter sodium hypochlorite solution was prepared by adding 66.7 ml of commercial unscented bleach (6.00% sodium hypochlorite; Clorox Co., Oakland, Calif.) to 1933.3 ml of sterile distilled water. The pH of the chlorine solution was adjusted to 6.9 ± 0.1 using a pH meter (model 612; Markson Science, Inc., Henderson, N.C.). The effectiveness of the chlorine decreases with increasing pH, and at a pH close to neutral (7.0) approximately 75 to 80% of chlorine is in the form of hypochlorous acid, which is the form with the strongest antibacterial activity (26). The 10-g parsley bunch was then

placed in a stomacher bag with 90 ml of sterile 0.1% peptone water and pummeled for 2 min. Appropriate decimal dilutions of the homogenate were then surface plated onto LBA-amp plates, which were incubated at 37°C for 16 to 24 h. Following incubation, fluorescent colonies were identified and counted by examining the plates under UV light (365 nm).

To evaluate the background microflora, aerobic plate counts and yeast and mold counts were determined for the control samples. Ten grams of parsley was placed in a stomacher bag with 90 ml of sterile 0.1% peptone water and pummeled for 1 min. The decimal dilutions of the homogenate were plated onto Petrifilm Aerobic Count Plates (3M Microbiology Products, St. Paul, Minn.) and Petrifilm Yeast and Mold Count Plates (3M Microbiology Products). The aerobic count plates were incubated at room temperature (25°C) for 48 h. Following incubation, all colonies were counted. The yeast and mold count plates were incubated at 25°C for 72 ± 3 h, and yeasts were identified as small colonies with defined edges and pink-tan to blue-green color that were usually raised and usually without a focus (dark center in the middle of the colony). Molds appeared as large, flat colonies with diffuse edges and variable color and usually with a focus in the center of a colony.

Visualization of *Salmonella*/pEGFP on parsley. Four leaf sections (0.5 by 0.5 cm) obtained from samples from day 0, 1,

TABLE 2. Mean counts of loosely attached, strongly attached, and internalized and/or entrapped *Salmonella*/pEGFP on parsley stored at 25°C stratified by dip temperature, duration of dip, and days of storage^a

Effect	<i>n</i>	Loosely attached ^b	Strongly attached ^c	Internalized/entrapped ^d
Temperature of dip (°C)	36	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.1581
5		5.5 A	5.2 A	4.6 A
25		4.9 B	4.9 B	4.3 A
35		5.6 A	5.6 A	4.5 A
Duration of dip (min)	54	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001
3		5.0 B	4.9 B	4.1 B
15		5.6 A	5.5 A	4.8 A
Storage (days)	36	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001
0		4.3 C	3.9 C	2.7 C
1		5.4 B	5.4 B	4.8 B
7		6.2 A	6.3 A	5.9 A
Dip time × storage	18	<i>P</i> = 0.0169	<i>P</i> = 0.1410	<i>P</i> = 0.0130
3 min × 0 days		4.1 D	3.6 D	2.4 D
3 min × 1 day		4.9 C	5.0 C	4.2 C
3 min × 7 days		6.1 AB	6.1 AB	5.6 AB
15 min × 0 days		4.6 CD	4.2 D	3.0 D
15 min × 1 day		5.9 B	5.9 B	5.4 B
15 min × 7 days		6.4 A	6.5 A	6.1 A

^a Counts are given as log CFU per gram. Within a column and for each effect, means with different letters are significantly different (*P* < 0.05).

^b Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml of 0.1% peptone water.

^c Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (prerinsed with 90 ml of 0.1% peptone water) with 90 ml of 0.1% peptone water.

^d Internalized/entrapped *Salmonella* were enumerated on parsley that was surface disinfected with a 2,000 mg/liter sodium hypochlorite solution.

and 7 treatment groups were placed on a Rite-on microslide (GoldSeal Products, Portsmouth, N.H.), and a coverslip (VWR Scientific) was gently placed over the leaf sections. Each coverslip was secured by applying clear nail polish to the edge of the coverslip. Slides were transported to the Image Analysis Laboratory at the College of Veterinary Medicine at Texas A&M University (College Station, Tex.) where they were observed using a Radiance 2000 scanning laser confocal microscope (Bio-Rad) equipped with a krypton/argon laser interfaced with a fully equipped T300 inverted microscope (Nikon, Tokyo, Japan). EFGP-expressing *Salmonella* cells were detected using an excitation wavelength of 488 nm and observed using the 60× oil immersion objective. Images of fields that contained cells and in which the leaf structure was apparent were obtained and recorded using the Lasersharpe Capture Software (Bio-Rad).

