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Effects of Tomato Variety, Temperature Differential, and Post–Stem Removal Time on Internalization of *Salmonella enterica* Serovar Thompson in Tomatoes†

XIAODONG XIA,[1,2] YAGUANG LUO,[2]* YANG YANG,[2] BRYAN VINYARD,[3] KEITH SCHNEIDER,[4] AND JIANGHONG MENG[5]

[1]*School of Food Science and Engineering, Northwest A&F University, 28 Xinong Road, Yangling, Shaanxi, China 712100;* [2]*U.S. Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Environmental Microbiology and Food Safety Laboratory, 10300 Baltimore Avenue, Beltsville, Maryland 20705, USA;* [3]*U.S. Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Biometrical Consulting Service, 10300 Baltimore Avenue, Beltsville, Maryland 20705, USA;* [4]*Department of Food Science and Human Nutrition, University of Florida, Gainesville, Florida 32611, USA; and* [5]*Joint Institute for Food Safety and Applied Nutrition and Department of Nutrition and Food Science, University of Maryland, 0112 Skinner Building, College Park, Maryland 20742, USA*

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ABSTRACT

Tomatoes have been implicated in salmonellosis outbreaks due to possible contamination through bacterial internalization during postharvest handling. This study was conducted to determine the effects of tomato variety, temperature differential between tomato pulp and bacterial suspension, and the time delay between stem removal and immersion in bacterial suspension on internalization of *Salmonella enterica* serovar Thompson in tomato fruit. Mature green tomatoes at 32.2°C were immersed in water containing approximately 10^6 CFU/ml *S. enterica* bacteria. Different tomato varieties (Mountain Spring, Applause, and BHN961), temperature differentials (−10, 0, and 10°F, or −5.6, 0, and 5.6°C, respectively), and post–stem removal times (0, 2, and 16 h) were evaluated for their effects on *S. enterica* internalization. The incidence and density of internalized cells were determined by culture enrichment and most-probable-number methods, respectively. Overall, variety and post–stem removal time by variety interaction significantly affected the incidence of *S. enterica* internalization ($P < 0.0001$), while temperature differential had no significant effect ($P = 0.36$). Mountain Spring tomatoes were less susceptible to *S. enterica* internalization than were Applause and BHN961. Increasing the time interval between stem removal and immersion greatly reduced pathogen internalization in BHN961 and Applause, while it had no effect in Mountain Spring tomatoes. The variety and interactions between varieties and post–stem removal times ($P = 0.0363$) and between temperature differentials and post–stem removal times ($P = 0.0257$) had significant effects on the populations of internalized *S. enterica*. Furthermore, all internalized *S. enterica* cells were found within the core tissue segments immediately underneath the stem scars.

Salmonella enterica is a common etiologic agent of foodborne illness in the United States. It is estimated that foodborne *S. enterica* causes 1 million infections, 19,533 hospitalizations, and 378 deaths annually in the United States *(19)*. Traditionally, most salmonellosis cases and outbreaks were attributed to foods of animal origin, such as meat and poultry products, eggs, and dairy products; however, *S. enterica* outbreaks linked to consumption of fresh fruits and vegetables have been increasing recently *(12)*. Tomato fruit is a common vehicle of produce-associated salmonellosis in the United States and has been linked with at least 12 outbreaks since 1998 *(3)*.

Outbreak investigations linked to tomatoes suggest that contaminated wash water in tomato packing facilities may have been one of the factors contributing to microbial contamination *(13, 21)*. Other possible sources include irrigation water and the food preparation environment *(12)*. Pathogen internalization can occur through the porous tissues of the stem scar. Once internalized, bacteria are sheltered by tomato tissues and become difficult to remove or inactivate. Thus, preventing pathogen internalization is critical to reduce food safety risks. Current postharvest handling practices in typical tomato packinghouses include immersion of tomatoes for up to 2 min in a heated, chlorinated water dump tank, with water temperature maintained at least 5.6°C (10°F) higher than the incoming tomato pulp temperature *(23)*. This practice, intended to reduce the risk of pathogen internalization, was developed based on previous research on tomato soft rot development which suggested that immersing fruit in cooler water could allow gases inside the fruit to contract, thus creating negative pressure that allows water, along with the spoilage microorganisms, to be drawn into the fruit *(7, 25)*.

