Colonization of Tomatoes by *Salmonella* Montevideo Is Affected by Relative Humidity and Storage Temperature[†]

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ABSTRACT

The influences of the relative humidity (RH) and storage temperature on the colonization of tomato surfaces by *Salmonella* Montevideo were studied. Red, ripe tomatoes (*Lycopersicon esculentum*) were spot inoculated in three separate trials with 100 μl (approximately 10^6 CFU) of *Salmonella* Montevideo and stored for 90 min at 22°C under 97% RH to facilitate attachment of cells to the blossom end of tomato surfaces. Following this attachment step, tomatoes were washed to remove loosely adhered cells and then stored at 22 or 30°C for up to 10 days under RH of 60, 75, 85, or 97%. At 0, 0.4, 1, 4, 7, and 10 days of storage, three tomatoes were individually hand massaged in 50 ml of 0.1% peptone water and the washes were separately analyzed to enumerate populations of *Salmonella* Montevideo. The number of *Salmonella* Montevideo cells attached after 90 min at 22°C was 3.8 log CFU per tomato; this level was determined to be the initial colonizing population. After 10 days of storage at 30°C, the *Salmonella* Montevideo population increased to 0.7, 1.0, 1.2, and 2.2 log CFU per tomato at 60, 75, 85, and 97% RH, respectively. A similar trend was observed at 22°C, although populations were lower than at 30°C. Scanning electron micrographs of tomato cuticles after storage revealed a well-defined biofilm containing bacteria. These findings reinforce the importance of maintaining stored tomatoes at temperatures that do not support growth of pathogenic bacteria and demonstrate the growth-promoting effects of high humidity.

Microbial attachment to surfaces and the development of biofilms are known to occur in many environments. A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material *(7)*. It has been well documented that microorganisms colonizing surfaces as part of a biofilm are more resistant to environmental challenges than their planktonic counterparts in suspension *(10)*.

Such conditions have been implicated in outbreaks of salmonellosis associated with the consumption of produce *(6, 13)* and have lead to increased emphasis on good agricultural practices and hazard analysis critical control point (HACCP) systems to reduce the risk of produce contamination and pathogen growth *(2, 24)*. However, the design of HACCP plans is limited by inadequate information about the effects of commercially relevant storage conditions on the survival and growth of *Salmonella.*

The ability of *Salmonella* to attach and produce extracellular polymers on stainless steel has been observed *(23, 25)*. Other human pathogens, such as *Listeria monocytogenes* and *Escherichia coli* O157:H7, are also able to develop a biofilm on inert surfaces *(16, 17, 20)*.

Since contamination of raw produce with pathogenic and nonpathogenic microorganisms may occur at any point from the field until consumption, the adherence of pathogenic bacteria to produce and biofilm formation are sources of concern for food safety. Biofilms have been observed on leaf surfaces of spinach, lettuce, Chinese cabbage, celery, leek, basil, parsley, and endive *(18)*. However, little is known regarding biofilm formation on fruits. In a previous study, we demonstrated that *Salmonella* Montevideo is able to attach to the surface of tomatoes and tomatillos *(14)*. Once *Salmonella* has attached, it could colonize the fruit under suitable conditions.

The presence of biofilms on the surface of fruits and vegetables may provide protected colonization sites for human pathogens, rendering their eradication with antimicrobial compounds difficult *(9)*. A better understanding of the factors involved in the attachment and colonization of bacteria on produce surfaces could be useful to design improved methods for washing and disinfection treatments of produce. The objective of this research was to evaluate the effects of relative humidity (RH) and storage temperature on the colonization of *Salmonella* Montevideo on the surface of tomatoes.

MATERIALS AND METHODS

Source of tomatoes. Red, ripe tomatoes (*Lycopersicon esculentum*) were picked by hand in a greenhouse on the Universidad Autónoma de Querétaro (Querétaro, Querétaro, Mexico). Only tomatoes free of visible defects, such as bruises, cuts, and abrasions, were used. Tomatoes were stored at 12°C for no more than 2 days; before inoculation, fruits were tempered to 22°C in an incubator.

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Preparation of inoculum. The *Salmonella* Montevideo strain used in this study was isolated from a patient in a U.S. outbreak associated with consuming raw tomatoes; it was obtained from the culture collection of the University of Georgia, Center for Food Safety (Griffin). For the present studies, resistance to rifampin (Sigma Chemical Co., St. Louis, Mo.) was induced in the strain *(15)*. Briefly, 10 ml of a 24-h culture was centrifuged and the pellet was resuspended in 1 ml, then the cell suspension was surface plated (0.1 ml per plate) on tryptic soy agar (Difco, Becton Dickinson, Sparks, Md.) supplemented with rifampin (TSAR; 100 mg/liter) and incubated for up to 72 h.