Data analysis. Microbiological plate count data were converted to log CFU per gram before analysis. Data were analyzed using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, N.C.). The Bonferroni least significant difference method was used to determine whether there were significant differences (*P* < 0.05) between means.

RESULTS AND DISCUSSION

Stability of EGFP expression by *Salmonella* strains. Transformed *Salmonella* strains continued to express the pEGFP through 2 consecutive cycles of subculturing in LBB-amp followed by 10 cycles of subculturing onto LBA-amp plates. Other studies have also demonstrated that at

37°C, recombinant strains are able to maintain plasmid and express fluorescence when grown with antibiotic selection (14, 36). *Salmonella*/pEGFP strains were only grown on media supplemented with antibiotic. Prachaiyo and McLandsborough (27) found that when grown without antibiotic selection, approximately 90% of the cell population lost the plasmid over the course of an 8-h culture. Transformed *Salmonella* strains inoculated onto LBA-amp plates, incubated for 12 to 16 h, and stored at 4°C stably maintained the pEGFP plasmid for over 30 days. This result is in contrast with that of Takeuchi and Frank (33), who found that a percentage of *E. coli* O157:H7 transformed to express GFP lost or failed to express the pEGFP during refrigeration. Vialette et al. (36) reported that only 0 to 10% of *E. coli* O157:H7/pEGFP transformed strains lost the plasmid during storage at 10°C for 5 days. Fratamico et al. (14) found that freezing recombinant strains at −70°C in 20% glycerol did not affect the expression of GFP when the bacteria were subsequently cultured.

Growth comparison of transformed *Salmonella* versus parent strains. Several researchers have concluded that the growth and/or survival of transformed strains does not differ from that of their parent strains (6, 14, 19, 27, 35, 36). However, Oscar (25) found that the growth kinetics (i.e., lag time, specific growth rate, and maximum population density) of the GFP strains tested were different from those of the parent strains.

TABLE 3. Three-way interactions of dip temperature, duration of dip, and length of storage for mean counts of loosely attached, strongly attached, and internalized and/or entrapped *Salmonella*/pEGFP on parsley stored at 4°C^a

Dip temp × dip time × storage (n = 6)	Loosely attached (P = 0.0228) ^b	Strongly attached (P = 0.0015) ^c	Internalized/ entrapped (P = 0.0001) ^d
5°C × 3 min × 0 days	4.4 AB	3.6 BCD	1.9 EF
5°C × 3 min × 1 day	2.7 DEF	2.7 DE	2.3 DE
5°C × 3 min × 7 days	<1.0 HIJ	<1.0 IJ	<1.0 G
5°C × 3 min × 14 days	<1.0 J	<1.0 J	<1.0 G
5°C × 3 min × 30 days	<1.0 IJ	<1.0 J	<1.0 G
5°C × 15 min × 0 days	4.7 AB	4.1 ABC	2.8 ABCD
5°C × 15 min × 1 day	3.3 CD	3.2 CD	2.7 BCD
5°C × 15 min × 7 days	1.7 FGHI	1.6 GHI	<1.0 G
5°C × 15 min × 14 days	<1.0 HIJ	<1.0 J	<1.0 G
5°C × 15 min × 30 days	<1.0 J	<1.0 J	<1.0 G
25°C × 3 min × 0 days	2.9 CDE	2.7 DEF	1.6 F
25°C × 3 min × 1 day	1.8 FGHI	2.1 EFG	1.7 F
25°C × 3 min × 7 days	<1.0 HIJ	<1.0 IJ	<1.0 G
25°C × 3 min × 14 days	<1.0 J	<1.0 IJ	<1.0 G
25°C × 3 min × 30 days	<1.0 J	<1.0 J	<1.0 G
25°C × 15 min × 0 days	4.7 AB	4.1 ABC	3.0 ABC
25°C × 15 min × 1 day	3.0 CDE	3.2 CD	1.6 F
25°C × 15 min × 7 days	<1.0 HIJ	1.0 IJ	<1.0 G
25°C × 15 min × 14 days	<1.0 J	<1.0 J	<1.0 G
25°C × 15 min × 30 days	<1.0 J	<1.0 J	<1.0 G
35°C × 3 min × 0 days	4.6 AB	4.5 AB	2.3 CDE
35°C × 3 min × 1 day	4.1 BC	3.6 BC	1.8 EF
35°C × 3 min × 7 days	2.0 EFG	1.8 FGH	<1.0 G
35°C × 3 min × 14 days	1.1 GHIJ	1.0 IJ	<1.0 G
35°C × 3 min × 30 days	<1.0 HIJ	<1.0 J	<1.0 G
35°C × 15 min × 0 days	5.4 A	5.1 A	3.2 AB
35°C × 15 min × 1 day	4.4 AB	3.9 BC	3.5 A
35°C × 15 min × 7 days	1.8 FGH	1.3 GHIJ	<1.0 G
35°C × 15 min × 14 days	1.0 GHIJ	1.0 HIJ	<1.0 G
35°C × 15 min × 30 days	<1.0 HIJ	1.0 IJ	<1.0 G