* Author for correspondence. Tel: 301-504-6186; Fax: 301-504-5107; E-mail: Yaguang.Luo@ars.usda.gov.

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Conversely, higher water temperatures should help prevent such infiltration *(7, 16, 25)*. However, whether such conditions are effective for controlling *Salmonella* contamination remains undetermined *(7)*. Contradictory findings which showed that heating water would not help prevent infiltration of spoilage microorganisms and might be detrimental to tomatoes have also been reported *(20)*. Additionally, heating dump tank water not only requires a large amount of energy and increases cost but also decreases chlorine efficiency *(23)*.

Previous studies also demonstrated that in addition to temperature differential, the tomato variety and the time between harvest and dump tank water immersion may affect tomato stem scar water uptake and soft rot development *(22)*. Smith et al. *(22)* reported that Florida 47 had significantly greater water uptake than Sebring when held under the same conditions and that greater water uptake occurred when the fruits of both varieties were dipped in water at 2 h after harvest than at 8, 14, and 26 h after harvest. However, no studies have evaluated the effects of these postharvest handling conditions on *S. enterica* internalization. Therefore, the aim of this study was to examine the effects of three major factors (tomato variety, temperature differential between tomato pulp and bacterial suspension, and post–stem removal time) on the internalization of *Salmonella enterica* serovar Thompson into tomato fruit.

MATERIALS AND METHODS

Bacterial culture. *S. enterica* serovar Thompson strain RM1987 transformed with plasmid pGT-KAN, which conferred green fluorescence and gentamicin resistance, was used in this study. The parent strain, RM1987, was a clinical isolate from a salmonellosis outbreak linked to consumption of cilantro in California in 1999 *(8, 9)*. *Salmonella* Thompson has also been previously associated with a tomato salmonellosis outbreak *(12)*. The transformed strain was maintained at −80°C in brain heart infusion broth containing 20% glycerol. Prior to experimentation, the bacterial stock was streaked onto a blood agar plate and incubated at 35°C overnight. The overnight culture was inoculated into tryptic soy broth (TSB) containing 50 μg/ml gentamicin and grown overnight at 35°C with shaking. The bacterial cell suspension was prepared by adding 1.5 ml of this culture into 4.5 liters of distilled water. The final concentration of the cell suspension was determined by plate counting to be approximately 10^6 CFU/ml.

Tomato harvesting. For studies investigating the effects of tomato variety and postharvest handling practices on pathogen internalization, mature green tomatoes (*Lycopersicon esculentum* Mill.) of three varieties (Applause, BHN961, and Mountain Spring) were harvested from Spicknall's Farm (Beltsville, MD). Fruits were harvested by cutting the petiole about 3 cm from the calyx with clippers, so that part of the stem remained on the fruit until testing time (within 0 to 16 h postharvest). Tomatoes were sorted to remove samples with visible defects or damage and to ensure uniform size and shape of the samples from each variety. All tomatoes were incubated overnight at 32.2°C to ensure uniform tomato pulp temperature. Experiments involving the determination of pathogen distribution inside tomatoes were conducted during a period when locally grown tomatoes were not available, and therefore, recently harvested stemless mature green tomatoes (cv. Soraya) grown in Florida were obtained from Coastal Sunbelt Produce Co. (Savage, MD) via an in-kind donation.