Cultures were maintained at 4°C on tryptic soy agar slants. Three successive loop transfers to tryptic soy broth (Difco, Becton Dickinson) supplemented with rifampin (100 mg/liter) were made at 35°C and 24-h intervals before cells from a 20-h culture were collected by centrifugation (3500 × *g*, 15 min, 22°C). The pellet was washed twice with 0.85% NaCl (saline) and resuspended in distilled water. Serial dilutions of the suspension were prepared in distilled water to obtain the desired cell concentration. The population in the inoculum was determined by pour-plating samples on TSAR. Plates were incubated at 35°C for 24 h before colonies were counted.

Inoculation procedure. Tomatoes were spot-inoculated (ca. 5 log CFU per fruit). The cell suspension (100 μl) was distributed in 10 drops (10 μl each) on the tomato surface near the blossom end of each tomato within a 2- to 3-cm diameter circle.

Microenvironments. Saturated solutions of NaBr, NaCl, KCl, and K$_2$SO$_4$ (J. T. Baker, Xalostoc, México) were prepared to equilibrate the atmosphere of containers to approximately 60, 75, 85, and 97 ± 2% atmospheric RH, respectively *(19, 22)*. RH values were measured with a calibrated Aqualab device (model CX2, Decagon Device, Inc., Pullman, Wash.). The solutions were dispensed in 2-cm layers in separate polyethylene containers (30 by 40 by 14 cm). The atmospheric RH was monitored by placing a digital RH and temperature meter (model 4096, Control Company, Friendswood, Tex.) inside of each container. Containers were adjusted to 22 or 30°C.

Colonization assay. Inoculated tomatoes were placed in 150-ml plastic beakers located inside containers equilibrated at 97% RH. The containers were covered, hermetically sealed with high-vacuum grease (Dow Corning Corp., Midland, Mich.), and stored at 22°C for 90 min (attachment step).

At 0 min (≤30 s) and after 90 min at 22°C, tomatoes were removed from containers and individually placed in resealable polyethylene bags (17.8 by 23.3 cm), containing 200 ml of distilled water. Bags were sealed and gently shaken by hand for 20 s. Tomatoes were transferred from the bags with sterile stainless steel tongs and placed in 150-ml plastic beakers located inside containers equilibrated at 60, 75, 85, or 97% RH. The containers were covered, hermetically sealed, and stored at 22 or 30 ± 0.2°C for up to 10 days (colonization step).

***Salmonella* Montevideo enumeration.** Populations of *Salmonella* Montevideo were enumerated after 0, 0.4, 1, 4, 7, and 10 days of storage. Three tomatoes were removed from all combinations of treatments; each was placed individually into a polyethylene bag, containing 50 ml of sterile 0.1% peptone water, and rubbed by hand with firm pressure for 1 min. Populations of *Salmonella* were determined by pour-plating samples serially diluted in 0.1% peptone on TSAR. Plates were incubated at 35°C for 24 h. From each sample, 3 to 5 presumptive colonies of *Salmonella* Montevideo were picked and subjected to confirmation by bio-

chemical and agglutination tests using commercial antiserum (Difco, Becton Dickinson) *(1)*.

Preparation of samples for scanning electron micrographic examination. Colonization of *Salmonella* Montevideo on tomatoes was determined after storing inoculated fruits for up to 10 days. Samples were prepared for scanning electron microscopy, according to the procedure described by Getz et al. *(11),* with some modifications. Sections (5 by 5 by 1 mm) of tomato skin were removed and fixed for 3 h in 3% glutaraldehyde, followed by treatment for 3 h in 1% osmium tetroxide. Both solutions were prepared in 0.1 M sodium cacodylate (pH 7.36). Fixed tissues were dehydrated in a graded series of ethanol concentrations at 4°C. Following dehydration, tissues were critical-point dried, mounted, sputter coated with gold, and examined with a Zeiss DSM-950 (Carl Zeiss, Jena, Germany) scanning electron microscope operating at 15 to 20 kV.

Measurements of growth parameters. The model described by Baranyi et al. *(4)* was used to fit curves to the experimental data and to estimate values for the primary growth parameters of lag phase duration (h), growth rate (ln h^{-1}) and maximum population density (MPD) (log CFU per gram) using the DMFit curve-fitting software (courtesy of J. Baranyi, Institute of Food Research, Norwich, UK). Table curve 2D version 5.0 (SPSS, Inc., Chicago, Ill.) was used to fit a cubic model to the distribution of MPD versus RH. The Ratkowsky square root model was used to describe specific growth rate (SGR) as a function of the RH *(21)*.