^a Counts are given as log CFU per gram. Within a column, means with different letters are significantly different ($P < 0.05$).
^b Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml of 0.1% peptone water.
^c Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (prerinsed with 90 ml of 0.1% peptone water) with 90 ml of 0.1% peptone water.
^d Internalized/entrapped *Salmonella* were enumerated on parsley that was surface disinfected with a 2,000 mg/liter sodium hypochlorite solution.

Growth curves comparing the growth of the parent *Salmonella* strains with that of their respective *Salmonella*/pEGFP strains were constructed (not shown), and from these curves the generation times were determined for each of the recombinant and parent strains to serve as indices of their growth rates. Generation times for *Salmonella* Anatum, *Salmonella* Javiana, and *Salmonella* Rubislaw were all 0.3 h. Doubling times for the EGFP-expressing *Salmonella* strains were 0.4, 0.4, and 0.3 h, respectively. In this study, there were only minimal differences between the growth of two of the *Salmonella* strains (Anatum and Javiana) and their accompanying transformed strains. There was no difference between the growth of *Salmonella* Rubislaw/pEGFP and its parent strain.

Attachment of *Salmonella*/pEGFP to parsley. Studies have demonstrated that a negative temperature differential increases the infiltration of microorganisms into the stem scar of fruits (3, 8, 37). In this study, we examined

whether a temperature differential affected the attachment and internalization of pathogens on leafy vegetables with stems, such as parsley. Populations of loosely attached and/or associated, strongly attached and/or associated, and internalized and/or entrapped *Salmonella*/pEGFP cells were enumerated for each treatment. Internalized and/or entrapped cells were enumerated by disinfecting the surface of the parsley with a 2,000 ppm chlorine solution, which is an effective surface disinfectant. Seo and Frank (30) observed that a 20 ppm chlorine treatment destroyed bacteria on the surface of lettuce and some bacteria inside the stomata but had little effect on cells that had penetrated the lettuce tissue, and Zhuang et al. (37) reported that chlorine was effective at killing *Salmonella* Montevideo on the surface but did not significantly ($P < 0.05$) reduce the number of viable cells in the core tissue.

