Growth or penetration of *Salmonella* into citrus fruit is not facilitated by natural-light labels

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**A R T I C L E I N F O**

Article history:
Received 17 October 2012
Received in revised form 12 April 2013
Accepted 27 April 2013

Keywords:
Laser
*Salmonella*
Citrus *sinensis*
Fruit wax
Citrus postharvest

**A B S T R A C T**

In natural-light labeling of fruits and vegetables, the desired information is etched onto the produce surface using a low-energy carbon dioxide laser beam (10,600 nm). Etched characters are formed by surface depressions in the epidermis that may facilitate entrance of decay and pathogenic organisms. The objective of this study was to determine the effects of natural-light labeling and different postharvest treatments on *Salmonella* populations’ ability to survive/grow and penetrate into citrus fruit. A five-strain cocktail of *Salmonella* was spot inoculated onto Valencia orange in different application sequences with wax and natural-light etching. Samples were stored at 10, 26 °C, or combinations of both, for up to 42 days. Etched peels and corresponding juices were extracted from whole oranges following storage and enumerated for *Salmonella*. No set of conditions involving natural-light labeling promoted the growth of *Salmonella* on the fruit surface or resulted in the detection of *Salmonella* from the juice of sound fruit. Survival of *Salmonella* populations on the peel surface did not differ between any of the treatment and control samples. In all cases, *Salmonella* declined between 1.5 and 3.0 log CFU/orange after 30 days, with faster decline noted at 10 °C. Based on the data obtained from all treatments and under conditions extremely unfavorable and unrealistic in terms of fruit storage, natural-light labeling citrus fruit peels and subsequent waxing in any order did not allow for the growth or influence the natural decline of *Salmonella* populations on citrus fruit surfaces, or movement into juices, as compared to controls.

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1. Introduction

Most produce items are currently labeled with Price Look-Up (PLU) stickers that contain a four number code used to identify product groupings (Produce Electronic Identification Board, 1995). PLU stickers are commonly adhered to the surface of individual fresh fruits and vegetables during the packing process, typically after washing, culling and waxing, and prior to final packaging (Varon and Paddock, 1978). PLU stickers can easily detach during postharvest handling following sticker application, eliminating any information the PLU stickers may provide related to traceability and may also leave sticky residues or damage on produce surfaces (Etxeberria, Miller, & Achor, 2006a).

Imprinting an alphanumeric code directly onto the surface of produce, at the same point in production as PLU stickers, is currently being explored as an alternative way of permanently labeling fresh fruits and vegetables. A natural-light etching device, designed for labeling citrus surfaces with a low energy carbon dioxide laser beam (10,600 nm; Drouillard & Rowland, 1997), is a promising technology. The code produced is permanent, requires no adhesive and labeling information can be easily modified prior to application to individual citrus fruits (Etxeberria, Miller, & Achor, 2006b). In previous tests, penetration depression diameter and depth due to natural-light labels were similar on tomato and avocado fruits surfaces, averaging 200 μm and 25 μm, respectively, and affecting only the outermost 2–5 epidermal cells which are much smaller than those of the underlying endocarp (Etxeberria et al., 2006a).

When examined immediately after labeling, label markings are formed by pinhole depressions (Etxeberria et al., 2006b).

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**Abbreviations:** PLU, Price Look-Up; TBZ, thiobendazole; XLD, xylose lysine deoxycholate.

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0956-7135/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodcont.2013.04.036
Interruption to the natural cuticular barrier of the citrus surface potentially opens a route that could allow for the penetration of spoilage and pathogenic microorganisms. Studies evaluating the susceptibility of natural-light labeled tangerines (Sood, Ference, Narcisco, & Etxeberria, 2008) and grapefruits (Sood, Ference, Narcisco, & Etxeberria, 2009) to Penicillium digitatum spoilage failed to demonstrate the ability of decay microorganisms to colonize etched surfaces of citrus fruits. The lack of colonization of decay organisms into citrus fruit was attributed to the unique anatomical organization of the citrus peel which contains numerous oil glands and a loosely packed thick mesoderm (Bain, 1957). In addition, anatomical studies of etched citrus peel indicated the cauterization of the label markings (Etxeberria et al., 2006b; Etxeberria, Narcisco, Sood, Gonzalez, & Narciso, 2009), unsuitable conditions for spore germination.