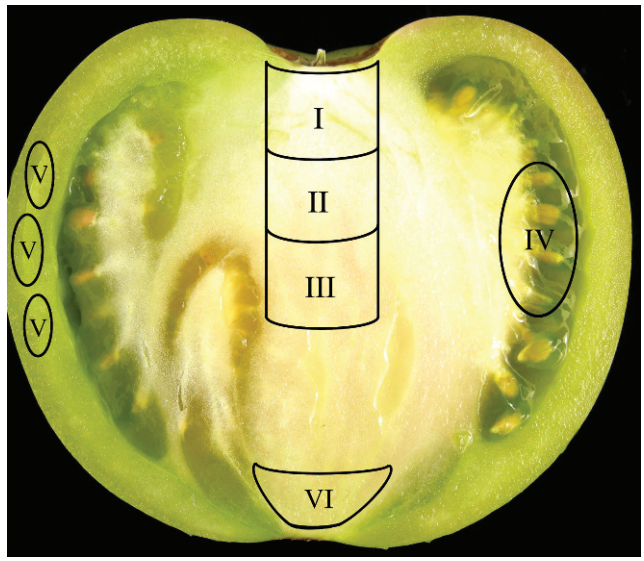

FIGURE 1. *Photograph and schematic drawing of a tomato showing various sample locations. I, tomato core tissue segment within 2 to 12 mm below stem scar; II, tomato core tissue segment within 12 to 22 mm below stem scar; III, tomato core tissue segment within 22 to 32 mm below stem scar; IV, tomato locular cavity; V, tomato pericarp tissues; VI, tomato internal tissues below the blossom end.*

Determining *S. enterica* distribution profile inside tomatoes. Tomatoes were incubated overnight at 32.2°C. The cell suspension of *S. enterica* was adjusted to 2.3°C to generate a temperature differential of −30°C between the fruit pulp and the bacterial suspension. Batches of tomatoes (five tomatoes per batch) were submerged in 4.5 liters of the bacterial suspension in a plastic tray with the submerging depth held at approximately 2 layers of tomatoes (a depth of approximately 12 cm). All tomatoes were taped to the bottom of the tray and held for 30 min to ensure that *S. enterica* infiltrated into most of the tested tomatoes. After removal from the bacterial suspension, each tomato (with stem scar pointing downward) was sprayed with 70% ethanol, followed by wiping with 70% ethanol-saturated paper towels to ensure that surface-attached *S. enterica* bacteria were killed to avoid cross-contamination. After drying on sterile petri dishes, the internal tissues from six different locations in the tomatoes, as shown in Figure 1, were excised for microbial analysis. Excision proceeded as follows: a longitudinal cut was first made at the shoulder of the tomato distant from the core tissue to expose the internal tissue. This was followed by two latitudinal cuts initiating from the first cut surface at approximately 2 mm and 32 mm below the stem scar. A cylindrical plug of tomato core tissues was obtained from the resulting slab of tomato by pushing a cork borer from the side farthest from the stem scar (less likely to be contaminated) toward the stem scar side. The core tissue plug was then pushed out of the cork borer with a sterile cotton swab and sequentially cut into three 10-mm segments (sample locations I, II, and III). An incision close to the locule was then made through the edge of the placental tissue in order to free the gel in the locular cavity (IV); a small amount of the placental tissue was taken along with the gel due to the difficulty of separating them. The outer wall of the pericarp (V) was sampled by cutting out three wedge segments with a scalpel. A

latitudinal cut approximately 10 cm above the blossom end of the tomato was then made, and a conical segment of internal tissue (VI) was excised using a scalpel. In order to avoid any potential for contamination, all items that came into contact with tomato fruit were either presterilized or flamed after dipping in 70% alcohol if reuse was necessary, and each cut was made with a separate sterile knife or scalpel. Additionally, two different surface sterilization methods were tested for their efficacy in eliminating *S. enterica* from the tomato surface. One included a 70% ethanol spray and wipe, and the other was to flame the surface following exposure to 90% ethanol *(14)*. The tomato skins were stomached and enriched in TSB. No live bacterial cells were detected on the tomato surface following any of these methods. Spraying and wiping tomatoes with 70% ethanol was selected because of its ease of handling and avoidance of incidental killing of internalized bacterial cells.

Assessing the effects of tomato variety and postharvest handling conditions on *S. enterica* internalization. Three major factors were evaluated. These were the tomato variety (Applause, BHN961, and Mountain Spring) and two postharvest handling conditions, the temperature differential (−5.6, 0, and 5.6°C) between tomato pulp and bacterial suspension and the post–stem removal time (0, 2, and 16 h). The entire study was completed in 24 experimental trials, each of which consisted of 15 tomatoes that were of the same variety, had the same post–stem removal time, and were tested under three temperature differentials. The tomato temperature was adjusted to 32.2°C overnight as described above. An *S. enterica* suspension was prepared and divided into three basins with the temperature adjusted to 80°F (26.7°C), 90°F (32.2°C), and 100°F (37.8°C). A batch of five tomatoes was then submerged in one of those *S. enterica* suspensions for 2 min using gloved hands. The tomatoes were then removed from the bacterial suspension and surface sterilized individually as described above. The core tissues (Fig. 1, combined sections I and II) were excised for subsequent *S. enterica* enrichment and enumeration following a procedure similar to that described in the previous section. Both negative (tomatoes submerged in sterile distilled water) and positive (tomatoes with pin holes on the stem scar area) controls were included for each batch of tomatoes tested as a validation procedure. Each combination of variety, post–stem removal time, and temperature differential was repeated two to five times (replication numbers were not uniform due to insufficient sample quantities for some of the treatments).