Statistical analysis. All experiments were performed in triplicate and each replicate experiment consisted of three fruits subjected to each combination of treatments. Mean and standard deviation were calculated using the Statgraphics program (Manugistics, Inc., Rockville, Md.). Comparisons of MPD prediction to the observations were computed by the method of Baranyi et al. *(3),* where bias and accuracy values of 1 equal perfect agreement between prediction and observation. The *F* test was used to test for statistical differences in the standard deviation between populations.

RESULTS AND DISCUSSION

During production (cultivation and harvesting), tomatoes are exposed to numerous opportunities for contamination. After harvesting, the product is frequently submerged in a rinse tank to eliminate field heat and visible soil contamination. In this study, we evaluate the potential of *Salmonella* Montevideo cells to grow and eventually produce biofilms once they have been attached to the surface of tomatoes. After the attachment of *Salmonella* Montevideo cells, a rinsing step with water was applied to the tomatoes to eliminate weakly attached cells; this rinsing would simulate the treatment used in tomato processing plants.

Populations of *Salmonella* Montevideo attached after 90 min (mean = 3.8 log CFU per fruit) were considered as the initial inoculum during the colonization process. At 10 days of storage, colonization of the pathogen on tomato surfaces was greater at 30 than at 22°C, and greater with increasing humidity (Fig. 1). At 10 days of storage at 30°C, the population of *Salmonella* Montevideo increased to 0.7, 1.0, 1.2, and 2.2 log CFU per tomato at 60, 75, 85, and 97% RH, respectively (Fig. 1). A similar trend was observed at 22°C, although populations were not as high as
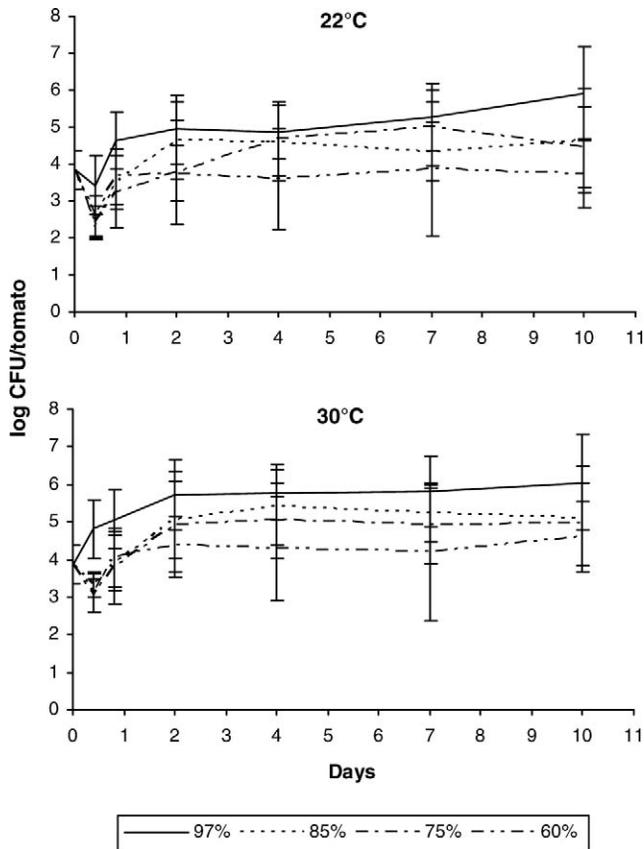
FIGURE 1. *Colonization of* Salmonella *Montevideo on the surface of red tomatoes during storage at different temperature and relative humidity conditions. The values are the mean of nine tomatoes analyzed for each combination of test parameters.*

FIGURE 2. *Plots of MPD as a function of RH at 30°C (A) and 22°C (B).* □ = *observed MPD; solid line = predicted MPD with* R^2 = 0.999 (A) and 0.825 (B).