Sensitivity to food safety risks associated with Salmonella exists within the citrus production and processing industries due to outbreaks associated with fresh orange juice that occurred in the mid-1990s (Vojdani, Beuchat, & Tauxe, 2008). Although previously reported studies indicate Salmonella is unable to colonize natural-light labels on mature green (Danyluk, Interiano, Friedrich, Schneider, & Etxeberria, 2010) or mature red (Yuk, Warren, & Schneider, 2007) tomato fruits, even in the presence of soft-rot organisms (Danyluk et al., 2010), colonization of citrus fruit by enteric pathogens through natural-light labels remains undetermined.

The objective of the present study was to determine if differences in Salmonella survival and/or growth on orange peel surfaces exist between non-light labeled oranges and those that are natural-light labeled, and to evaluate the potential for Salmonella penetration through the flavedo and albedo of the citrus peel into juice vesicles, resulting in internalization of Salmonella and its presence in orange juice.

2. Materials and methods

2.1. Plant material

Valencia oranges (Citrus sinensis) were shipped overnight from Sunkist LTD (Ventura, CA, USA) to the University of Florida’s research facilities located at the Citrus Research and Education Center in Lake Alfred, Florida, USA. Fruit was packed the day of shipment following standard commercial protocol, which included waxing the fruit with 5 ppm thiobendazole (TBZ). Fruit was stored at 10°C immediately after arrival and transferred to 21°C the day before experimental use.

2.1.1. Selection of strains and inoculum preparation

A cocktail of five Salmonella serovars isolated from orange juice was used. Salmonella serovars included: Gaminara (CDC HO662); Rubislaw (F2833); Typhimurium (ATCC14028); Hardford (CDC H0778); and Muenchen (LJH 0592).

Prior to each replication, frozen stock cultures of each strain were streaked onto nutrient agar (Difco: Becton, Dickinson and Co.) and incubated at 35°C for 24 h. One isolated colony from each strain was transferred to 10 mL of nutrient broth with 1% glucose (Difco: Becton, Dickinson and Co.) and incubated at 35°C. After 24 h, 1 mL of culture was spread over a nutrient agar + 1% glucose and incubated at 35°C for 24 h, when a lawn of stationary cells had formed. For acid adaption reasons, nutrient broth and agar was supplemented with 1% glucose (Buchanan & Edelson, 1996). Cells from each individual strain of the cocktail were harvested from five plates using a sterile hockey stick and 10 mL of 0.1% peptone water (Difco: Becton, Dickinson and Co.). Serial dilutions were carried out in 0.1% peptone water (9 mL) to prepare final inocula concentrations (ca. 9.6 log CFU/mL), verified for each strain by enumeration on tryptic soy agar (Difco: Becton, Dickinson and Co.). Equal volumes (1 mL) of each Salmonella strain were combined to obtain the final inoculum. Final inoculum cocktails were stored on ice prior to and during citrus inoculation.

2.1.2. Inoculation treatments

Fruit were inoculated, punctured, labeled and/or waxed in 11 different sequence combinations (Fig. 1), including two control sets of fruit where no Salmonella was inoculated (treatments A and B), four controls where fruit were inoculated but not labeled (treatments C, D, E, and F), and one control where fruit were punctured (treatment G).

2.1.3. Fruit peel inoculation

Oranges were inoculated with 20 μL of inoculum distributed in 4–6 droplets over the designated inoculated area of the fruit (Fig. 2). Samples were held in a biological safety cabinet (NU4625600, Nuaire, Plymouth, MN, USA) for 20 min to allow the inoculum to dry prior to any further inoculation treatment steps.

2.1.4. Fruit peel labeling

Fruit labeling was conducted as previously described by Sood et al. (2008). Fruit were individually placed on a plastic ring with the orange surface 10 cm apart from the natural-light source. All fruit were labeled with the phrase “Citrus 35us USA” using a maximum energy level of 0.578 W per character with a time of exposure of 35 μs and a duty cycle of 25%. As a positive control, oranges were wounded by puncturing the peel. Wounds, in a similar location and area as the natural-light label, were 2 mm in diameter and 3 mm deep, and achieved by puncturing the peel with a metal puncturing device specifically designed and manufactured in house for puncturing citrus peels.