Microbial analysis. The internal tissue samples of tomatoes obtained as described above were placed in a sterile stomacher bag and mixed with gentamicin (50 μg/ml)–supplemented TSB at 10 times the sample weight. The samples were macerated for 1 min using a stomacher blender (Seward 400 Biomaster, Brinkmann Seward, Ontario, Canada). A portion of the filtrate was taken out of the bag and stored at 4°C, and the remaining mixture in the stomacher bag was incubated at 35°C overnight. If the solution in the stomacher bag showed signs of growth (turbidity), a loopful of the solution was further plated on gentamicin (50 μg/ml)–supplemented tryptic soy agar (TSA). Colonies were confirmed as RM1987/pGT-KAN by the presence of green fluorescence under UV light. The internalization incidence in each replication was calculated based on the number of confirmed positive samples over the total number of tomatoes ($n = 5$). For positive samples, the bacterial concentration in the corresponding 4°C stored sample was enumerated with a microplate-based eight-well most-probable-number (MPN) method as previously described *(15),* with modifications. In brief, eight 3-ml aliquots were serially diluted in gentamicin (50 μg/ml)–supplemented TSB in a deep-well microplate and incubated at 35°C overnight. This was followed by plating 3-μl droplets of all dilutions on gentamicin (50 μg/ml)–supplemented TSA medium. After incubation for 6 h, the growth of *S. enterica* was confirmed by fluorescence and the growth pattern was recorded and analyzed using MPN calculator software available online *(11)*.

Experimental design and statistical analysis. The experimental design was a split plot with variety and post–stem removal time applied to the whole plot (i.e., trial) and temperature applied to subplots within each trial. A 3^3 factorial split-plot analysis of variance model was fit separately to the incidence and the MPN data. The MPN data were log transformed to meet the requirement for normality and homogenous variance, and a variance grouping technique was used to model the observed variance heterogeneity using PROC MIXED in SAS 9.2. The percent infiltration data were modeled using PROC GLIMMIX in SAS 9.2 to fit a generalized linear model specifying a binary distribution for incidence and using a logit link function. When effects were statistically significant, means comparisons were conducted using Sidak-adjusted (MPN) and Shaffer-adjusted (incidence) P values to maintain experimentwise errors of ≤ 0.05.

RESULTS

Distribution of *S. enterica* inside tomatoes. The incidence and extent of *S. enterica* internalization varied significantly among different locations and/or tissue types (Figs. 1 and 2). Among all 25 tomato samples tested, *S. enterica* was only detected in the core tissue segments (Fig. 1, segments I, II, and III). The internalization incidence and populations in the core tissue samples declined steadily with distance from the stem scar. The highest internalization incidence (68% of tomatoes sampled) and *S. enterica* populations (average, 527 MPN/g) were found in the segment immediately beneath the stem scar (Fig. 2, segment I), while the internalization incidence and population declined to 52% and 44 MPN/g, respectively, on samples taken from the tissues at 12 to 22 mm below the stem scar (Fig. 2, segment II). At a distance of 22 to 32 mm below the stem scar (Fig. 2, segment III), 32% of tomatoes tested positive for *S. enterica* with the cell counts barely above the detection limit (0.38 MPN/g). *S. enterica* was not detected in any of the other internal tissues, including the locular cavity, tissues proximal to the blossom end, and pericarp tissue samples. This distribution pattern suggests that the stem scar is the major point of entry for *S. enterica* internalization. The high *S. enterica* populations and frequency of internalization in core tissue segments I and II prompted us to use these combined segments for subsequent studies investigating the effects of the tomato variety and post-harvest handling conditions on *S. enterica* internalization.