at 30°C. During storage at 22°C and 60% RH, no growth but survival of the pathogen was observed.
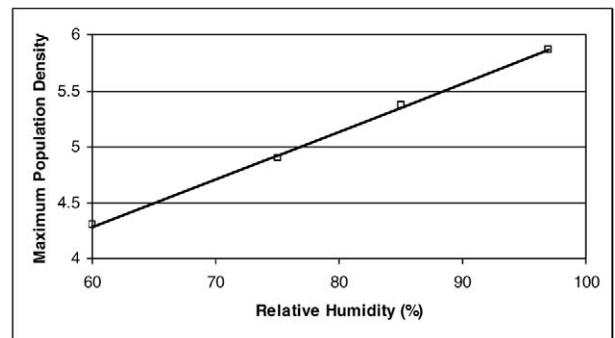
The Baranyi model was used to fit curves to *Salmonella* Montevideo growth data at each combination of storage temperature and RH. Examination of the growth parameters showed a linear relationship between MPD and RH (Fig. 2). This association could be described by the equation:

$$MPD = a + b \times RH$$

At 30°C, $a = 1.725$, $b = 0.043$, with $R^2 = 0.999$ and F value = 2.078. For 22°C, $a = 1.268$, $b = 0.043$, with $R^2 = 0.825$ and F value = 9.435. The model accuracy was 1.30 and 1.02 for 22 and 30°C, respectively. Such relationships between RH and MPD may be useful for estimating potential human exposure levels of *Salmonella* Montevideo from tomatoes.

After 10 h (0.4 days) of storage under all treatment conditions, except at 30°C and 97% RH, a decrease of approximately 0.4 to 1.5 log CFU per tomato occurred. These decrements were higher at 22°C (Fig. 1). During the attachment of *Salmonella* Montevideo to the surface of tomatoes, the drops containing the inoculum did not dry out, but after rinsing with water, all drops were removed. It is likely that the inoculum dryness, at the beginning of storage during colonization experiments, created an adverse environment for the bacteria, whereby some cells became
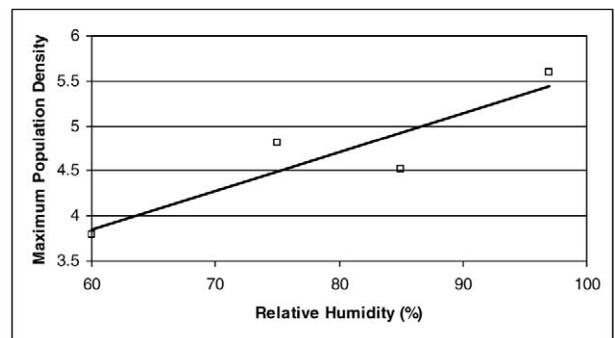
stressed and perhaps died. Apparently, it took a few hours for *Salmonella* Montevideo cells to adapt to the new environment before starting to grow. Although it was not possible to control the population that remained on tomatoes after rinsing, standard deviations oscillated between 0.4 and 1 log CFU during the first 4 days of storage. Thereafter, standard deviation values gradually increased up to 1.7.

Scanning electron micrographs of tomato cuticles after storage for 10 days at 22°C and 97% RH revealed biofilm development (Fig. 3) At 10 h of storage, thin fibers were observed that are generally associated with the production of extracellular polymers *(8)*. At 4 days, a well-defined biofilm containing numerous bacteria was present. After 10 days, fruit cuticles were covered with slime, although no bacteria could be detected.

