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INFLUENCE OF TEMPERATURE, HUMIDITY, AND DILUENT TYPE ON SURVIVAL OF *SALMONELLA* SPP. ON THE SURFACE OF RAW TOMATOES

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ABSTRACT

Tomatoes are an important commodity, placing fourth among most popular vegetables in the U.S. However, fresh tomatoes lack a final pathogen elimination step and have been implicated in *Salmonella*-related outbreaks. The purpose of the study was to evaluate survival of *Salmonella* post-drying in three diluents on the surface of green mature tomatoes at 12 °C or 25 °C. Additionally, low and high air relative humidity influence was evaluated at 25 °C on pathogen survival. A five *Salmonella* rifampin-resistant strain cocktail was double-washed in buffered peptone water (BPW) and resuspended in 0.1% peptone, BPW, or fresh tomato serum. Inoculum (0.1 mL) was allowed to dry on the surface of tomatoes. For study I, tomatoes were placed in 12 °C and 25 °C incubators with no humidity control and sampled on days 0, 1, 3, and 5. For study II, tomatoes were sampled on days 0, 1 (biosafety hood storage) and on day 5 after storage in two 25 °C incubators (low and high relative humidity). *Salmonella* was recovered from tomatoes (20 mL BPW) and plated (TSA-rif80, 37 °C, 48 hours). Post-drying *Salmonella* counts (ca. 4.5 – 5.0 $\log_{10}$ CFU.mL^{-1}) remained at 4.03 and 4.40 $\log_{10}$ CFU.mL^{-1} in serum after 5 days of storage at 12 °C and 25 °C, respectively. Conversely, corresponding counts in BPW and peptone were lower at ca. 1.4 to 1.8 and 2.2 to 2.8 log units at 12 °C and 25 °C, respectively. At low humidity, post-drying *Salmonella* counts showed highest decline for peptone (final 1.98 $\log_{10}$ CFU.mL^{-1}) compared to BPW (3.79 $\log_{10}$ CFU.mL^{-1}) and tomato serum (4.75 $\log_{10}$ CFU.mL^{-1}) on day 5. Counts declined rapidly to 0.03, 0.56, and 0.44 $\log_{10}$ CFU.mL^{-1} for peptone, BPW, and tomato serum, respectively, at high humidity on day 5. To summarize, it was shown that increased solutes have protective effect on *Salmonella* in desiccated conditions, while high humidity storage causes accelerated death of stationary culture within five days storage period.

Keywords: tomatoes; *Salmonella*; humidity; refrigeration; survival

INTRODUCTION

Tomatoes are an important commodity, placing fourth among most popular vegetables in the U.S. According to **FAOSTAT (2017)**, top ten tomato producing countries in the world were China, India, Turkey, USA, Egypt, Iran, Italy, Spain, Mexico, and Brazil, with Slovakia present in the top twenty and producing as much as 21,964 tonnes in 2017 alone. Enteric pathogens, such as *Escherichia coli* O157:H7 and *Salmonella*, may be present on fresh produce as contamination from environment and may persist on the surfaces **(Sreedharan et al., 2015; Tokarskyy et al., 2018)**. *Salmonella*-associated tomato outbreaks were recorded in the United States on numerous occasions **(CDC, 2002; Croby et al., 2005)**. It is generally believed that pathogen will grow in the tomato flesh at ambient temperature if introduced through stem scars, wounds, and abrasions **(Wei, 1995; Zhuang, Beuchat and Angulo, 1995; Shi et al., 2007; Beuchat and Mann, 2008)**. As for the fate of the pathogen on the healthy tomato surface, most studies agree that *Salmonella* populations decline over time, depending on bacterial strain, humidity, and tomato storage temperature **(Yuk, Warren and Schneider, 2007; Tokarskyy et al., 2018)**. Conversely, a study by **Iturriaga, Tamplin and Escartín (2007)** showed the potential for *Salmonella* Montevideo to colonize and grow on the surface of healthy undamaged tomatoes, but those results could have been due to the presence of micro abrasions on the surface where pathogen could have been introduced, or possibility of the pathogen introduction onto the stem part during wash-off step **(Wei et al. 1995)**. Therefore, *Salmonella* will likely to die on the surface of healthy tomatoes, and its behaviour might mimic survival rate on non-biological inanimate objects.