Internalization frequency of *S. enterica*. A total of 24 experimental trials were conducted to assess the frequency of *S. enterica* internalization in tomatoes as affected by tomato variety, temperature differential between tomato fruit pulp and bacterial suspension, and time delay between stem removal and immersion in bacterial suspension. As shown in Figure 3, the internalization frequency was significantly

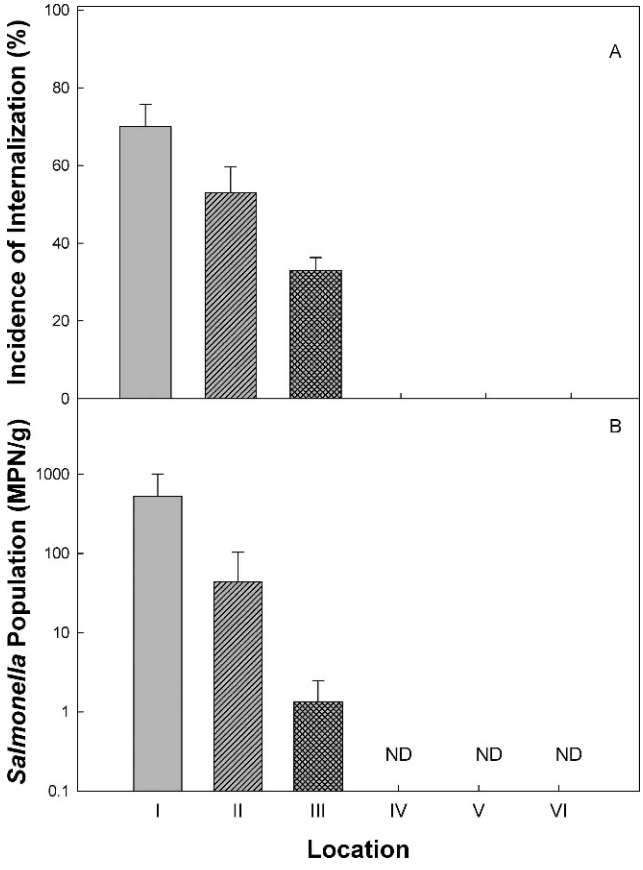

FIGURE 2. *Frequency and extent of* Salmonella *Thompson internalization at various locations within tomato fruit. I, tomato core tissue segment within 2 to 12 mm below stem scar; II, tomato core tissue segment within 12 to 22 mm below stem scar; III, tomato core tissue segment within 22 to 32 mm below stem scar; IV, tomato locular cavity; V, tomato pericarp tissues; VI, tomato internal tissues below the blossom end. ND, not detectable at a detection limit of 0.38 MPN/g.*