The effects of different processing and storage parameters, including the temperature of the dip suspension, du-

TABLE 4. Three-way interactions of dip temperature, duration of dip, and length of storage for mean counts of loosely attached, strongly attached, and internalized and/or entrapped *Salmonella*/pEGFP on parsley stored at 25°C^a

Dip temp × dip time × storage (n = 6)	Loosely attached (P = 0.0071) ^b	Strongly attached (P = 0.0034) ^c	Internalized/ entrapped (P = 0.0414) ^d
5°C × 3 min × 0 days	4.5 FGHI	3.7 IJ	2.5 DE
5°C × 3 min × 1 day	5.1 DEFGH	5.2 CDEFGH	4.9 BC
5°C × 3 min × 7 days	6.6 AB	6.4 AB	5.7 AB
5°C × 15 min × 0 days	4.6 EFGHI	3.9 HIJ	2.8 DE
5°C × 15 min × 1 day	5.7 BCDEFG	5.7 BCDE	5.3 AB
5°C × 15 min × 7 days	6.4 ABC	6.5 AB	6.1 A
25°C × 3 min × 0 days	3.0 I	2.7 J	1.9 E
25°C × 3 min × 1 day	4.1 HI	4.3 FGHI	3.8 CD
25°C × 3 min × 7 days	6.0 ABCDE	6.1 ABC	5.9 AB
25°C × 15 min × 0 days	4.4 GHI	4.0 GHIJ	3.0 DE
25°C × 15 min × 1 day	5.8 ABCDEF	5.8 BCD	5.4 AB
25°C × 15 min × 7 days	5.9 ABCDE	6.1 ABC	5.8 AB
35°C × 3 min × 0 days	4.7 EFGH	4.4 EFGHI	2.7 DE
35°C × 3 min × 1 day	5.3 CDEFG	5.4 BCDEF	3.9 CD
35°C × 3 min × 7 days	5.7 ABCDEFG	5.7 BCDE	5.2 AB
35°C × 15 min × 0 days	4.8 DEFGH	4.7 DEFGHI	3.3 DE
35°C × 15 min × 1 day	6.2 ABCD	6.2 ABC	5.6 AB
35°C × 15 min × 7 days	6.9 A	7.0 A	6.3 A

^a Counts are given as log CFU per gram. Within a column, means with different letters are significantly different ($P < 0.05$).
^b Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml of 0.1% peptone water.
^c Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (prerinsed with 90 ml of 0.1% peptone water) with 90 ml of 0.1% peptone water.
^d Internalized/entrapped *Salmonella* were enumerated on parsley that was surface disinfected with a 2,000 mg/liter sodium hypochlorite solution.

ration of the dip, and days of storage at 4 or 25°C, are presented in Tables 1 and 2, respectively. Mean counts (log CFU per gram) of loosely attached and/or associated, strongly attached and/or associated, and internalized and/or entrapped *Salmonella*/pEGFP are presented, stratified by the individual effects. Each of these individual treatment effects, averaged over each of the other variables, were significant ($P < 0.05$). Although, the three-way interactions of the main effects were significant ($P < 0.05$), the main effects are discussed individually because of the inability to discern very many meaningful interpretations from the three-way interactions.

For both storage temperatures and across all effects, differences in *Salmonella*/pEGFP populations, as affected by the dip temperature, were slight. Mean counts for loosely and strongly attached cells on parsley dipped in the 25°C suspensions were lower than those for parsley dipped in 5 and 35°C suspensions for both storage temperatures ($P < 0.05$). Differences in the means ranged from 0.2 to 0.9 log CFU/g. The number of internalized and/or entrapped cells on parsley stored at 4°C was significantly lower for parsley dipped at 25°C (1.2 log CFU/g for 25°C versus 1.4 and 1.5 log CFU/g for 5 and 35°C, respectively; $P < 0.05$); however, for parsley stored at 25°C, there were no differences between the means for internalized and/or entrapped cells on parsley (4.6, 4.3, and 4.5 log CFU/g for 5, 25, and 35°C, respectively). Regardless of the temperature differential, *Salmonella*/pEGFP cells were entrapped or internalized in the parsley structure.

In packinghouses, parsley is likely to be immersed in a dump tank for less than 3 min, but there are situations that may cause the product to be exposed to longer immersion times (i.e., processing equipment malfunctions slowing down the line, employee breaks, or random chance). Parsley is not necessarily removed from the tank on a “first in, first out” basis. Bartz (2) reported that when keeping dipping times to less than 2 min and immersion depths to less than 17 cm, infiltration of *Erwinia carotovora* subsp. *carotovora* into tomatoes could be prevented. Parsley submerged for 15 min had higher populations of loosely attached, strongly attached, and internalized *Salmonella*/pEGFP cells (Tables 1 and 2), which agrees with Bartz’s (2) findings. However, when considering the three-way interactions of dip temperature, dip time, and days of storage, there were fewer differences seen between the 3- and 15-min dips (Tables 3 and 4). For parsley stored at 4°C, numbers of strongly attached *Salmonella* cells were significantly higher for parsley dipped for 15 min after 0 and 1 day of storage. After 1 day of storage at 4 or 25°C, there were no differences based on immersion time, regardless of the temperature of the dip suspension (Tables 3 and 4).