A systematic review by **Kramer, Schwebke and Kampf (2006)** suggested that nosocomial bacterial pathogens, including *Salmonella* and *Escherichia coli*, persist on inanimate objects longer at higher inocula, higher solute concentration, higher humidity, and lower temperature. Early studies on bacterial desiccation/drying on glass have shown that solutes overall protect bacteria in desiccated

state. For example, **Hirai (1991)** showed, using Rodac plate technique, that *Salmonella* counts in sterile distilled water dried on glass surface decreased to non-detectable level after 7 hours; however, *Salmonella* was detectable for over 5 days if suspended in 2% bovine serum albumen before glass inoculation.

Conversely, humidity effects might be more complicated as **Møretrø et al. (2010)** showed that shigatoxin-producing *Escherichia coli* dried on plastic or steel had highest inactivation rate at 85% relative air humidity, while survived the best at 98%. It can be argued that microorganisms in dried inoculum survive better at low humidity (low metabolic activity) compared to high humidity, where stationary culture, still metabolically active, slowly dies off. However, at low inoculation levels and high organic matter and high humidity might stimulate growth.

It is generally believed that *Salmonella* survives desiccation very well and may persist in dry and low water activity foods. Several *Salmonella* foodborne outbreaks involving dry/low water activity foods, such as peanut butter, were recorded. **Li, Megalis and Tortorello (2010)** has shown that dried (a_W = 0.21) *Salmonella* Typhimurium LT2 culture had 5-log reduction in numbers at 97% relative air humidity, while only 2 log reduction was observed at 33% relative humidity, with similar trend observed for *Salmonella* Tennessee. However, only desiccation-injured pathogens (drying to a_W = 0.21) were impacted by high humidity, while cells dried to higher water activity (a_W = 0.55) survived high relative humidity better.

Stine et al (2005) came to an overall conclusion that lower relative humidity stimulates survival of bacterial pathogens and indicators, such as *Escherichia coli*, *Salmonella enterica*, and *Shigella sonnei* on the surface of lettuce, and bell pepper. However, controversial data exist for *Salmonella* survival on tomatoes, which might be attributed to the phenotypic strain variations. For example, **Rathinasabapathi (2004)** showed that *Salmonella* Montevideo spiked on the surface of pericarp discs cut from green mature tomato can survive with little reduction for at least 6 days at 100% relative humidity. However, **Wei at al. (1995)** have shown that *Salmonella* Montevideo introduced on the surface of unbroken skin at 4 $\log_{10}$ CFU per site survived for at least 48 hours but could not be consistently detected after 5 days. **Lang, Harris and Beuchat (2004)** showed that *Salmonella* counts in 5% horse serum on the spot-inoculated tomatoes decreased 0.8 log after 1 hour drying and 2.2 $\log_{10}$ 24 hours post-drying from initial 7.22 $\log_{10}$ CFU per tomato. However, **Guo et al. (2002)** found that a cocktail of five *Salmonella* strains diluted in sterile tap water and inoculated on the surface of green tomato at 7.72 $\log_{10}$ CFU per tomato experienced only 1 $\log_{10}$ CFU per tomato reduction on day 1 and 3 log reduction on day 7 at 20 °C and 70% relative air humidity. Similarly, **Allen et al. (2005)** showed persistence of *Salmonella* in phosphate buffered saline dried on the surface of tomatoes for at least 14 days at 30 °C/80% RH, 20 °C/60% RH, and 20 °C/90% RH.

Other studies have used deionized water, tryptic soy broth, 5% sterile horse blood, tomato serum, soil, 0.1% peptone water, buffered peptone water, phosphate buffered saline, among others, to dilute *Salmonella* culture before placing on the surface of tomatoes. As noted by **Wei et al. (1995)**, TSB as a diluent supported better bacterial survival on tomato surface and provided protection against chlorine treatment. According to the researchers, *Salmonella* Montevideo grew in TSB, but died rapidly in Butterfield's buffer or tomato serum, while death rate in deionized water was slower. **Guo et al. (2002)** showed that *Salmonella* on tomato in contact with soil was capable to grow up to day 4 and persisted thereafter up to day 10.