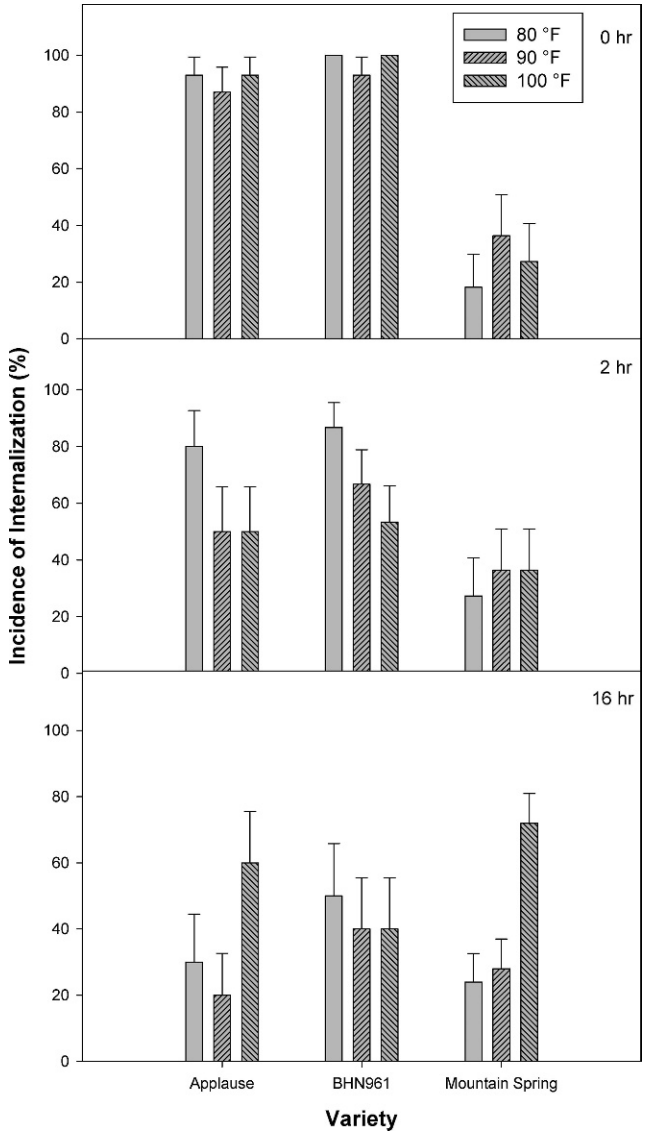

FIGURE 3. *Frequency of internalization of* Salmonella *Thompson in tomatoes (*n *= 360) as affected by variety, temperature differential between tomato pulp and bacterial suspension, and post–stem removal time. Vertical bars show means ± standard errors.*

($P < 0.0001$) affected by tomato variety but not by temperature differential ($P = 0.3131$) under the testing conditions. There was also a significant ($P < 0.0003$) interaction between tomato variety and post–stem removal time. Among the three tomato varieties tested, Applause and BHN961 had significantly higher internalization frequencies than Mountain Spring at 0 and 2 h post–stem removal times. Removing the stems immediately before immersion resulted in the highest pathogen internalization incidences, with averages of 91.1 and 97.8% for the Applause and BHN961 varieties, respectively, although the average was only 27.8% for Mountain Spring tomatoes. Applause and BHN961 had significantly lower incidences of *S. enterica* internalization when post–stem removal time was increased from 0 to 2 h and from 2 to 16 h, with the average incidences declining to 60.0 and 68.9% at 2 h and to 36.7 and 43.3% at 16 h, respectively. Interestingly, Mountain Spring tomatoes did not follow the same pattern as Applause and BHN961; they exhibited no significant difference in internalization incidence among the three post–stem removal times.

Populations of internalized *S. enterica*. The concentration of internalized *S. enterica* cells in each of the 209 positive tomatoes was further analyzed. Cell populations in the positive samples varied greatly, from below the detection limit of 0.38 MPN/g to 1,200 MPN/g, and most samples (204 of 209) had fewer than 100 MPN/g recoverable *S. enterica* cells. Analysis of all log-transformed MPN data from positive samples only showed that none of the three factors (variety, temperature differential, and post–stem removal time) had a significant effect on the number of internalized cells (data not shown). However, when all tomato samples were included (Fig. 4), all three factors had a significant effect on internalized bacteria populations, with significant effects among varieties ($P = 0.0105$) and for the interactions between variety and post–stem removal time ($P = 0.0363$) and between temperature differential and post–stem removal time ($P = 0.0257$). Examining the variety by post–stem removal time effect in more detail indicates a significant difference among stem-removal times