Growth and survival of *Salmonella*/pEGFP on parsley as affected by immersion time in the dip suspension and the changes in the population during storage at 4 or 25°C are illustrated in Figures 1, 2, and 3 for loosely attached, strongly attached, and internalized and/or entrapped cells, respectively. Populations of loosely attached, strongly attached, and internalized and/or entrapped cells significantly

FIGURE 1. Effect of immersion time in dip suspension (3 or 15 min) and storage temperature (4 or 25°C) on the growth and survival of *Salmonella*/pEGFP (loosely attached) cells on parsley. Parsley was dipped for 15 min with storage at 25°C (○), 15 min with storage at 4°C (□), 3 min with storage at 25°C (●), and 3 min with storage at 4°C (■). The minimum detectable limit was 0.7 log CFU/g.

increased ($P < 0.05$) during storage at 25°C over days 0, 1, and 7, regardless of the duration of immersion (Figs. 1 through 3). In contrast, during storage at 4°C, all populations significantly decreased over days 0, 1, and 7, regardless of immersion time ($P < 0.05$). For parsley stored at 4°C, numbers of *Salmonella* were significantly higher for parsley dipped for 15 min ($P < 0.05$); however, after day 7 there were no differences based on immersion times ($P > 0.05$). For parsley stored at 25°C and sampled after days 0 and 1, populations of loosely attached cells were higher on parsley dipped for 15 min, but (as seen on parsley stored at 4°C) there was no differences on and following day 7. After 7 days of storage, no internalized cells were detected on parsley stored at 4°C, but viable loosely and strongly attached *Salmonella* cells were detected after 30 days of storage at 4°C. *Salmonella* cells rapidly died on parsley stored at 4°C.

For parsley dipped for 15 min, after 1 day of storage at 25°C the mean counts for loosely attached, strongly attached, and internalized and/or entrapped *Salmonella* cells were 5.9, 5.9, and 5.4 log CFU/g, respectively (Table 2). However, after only 1 day of storage at 4°C, the mean counts for loosely attached, strongly attached, and internalized and/or entrapped cells were reduced to 3.5, 3.4, and 2.6 log CFU/g, respectively (Table 1). Storage temperature is an important control measure for maintaining the safety of fresh parsley. As the parsley ages and begins to decay, *Salmonella* cells may be exposed to more of the phenolic acids, which are known to have antimicrobial effects. Phenolic compounds such as photoreactive furocoumarins that have been extracted from parsley can inhibit human pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, and various spoilage organisms, such as *E. carotovora*, and *Listeria innocua* (22). Elgayyar et al. (13) reported that oil extracted from parsley was strongly inhibitory against *Staphylococcus aureus*, *Yersinia enterocolitica*, and fungi and moderately inhibitory against *Salmonella* Typhimurium.

FIGURE 2. Effect of immersion time in dip suspension (3 or 15 min) and storage temperature (4 or 25°C) on the growth and survival of *Salmonella*/pEGFP (strongly attached) cells on parsley. Parsley was dipped for 15 min with storage at 25°C (○), 15 min with storage at 4°C (□), 3 min with storage at 25°C (●), and 3 min with storage at 4°C (■). The minimum detectable limit was 0.7 log CFU/g.