Many of the *Salmonella* spot-inoculation studies were done with high level of inoculum (~10^7 CFU per tomato). Low level of inoculation resulted in quick bacterial die-off. It can be argued that such high inoculum might mimic high solute diluent, where solutes from bacteria themselves might protect them in bulk. For example, **Wei et al. (1995)** showed that *Salmonella* Montevideo die-off at <5.12 $\log_{10}$ CFU.mL^{-1} was significantly faster in Butterfield's buffer, tomato serum, and deionized water, compared to 8.15 $\log_{10}$ CFU.mL^{-1} culture. When bacteria were inoculated at 2.85 – 3.86 $\log_{10}$ CFU per tomato in deionized water, the bacterial die off occurred overnight, while survival for 3 days was observed at 9.48 $\log_{10}$ CFU per tomato level. According to **Kusumaningrum et al. (2003)**, *Salmonella* Enteritidis was recovered from inoculated steel squares after drying for at least 4 days at high contamination level (10^5 CFU.cm^{-2}), while at moderate level (10^3 CFU.cm^{-2}) and low level (10 CFU.cm^{-2}) inoculation counts went below detection limit within 24 hours and 1 hour, respectively. In addition, milk residue and chicken fillet suspension improved survivability compared to 0.1% peptone +0.89% saline solution **(Kusumaningrum et al., 2003)**.

Scientific hypothesis

It is hypothesized that high organic solute concentration in inoculation diluent (buffered peptone water and natural tomato serum), as well as low humidity and low temperature, may improve survival of *Salmonella* on the surface of undamaged tomatoes. It is expected that stationary phase *Salmonella* culture on spot inoculated tomatoes stored in high humidity will experience rapid die-off as compared to tomatoes held at low humidity.

MATERIALS AND METHODOLOGY

Rifampin preparation and microbiological media supplementation

Rifampin stock solution (10,000 ppm) was prepared by dissolving 0.4 g of rifampin (Fisher Scientific, BP26795, Pittsburgh, PA) in 40 mL HPLC grade methanol (Fisher Scientific), filter-sterilized (0.2 μm nylon filter), and stored in the dark at 2 °C for no longer than 1 month. Tryptic Soy Broth (TSB) with rifampin at 100 ppm was prepared by aseptic addition of 0.1 mL of rifampin stock solution to 9.9 mL of sterile TSB (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Tryptic Soy Agar (TSA) with rifampin 80 ppm was prepared by aseptically addition of 8 mL of rifampin stock solution to 1 L of sterilized and cooled to 45 °C TSA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). TSA-rif80 was used either for pour plate method (1 mL of analyte with 15 – 18 of liquid TSA-rif80) or spiral plating on pre-poured TSA-rif80 plates (WASP2

spiral plater, Don Whitley Scientific Limited, West Yorkshire England).

Tomatoes and tomato serum preparation

Green mature unwashed and unwaxed round tomatoes (variety Florida 47) for inoculation studies were acquired from local packinghouses. Eight tomatoes were ripened, washed, trimmed, chopped, and 945 grams were homogenized into slurry (1-minute, Waring blender, Waring Products Inc., Torrington, CT, USA). Approximately 100 mL of slurry was centrifuged (10 minutes, 5,000 rpm, SG-3 rotor, Sorvall RC-5B, DuPont Instruments, Corp, Parkersburg, WV, USA) and the supernatant was filter sterilized (0.2 μm nylon filter), stored at 2 °C, and used as a "tomato serum" diluent for *Salmonella* within 24 hours of preparation. Absence of rif resistant microflora in the serum was confirmed on day 0 and day 5 (25 °C) using pour plating using TSA-rif80.