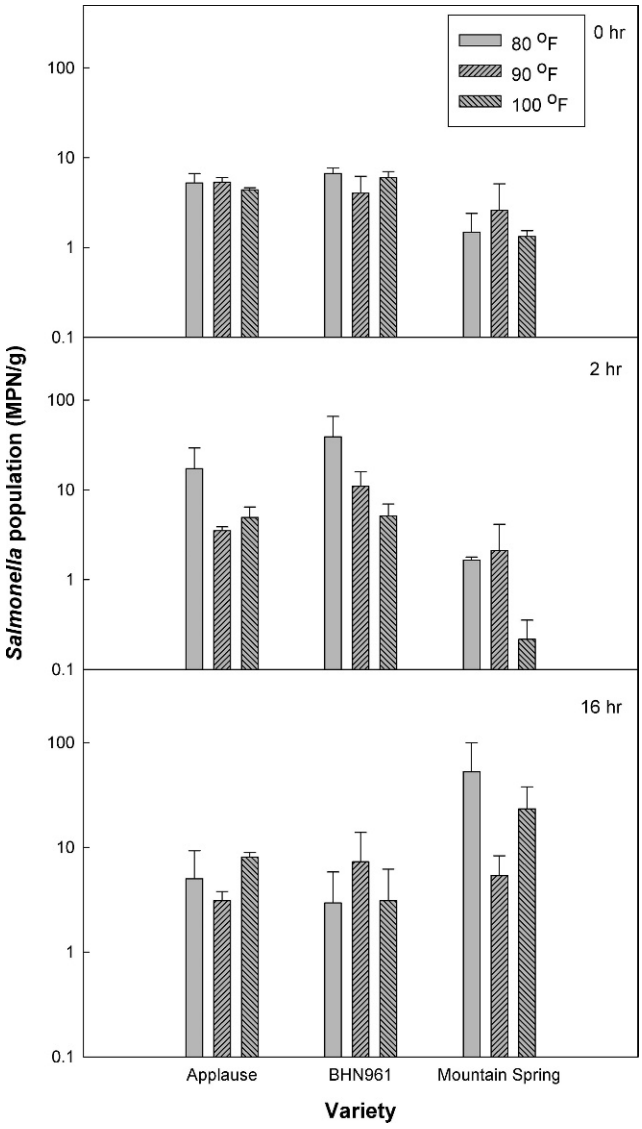

FIGURE 4. Salmonella *Thompson populations recovered in tomatoes positive for pathogen internalization as affected by variety, temperature differential between tomato pulp and bacterial suspension, and post–stem removal time. Vertical bars show means ± standard errors.*

for BHN961 ($P = 0.0417$) and a significant difference among varieties for both the 0 h ($P = 0.0108$) and 2 h ($P = 0.0093$) post–stem removal times. Examining the post–stem removal time by temperature differential interaction in more detail indicates a significant difference among temperature differentials at a post–stem removal time of 2 h ($P = 0.0129$), when Applause and BHN961 tomatoes had significantly larger amounts of *S. enterica* internalized.

DISCUSSION

In this study, the distribution of internalized *S. enterica* in tomatoes and the effects of tomato variety, temperature differential, and post–stem removal time on *S. enterica* internalization were investigated. All internalized *S. enterica* bacteria were found in the tomato core tissue segments underneath the stem scar, with the concentration gradient negatively correlating with the distance from the stem scar. This distribution pattern suggests that the stem scar is the major point of entry for *S. enterica* internalization. Samish et al. *(17, 18)* evaluated the distribution of aerobic bacteria inside tomatoes and also reported that the vast majority of bacteria were found in the region below the stem scar. Experiments on the effects of tomato varieties and postharvest handling conditions in this study indicated that different varieties of tomatoes varied largely in their susceptibility to *S. enterica* internalization. While the temperature differential had no significant effect on *S. enterica* internalization frequency and limited effect on the population of internalized cells under the testing conditions, the tomato variety and the time delay between stem removal and immersion significantly affected internalization frequency.

The differential between tomato dump tank temperature and tomato pulp temperature has been considered a critical factor for preventing pathogen internalization during packinghouse operations *(4, 5)*. A number of food safety guidelines have recommended the maintenance of at least a 5.6°C positive temperature differential, i.e., raising tomato dump tank water to at least 5.6°C above the pulp temperature of incoming tomatoes *(1, 2, 23, 24)*. These recommendations were established based on the conditions needed to prevent tomato soft rot development. However, many of those soft rot–related studies often used worst-case scenario conditions with a large temperature differential (−35°C to +33°C) and an extensive immersion time (10 to 30 min) *(4, 7)*. No direct scientific studies focusing on *S. enterica* internalization in tomatoes as affected by the temperature differential between the dump tank and tomato pulp were performed under the conditions matching the recommended operation parameters for food safety. Additionally, no studies examined the effects of postharvest handling conditions on *S. enterica* internalization exclusively to the internal tissues. In a study designed to evaluate the effects of chlorine treatment and storage temperature on the survival and growth of *Salmonella* Montevideo in tomatoes, Zhuang et al. *(25)* observed that there was more pathogen uptake by the core tissues when the tomatoes were submerged in a bacterial suspension that was 15°C colder than the tomato pulp than when tomatoes were submerged in a suspension that was 12°C warmer than the pulp. However, in that study, internalized cells (internal to the stem scar) were not separated from those attached to the stem scars. Burnett et al. *(10)* studied pathogen infiltration into apples as affected by water and pulp temperature differential. They reported that pathogens infiltrated into the core tissues of apples regardless of negative or positive temperature differential. Bartz and Showalter *(7)* submerged tomatoes in a *Serratia marcescens* suspension and were able to isolate the same organism from the internal tissues of tomatoes when there was a negative temperature differential but not when there was a zero or positive temperature differential. However, only a limited sample size was involved in that study. The present study demonstrates that *S. enterica* internalization could still occur under a zero or positive temperature differential *(16)*. Segall et al. *(20)* showed that the incidence of bacterial soft rot was higher in