The growth of *Salmonella* on whole ripe tomatoes during storage at 20 and 30°C and in an environment with 45 to 60% RH has been reported *(27)*. In our study, *Salmonella* Montevideo showed a similar pattern but a lower growth rate than that observed by Zhuang et al. *(27)*. Guo et al. *(12)* found that a population of *Salmonella* inoculated on mature green tomatoes, using water as a carrier, decreased by approximately 4 log CFU per tomato after storage for 14 days at 20°C and 70% RH. Differences in behavior of *Salmonella* on tomatoes could be associated with several factors, including the strains used in both studies. We used a single strain of *Salmonella* Montevideo, whereas Guo et al. *(12)* tested a mixture of five serotypes of *Salmonella enterica,* including the *Salmonella* Montevideo strain used
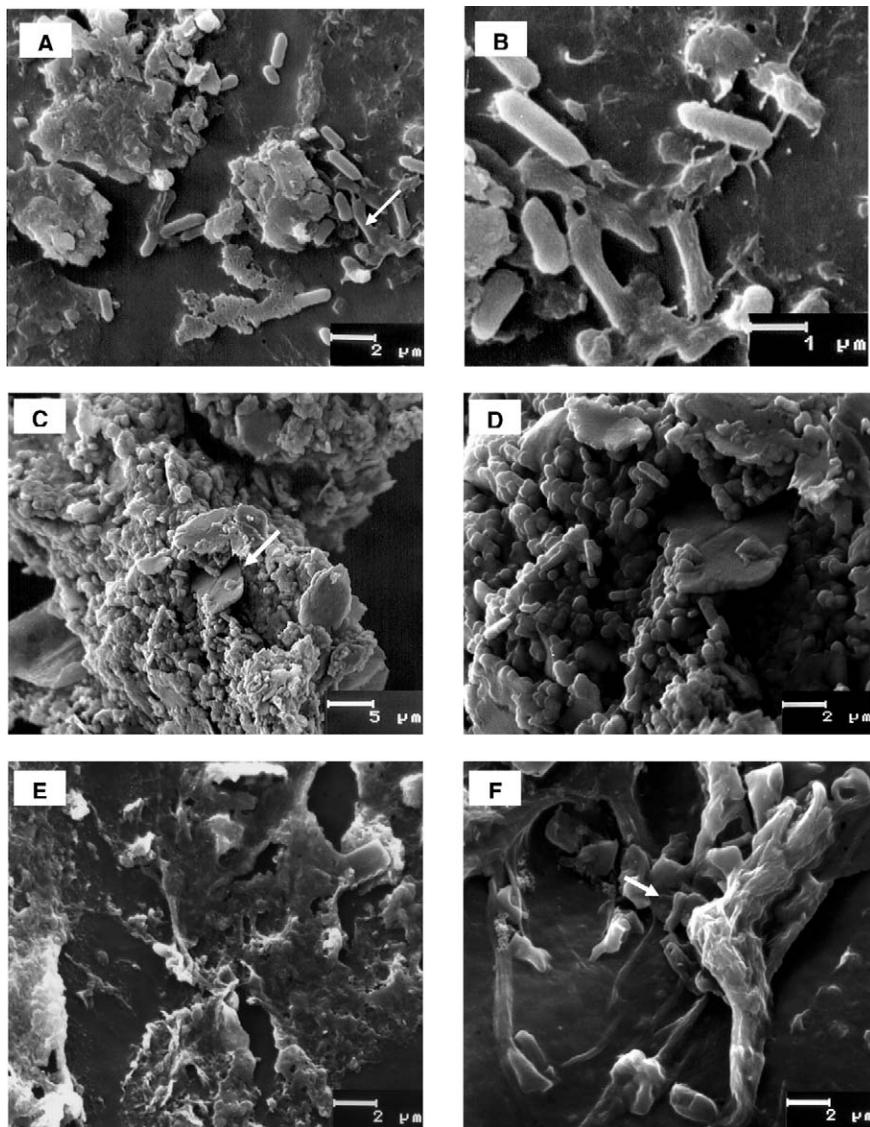
FIGURE 3. *Scanning electron micrographs of tomato cuticle inoculated with* Salmonella *Montevideo during storage for up to 10 days at 22°C and 97% RH. (A) Ten hours; micrograph shows initial extracellular material production. (B) Enlargement from panel A (arrow). (C) Four days; well-structured biofilms are shown with embedded bacteria. (D) Enlargement from panel C (arrow). (E) Ten days; structures apparently constituted by extracellular material. (F) Arrow shows forms resembling coated bacteria.*

herein. In the Guo et al. *(12)* study, *Salmonella* Montevideo was the most resistant strain, which supports the present results. The pathogen behavior could also be influenced by the grade of tomato ripeness (red vs. green); however, Wei et al. *(26)* found that the ripeness of wounded tomatoes had no apparent effect on *Salmonella* Montevideo populations after 24 h at 25°C. Additionally, during the rinsing step, the inoculum could be distributed to other areas (e.g., stem scar and flower end scar tissue) which could provide a better protective environment for bacteria and promote its growth *(26)*.

Survival and growth of a pathogen on or in raw produce are dictated by its metabolic capabilities. However, manifestations of these capabilities can be greatly influenced by intrinsic and extrinsic ecological factors naturally present in produce or imposed at one or more points during the entire system of production, processing, and distribution *(5)*. During growth of tomatoes in greenhouses or during postharvest handling, environmental conditions evaluated in this study could promote biofilm development on the surface of the fruit. These biofilms can provide a protective environment for pathogens and reduce the effectiveness of sanitizers and other inhibitory agents *(9)*.

In spite of the apparent nutrient scarcity, *Salmonella* Montevideo appears to be able to grow on tomato surface at all combinations of temperature and RH, except at 22°C and 60% RH. Colonization of the pathogen was greater at 30°C and biofilm development was apparent during the storage at both temperatures and 97% RH. These findings reinforce the importance of maintaining tomatoes at storage temperatures which do not support the growth of pathogenic bacteria and underline the growth-promoting effects of higher humidities. In addition, the resulting models that correlate the RH with the MPD can be used to determine storage conditions that minimize *Salmonella* Montevideo growth.

ACKNOWLEDGMENTS

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