Bacterial strains and inoculation

Salmonella rif-resistant strains (derivatives of Typhimurium ATCC 13311, Braenderup ATCC BAA-664, Enteritidis ATCC 4931, Newport ATCC 6962, and Javiana ATCC BAA-1593, American Type Culture Collection, Manassas, VA, USA), grown in three consecutive TBS-rif100ppm (37 °C, for 12 hours, 1st broth; 12 hours , 2nd broth; and 18 hours, final transfer), were combined 2 mL each, double-washed in BPW (4,000 g, 10 minutes), and finally resuspended in 10 mL of 0.1% Bacto™ peptone **(**Becton, Dickinson and Company, Franklin Lakes, NJ, USA), buffered peptone water (BPW, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), or tomato serum. Total solids and pH of the uninoculated diluents were measured (Brix, refractometer; pH meter). The resulting cocktails were diluted 1:10 in corresponding diluent and 0.1 mL of inoculum was spotted as ten 10 μL drops around blossom end of each green tomato. Inoculated spots were allowed to completely dry on tomatoes for 90 minutes in a biosafety hood.

Tomato storage

For study I, inoculated green tomatoes were placed in 12 °C and 25 °C incubators for 5 days after initial inoculum pre-drying in biosafety hood for 90 minutes, with no humidity control or monitoring.

For study II, green tomatoes were placed in two 25 °C incubators after initial inoculum pre-drying in biosafety hood for 24 hours. First incubator was maintained with low air relative humidity (LH) (~20 – 30% RH), and the atmosphere of the second one was humidified with a shallow pan filled with deionized water and soaked paper towels (HH) (~70 – 90% RH). Humidity and temperature were recorded at 10-minutes intervals (Hobo U12 dataloggers, Onset Computer Corporation, Bourne, MA, USA; software Hoboware Lite v. 3.1.0) throughout the study.

Salmonella enumeration

For study I three inoculated tomatoes and one negative control tomato were randomly pulled on day 0 (90 minutes dry) and afterwards from each incubator (12 and 25 °C) on days 1, 3, and 5.

For study II same set of tomatoes was analysed immediately after inoculation, after 90 minutes pre-drying in biosafety hood, on day 1 (24 hours dry in biosafety hood), and day 5 (25 °C, LH incubator and HH incubator). Each tomato was transferred to 20 mL BPW in sterile stomacher bag and pathogen was recovered by 20 seconds shake, followed by 20 seconds rub, and 20 seconds shake. The rinsate was either plated directly or serially diluted (BPW) and spiral plated (WASP2 spiral plater) or pour plated (TSA-rif80ppm, 37 °C, 24 to 48 hours).

Data and statistical analysis

Each experiment (Study I and Study II) was repeated three times on three different days. Statistical analysis was performed using commercially available software Statistica ver. 10.0 (StatSoft, Inc., Tulsa, OK, USA).

Study I. A multi-factorial design was utilized to determine the influence of diluent type (BPW, 0.1% peptone, and tomato serum), storage temperature (12 and 25 °C), and storage day (day 0, 1, 3, and 5), as well as their interactions, on *Salmonella* counts on tomato surface. Fisher's Least Significant Difference (LSD) test was utilized to separate treatment means when differences (p <0.05) occurred among factors.

Study II. A two-factorial design was used to test the effects of treatment-storage factor (immediately, 90 minutes dry, 24 hours dry, day 5 LH, day 5 HH) and diluent type (BPW, 0.1% peptone, and tomato serum), as well as their interaction, on *Salmonella* counts on tomato surface. Fisher's Least Significant Difference (LSD) test was utilized to separate treatment means when differences (p <0.05) occurred among factors.

Total solids of the uninoculated diluents (BPW, 0.1% peptone, and tomato serum) were evaluated three times, once for each replication, indirectly using handheld refractometer and expressed as average value of degree Brix with standard deviation among three measurements.

Similarly, pH value of the uninoculated diluents (BPW, 0.1% peptone, and tomato serum) were evaluated three times, once for each replication, and expressed as average pH value with standard deviation among three measurements.

Temperature and humidity measurements for each incubator for each replication (Study II) were averaged for all 10-minutes intervals and expressed as average value ±standard deviation.

RESULTS AND DISCUSSION

The measured solids in the diluents, indirectly expressed as ^{o}Brix, were 0.20 ±0.00, 2.50 ±0.00, and 4.93 ±0.15, for 0.1% peptone, buffered peptone water, and tomato serum, respectively. The corresponding pH values were 6.99 ±0.23 (0.1% peptone), 7.16 ±0.04 (BPW), and 4.29 ±0.10 (tomato serum).