Background or natural microflora on noninoculated parsley was monitored over 0, 1, and 7 days of storage at 25°C and over 0, 1, 7, 14, and 30 days of storage at 4°C (Table 5). Counts for aerobic bacteria on parsley stored at 4 and 25°C were not significantly different ($P > 0.05$) and remained constant over the storage period. During the course of the storage period, parsley dehydrated considerably, possibly inhibiting the growth of the normal flora. The average aerobic bacterial counts for parsley at both storage temperatures and across all days of storage was 6.4 log CFU/g. Garg et al. (16) reported similar counts for spinach received from the grower. Average populations of yeasts and molds and for aerobic bacteria remained constant over the storage period. Only one significant difference was observed. On day 7, numbers of molds on parsley stored at

FIGURE 3. Effect of immersion time in dip suspension (3 or 15 min) and storage temperature (4 or 25°C) on the growth and survival of *Salmonella*/pEGFP (internalized) cells on parsley. Parsley was dipped for 15 min with storage at 25°C (○), 15 min with storage at 4°C (□), 3 min with storage at 25°C (●), and 3 min with storage at 4°C (■). The minimum detectable limit was 0.7 log CFU/g.

TABLE 5. Mean counts for aerobic bacteria, yeasts, and molds on noninoculated parsley samples over 0, 1, 7, 14, and 30 days of storage at 4 or 25°C^a

Organism	Storage temp (°C)	Counts on storage day:				
		0	1	7	14	30
Aerobic bacteria	4	6.4 AY	6.0 AY	6.4 AY	6.2 A	6.4 A
	25	7.0 AY	6.1 AY	6.6 AY	— ^b	—
Yeasts	4	5.2 AY	5.4 AY	5.3 AY	5.2 A	5.6 A
	25	5.5 AY	5.5 AY	5.5 AY	—	—
Molds	4	4.5 AY	4.4 AY	3.9 AY	4.3 A	4.2 A
	25	4.1 AY	4.1 AY	4.8 AZ	—	—

^a Counts are given as log CFU per gram, *n* = 3. Within rows, means (A) are not significantly different (*P* > 0.05). Within columns, means with different letters (Y and Z) are significantly different (*P* < 0.05).
^b Parsley samples stored at 25°C were determined to be spoiled after 7 days of storage.

FIGURE 4. Confocal scanning laser microscope images (×600) of a section of parsley leaf inoculated with *Salmonella*/pEGFP, showing attachment to leaf structures. Red arrows indicate the stomata, and yellow arrows indicate *Salmonella* cells.

4°C were significantly lower than those on parsley stored at 25°C; however, this finding may be due to sample variability. Babic et al. (1) also reported relatively constant yeast populations over storage for spinach leaves.

Visualization of *Salmonella*/pEGFP on parsley. *Salmonella*/pEGFP cells were observed on leaf sections collected from samples of parsley after 0, 1, and 7 days of storage from each treatment. Observations on leaves submerged at different temperatures did not reveal any obvious difference in cell populations, although populations were not enumerated. Higher populations of *Salmonella* were observed on leaves that had been submerged in the dip suspension for 15 min compared with leaves dipped for 3 min. Cells were observed near the stomata of the leaf and in fissures on the leaf surface, as visible in confocal scanning laser microscopic images in Figure 4. Cells were only observed near stomata and within cracks of the waxy cuticle. No cells were observed near the leaf edges or on intact leaf surfaces. These observations agree with those of *E. coli* O157:H7 on lettuce surfaces (30–32, 34). For leaf surfaces that had been surface disinfected with a 2,000 mg/liter sodium hypochlorite solution, no cells were observed regardless of immersion time. Seo and Frank (30) reported that a 20 mg/liter chlorine treatment easily killed bacteria on the surface of lettuce leaves. After 7 days of storage at room temperature, it was difficult to observe cells because of a high level of plant autofluorescence, which may be attributed to the decay of the plant structure and increased exposure of phenolic acids.

In this study, the temperature of a simulated dump tank or dip suspension had very little effect on the attachment, internalization, and survival of *Salmonella* on fresh Italian parsley. Regardless of the temperature of the dip suspension, *Salmonella* cells were internalized in the structure of parsley. Immersion for longer times in the dip suspension resulted in higher numbers of loosely attached, strongly attached, and internalized *Salmonella* cells. Observation of leaf sections using confocal scanning laser microscopy confirmed these findings. Storage temperature had the greatest impact on the survival of *Salmonella* on parsley and therefore represents an important control point for the safety of fresh parsley.

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