tomatoes submerged in contaminated dump tank water at 32.2°C than at 15.6°C, while Bartz and Showalter *(7)* demonstrated that more soft rot decay caused by *Erwinia carotovora* subsp. *carotovora* occurred in fruit immersed in a bacterial suspension under a negative temperature differential than under a positive temperature differential *(4)*. Furthermore, studies have also shown that tomato immersion time and depth highly affected soft rot development *(7)*. Our current study investigated the effect of temperature differential on *S. enterica* internalization when other conditions were maintained under the recommended food safety practices, i.e., an immersion time of no more than 2 min and an immersion depth of no more than 2 layers of tomatoes. Under these testing conditions, the temperature differential showed no significant effect on *S. enterica* internalization incidence and a limited effect on the populations of internalized cells. It is worth noting that an enrichment procedure in combination with a modified eight-tube MPN method was employed in this study. With this high detection sensitivity, we found a high frequency of *S. enterica* internalization with low populations of internalized cells, with an absence of any appreciable weight gain. Additional studies comparing different inoculation levels indicate that reducing the inoculum concentration reduced infiltration incidence and cell density; however, the experiment trend in terms of temperature differential effect remained the same. This suggests that maintaining sufficient sanitizer levels in the tomato dump tank is critical to avoid pathogen internalization.

Tomatoes of different varieties have been demonstrated previously to have various susceptibilities to water uptake during submersion *(22)*, as well as various susceptibilities to disease due to plant pathogens, including *E. carotovora (6)*. This study demonstrated that tomato varieties vary largely in their susceptibility to *S. enterica* internalization. The Applause and BHN961 varieties were highly susceptible to *S. enterica* internalization immediately after stem removal, while Mountain Spring was less susceptible to *S. enterica* internalization even immediately after stem removal. The exact reason for tomato variety differences in susceptibility to pathogen internalization is unknown. The porosity of the stem scars and the physical and chemical characteristics of the vascular bundles of the tomato varieties may contribute to the differential susceptibility to pathogen infiltration.

The time delay between stem removal and water immersion had a significant impact on *S. enterica* internalization. Tomatoes with stems removed immediately before immersion had a significantly higher rate of internalization than tomatoes whose stems were removed 2 or 16 h before immersion. This result was in line with the findings from Smith et al. *(22)* that tomato fruit absorbed less water at 8 h than at 2 h postharvest. During experimentation, we observed that stem scars dried out gradually after stem removal, and the surface became more hydrophobic over time. Bartz and Showalter *(7)* also suggested that stem scars change from more water congested to more air congested with time, making water uptake more difficult. All these findings suggest that a delay between stem removal and immersion in the dump tank water can be a cost-effective way to reduce internalization of both plant and human pathogens into tomatoes.

In summary, this study demonstrates that tomato varieties and post–stem removal times significantly impact the frequency of *S. enterica* internalization in tomatoes and the internalized cell populations, while temperature differentials of up to 10°F have no effect on the internalization frequency and limited impact on the cell populations internalized. These findings are in line with those on tomato soft rot development in terms of varietal difference and post–stem removal interval, while the effects of temperature differential on soft rot versus *S. enterica* cannot be directly compared, primarily due to the different temperature differentials and immersion times used in these studies. Further worth noting is that this present study used a sensitive enrichment procedure that detects *S. enterica* populations as low as 1 cell per tomato, while traditional soft rot–related studies assessed the treatment conditions based on the development of symptoms rather than quantification of the causative agent, i.e., *E. carotovora*. Given the significant impact of tomato variety on the internalization of plant and human pathogens, the breeding and selection of resistant varieties and cultivars appears to be a promising approach in the endeavor to minimize pathogen internalization. Additionally, a time delay between stem removal and dump tank submersion can also reduce the incidence of pathogen internalization for sensitive varieties.

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