Peptone diluent used at concentration 1 $g.L^{-1}$ contained enzymatic digest of protein with no salt. Conversely, BPW used at recommended 20 $g.L^{-1}$ concentration contained enzymatic digest of protein (peptone) 10 $g.L^{-1}$, sodium chloride 5 $g.L^{-1}$, disodium phosphate 3.5 $g.L^{-1}$, monopotassium phosphate 1.5 $g.L^{-1}$ with claimed pH value as 7.2 ±0.2 by manufacturer. The composition of tomato serum remained unknown.

Table 1 Temperature and humidity variations in LH and HH incubators at 25 °C during 5 days tomato storage.

	LH incubator		HH incubator	
	Temp (ºC ±*SD*)	**Humidity (% ±*SD*)**	**Temperature (ºC ±*SD*)**	**Humidity (% ±*SD*)**
Replication 1	25.1 ±0.2	19.3 ±3.7	24.6 ±0.7	71.8 ±2.2
Replication 2	24.8 ±0.3	30.0 ±12.3	25.9 ±0.4	88.1 ±5.9
Replication 3	24.9 ±0.0	19.5 ±3.0	24.7 ±0.3	92.9 ±3.0

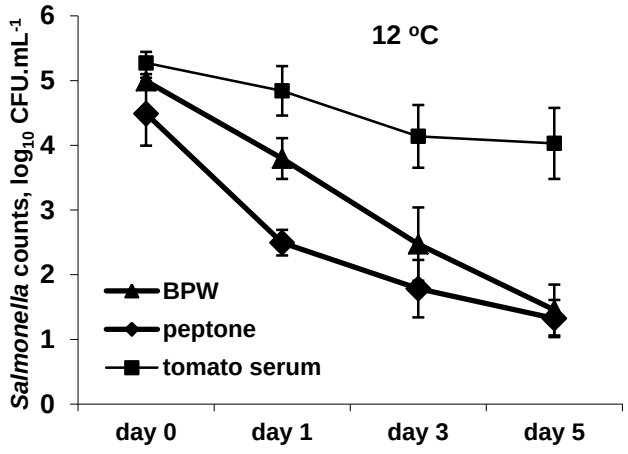

Figure 1 Survival of *Salmonella* on the surface of raw green tomatoes in BPW, 0.1% peptone and tomato serum for 5 days at 12 °C. Counts expressed as $\log_{10}$ CFU.mL^{-1} in 20 mL BPW rinsate. Error bars reflect standard deviation.

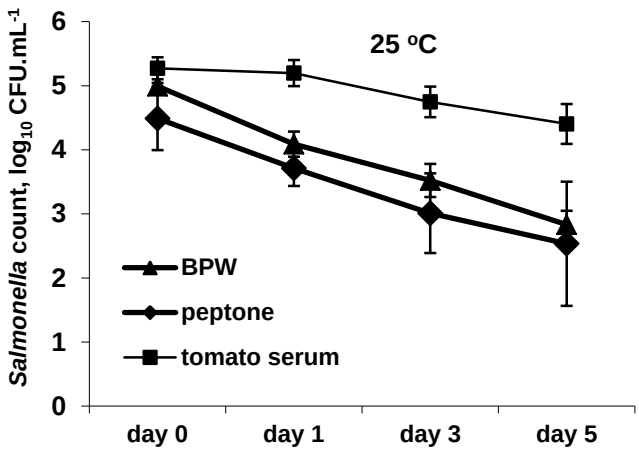

Figure 2 Survival of *Salmonella* on the surface of raw green tomatoes in BPW, 0.1 % peptone and tomato serum for 5 days at 25 °C. Counts expressed as log10 CFU.mL-1 in 20 mL BPW rinsate. Error bars reflect standard deviation.

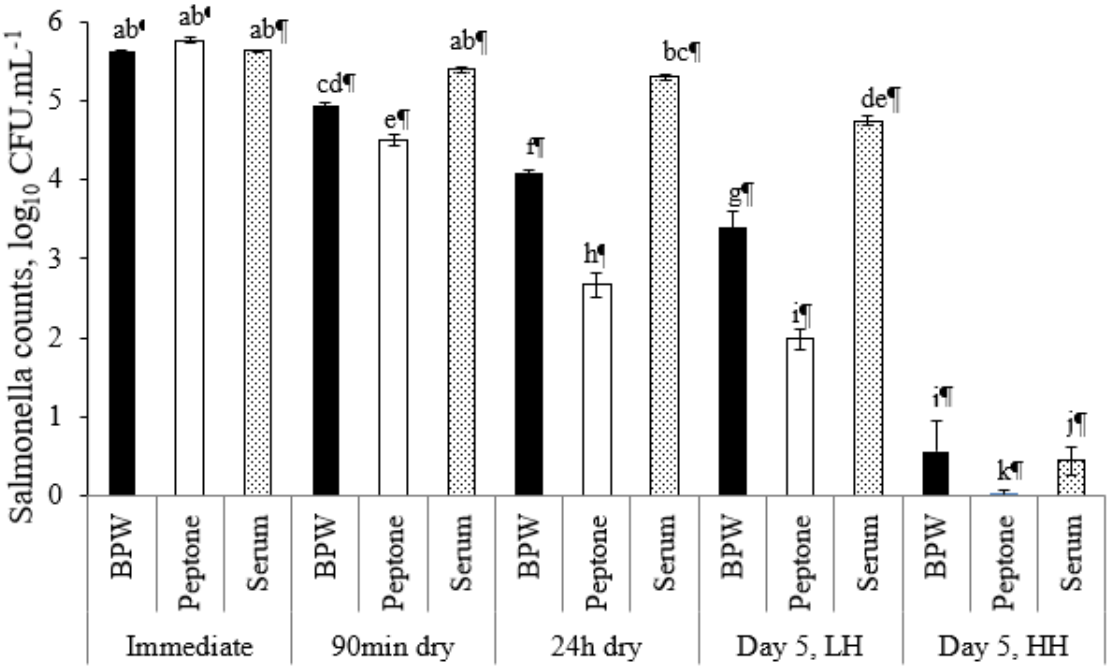

Figure 3 Survival of *Salmonella* on the surface of raw green tomatoes immediately after inoculation, 90 minutes postdrying and 24 hours postdrying in biosafety hood, and after 5 days in low humidity incubator and high humidity incubator at 25 ºC. *Salmonella* counts are expressed as mean values of $\log_{10}$ CFU.mL^{-1} in 20 mL BPW rinsate of three replications. Error bars reflect standard error of mean. Means with the same letters are not significantly different (p >0.05)

Study I. All three factors (diluent type, storage temperature, and storage day) had significant influence on *Salmonella* recovery in rinsate diluent (p <0.05).

Estimated inoculation level was ca. 5.6 $\log_{10}$ CFU.mL^{-1} per tomato as expressed by counts in BPW rinsate. *Salmonella* counts upon storage at two different temperatures is shown in Figures 1 and 2. Upon 90 minutes drying, *Salmonella* counts declined significantly the most in low-solute 0.1% peptone (4.49 $\log_{10}$ CFU.mL^{-1}, p <0.05), comparing to BPW (4.99 $\log_{10}$ CFU.mL^{-1}) and tomato serum (5.27 $\log_{10}$ CFU.mL^{-1}), which were not significantly different between each other (p >0.05). *Salmonella* counts remained at 4.03 $\log_{10}$ and 4.40 $\log_{10}$ CFU.mL^{-1} in tomato serum after 5 days of storage at 12 °C and 25 °C, respectively, with no significant difference between two values (p <0.05). *Salmonella* counts in BPW were significantly lower (p >0.05) comparing to tomato serum on day 5 at both storage temperatures, 1.45 $\log_{10}$ and 2.83 $\log_{10}$ for 12 °C and 25 °C, respectively. Moreover, there was a significant difference between two storage temperatures for BPW diluent on day 5 (p >0.05). Similarly, *Salmonella* counts in peptone were significantly lower (p >0.05) comparing to tomato serum, but not to BPW, on day 5 at both storage temperatures, 1.32 $\log_{10}$ and 2.53 $\log_{10}$ for 12 °C and 25 °C, respectively. The difference for *Salmonella* counts between 12 °C and 25 °C for peptone diluent on day 5 was significant as well (p >0.05).

Similarly, **Wei et al. (1995)** showed that *Salmonella* Montevideo dried on tomato surface in water at ca. 5.8 $\log_{10}$ CFU per area died after four days, while pathogen dried in TSB persisted and increased in numbers at 25 °C and 72% RH. Conversely, *Salmonella* died in tomato juice upon storage, which might be attributed to autoclaving **(Wei et al., 1995)**, as tomato pulp is suitable for *Salmonella* growth. **Guo et al. (2002)** showed *Salmonella* growth on tomato surface in contact with soil up to four days and persistence until day 10 thereafter, while *Salmonella* in water diluent declined by 3 $\log_{10}$ units on day 7. Similarly, **Hirai (1991)** showed that solutes overall protect *Salmonella* in desiccated state on inert glass surface. As expected, death in the presence of high solutes was slower. However, the lack of preservation effect of low temperature on *Salmonella* can be explained by rapid humidification of air in the incubator during sampling and inoculated spots temporary liquefaction, causing stress to bacteria.

Study II visual observations of inoculated tomatoes stored at high humidity suggested hygroscopic nature of diluent solids, as BPW and tomato serum inoculated spots, but not peptone water spots, liquefied. Average temperature and humidity values, both in LH and HH incubator, are shown in Table 1. *Salmonella* counts obtained during Study II experiments are shown in Figure 3. Both factors, namely, treatment-storage and diluent type, as well as their interaction, had significant influence on *Salmonella* counts (Figure 3, p >0.05). *Salmonella* counts post-drying in biosafety hood (24 hours) declined to 5.3 $\log_{10}$ CFU.mL^{-1} in tomato serum, comparing to 4.08 and 2.67 $\log_{10}$ in BPW and peptone, respectively. At this time frame, only *Salmonella* counts in tomato serum were not significantly different comparing to initial counts upon inoculation and 90 minutes post drying (Figure 3, p <0.05). At 5 days low humidity storage at 25 °C, *Salmonella* counts in tomatoes remained as high as 4.75 $\log_{10}$ comparing to decline in BPW (final 3.40 $\log_{10}$ CFU.mL^{-1}) and peptone (1.98 $\log_{10}$ CFU.mL^{-1}). Interestingly, 5 days high humidity storage at 25 °C accelerated *Salmonella* populations decline to close to below detection limit at 0.44, 0.56, and 0.03 $\log_{10}$ CFU.mL^{-1} for tomato serum, BPW, and peptone diluent, respectively. Those data further solidified the concept of solute protective effect in desiccated state. **Li, Megalis and Tortorello (2010)** has shown that day one dried (a_W = 0.21) *Salmonella* Typhimurium LT2 culture had 5-log reduction in numbers at 97% RH, while only 2-log reduction was observed at 33% RH. It has been shown that microorganisms in dried inoculum survive better at low humidity compared to high humidity. However, at a low inoculation level along with high organic matter, high humidity may stimulate growth **(Wei et al., 1995; Guo et al., 2002)**. As noted by **Wei at al. (1995)**, TSB as a diluent not only supported better bacterial survival on tomato surface, but also caused protection against chlorine treatment. According to the researchers, *Salmonella* Montevideo grew in TSB, but died rapidly in Butterfield's buffer or tomato serum.

These data can be compared to the temperature/humidity fluctuations during tomato growing season in major US tomato growing states, such as Florida and California, as well in European countries. According to the results, lower temperature storage was not more beneficial regarding rate of *Salmonella* die-off. A recommendation of high average relative humidity (>72%) within first three days of storage, might result in higher bacterial die-offs. Potentially more significant in terms of public health is that tomato surface cleanness may play an important role regarding *Salmonella* survival.

CONCLUSION

It has been shown that high solute diluent for inoculum preparation, mimicking naturally soiled tomato surface, improves survival of *Salmonella* on the surface of undamaged tomatoes at low humidity storage. Additionally, stationary phase culture on spot inoculated tomatoes stored in high humidity experiences rapid die-off. Cleanness is one of the key factors to keep tomato surface unsuitable for pathogen survival, as solutes can contribute to survival and growth.

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