Fig. 1. Flow diagram of the inoculation treatments. Fruit were inoculated, labeled and/or waxed in different sequence combinations before storage at 10 or 26°C. Inoculation was carried out by spotting 20 μL of inoculum in 4–6 droplets over the labeled surface or designated area of the fruit. Carnauba wax was used in those experiments requiring reapplication using a sterile swab.
Survival may be expected, a second set of three different Salmonella within recommended citrus storage conditions where extended storage temperatures and times that would approximate scenarios 1 day at 26°C; 2) 2 days at 10°C followed by 2 weeks at 26°C; and 3) 4 weeks at 26°C followed by 2 weeks at 26°C. Truplicate fruit were enumerated on day 0, at the time of transfer to 26°C and at the end of the experiment. All fruit in this treatment were inoculated as per treatments D or H (Fig. 1). Fruit were stored in containers as described previously. All storage experiments were run in duplicate, with fruit from different shipments.

2.1.5. Fruit peel waxing
Commercial carnauba wax (Carnauba 50S obtained from USDA-ARS, Winter Haven, FL, USA) without TBZ was used in those inoculation treatments requiring reapplication of wax. Application was carried out using sterile cotton-tipped applicators (Thermo-Fisher Scientific, Waltham, MA, USA) dipped in the wax and then wiped over the labeled surface or designated area (Danyluk et al., 2010; Sood et al., 2008, 2009).

Any effect of the applied wax on the Salmonella inoculum was investigated by combining wax and inoculum at different volume to volume ratios (inoculum:wax; 1:0; 1:0.5; 1:1 and 1:2) and enumerating following serial dilutions and plating as described below. To estimate Salmonella populations removed from the citrus peel during the waxing step, swabs used to apply the wax were enumerated by immediately placing in 10 mL of sterile 0.1% peptone water and vortexing vigorously for 1 min, followed by serial dilutions and plating as described below.

2.1.6. Sample storage
After drying, samples were placed into sterile filtered stomacher bags (Whirl-pak; Nasco, Modesto, CA, USA; Danyluk et al., 2010). Each bag was folded over once and placed in a large plastic container (Fashion Clears 4, Sensitech, Beverly, MA, USA). Stomacher bags were folded and not sealed to allow air movement and prevent the creation of an anaerobic environment due to fruit respiration. Lids were left off the large plastic storage containers. Inoculated oranges were stored at 10 ± 2°C or 26 ± 2°C and 95% relative humidity for up to 29 days, to approximate commercial storage at abusive temperatures where Salmonella growth may occur (10 ± 2°C) and ambient temperature 26 ± 2°C. Triplicate fruit samples were enumerated for Salmonella, as described below, from the different storage temperatures on days: 0, 3, 7, 10, 14, 17, 21, 25 and 29. At each time point, juice was also excised from fruit samples, as described below, and enumerated for Salmonella.

In an attempt to simulate minimal (10°C) and maximal (26°C) storage temperatures and times that would approximate scenarios within recommended citrus storage conditions where extended Salmonella survival may be expected, a second set of three different storage time temperature combinations was evaluated. Time and temperature combinations included: 1) 2 days at 10°C followed by 1 day at 26°C; 2) 2 days at 10°C followed by 2 weeks at 26°C; and 3) 4 weeks at 26°C followed by 2 weeks at 26°C. Triplicate fruit were enumerated on day 0, at the time of transfer to 26°C and at the end of the experiment. All fruit in this treatment were

### Table 1

<table>
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<tr>
<th>Treatment</th>
<th>C</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
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<td>6.4 ± 1.6</td>
<td>3.9 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>5.5 ± 1.2</td>
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<td>5.4 ± 1.7</td>
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<td>3.0 ± 1.2</td>
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<td>2.9 ± 0.2</td>
<td>-2&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>29</td>
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<td>3.5 ± 0.6</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Significant difference in treatments compared to control (Treatment C) on each day (i.e. across rows; P < 0.05).

#### Table 2

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<th>Time (days)</th>
<th>Treatment</th>
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<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
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<tr>
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<td>7.1 ± 0.2</td>
<td>6.0 ± 1.1</td>
<td>6.4 ± 1.0</td>
<td>6.1 ± 0.9</td>
<td>5.6 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6.1 ± 1.3</td>
<td>5.6 ± 1.1</td>
<td>3.7 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 1.0</td>
<td>3.9 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1 ± 1.2</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant difference in treatments compared to control (Treatment C) on each day (i.e. across rows; P < 0.05).

![Fig. 2. Diagram of natural-light etching/waxing, inoculation and excision methodology.](image-url)
2.1.8. Statistical analysis

A multivariate analysis of variance was performed in Excel (Microsoft, Redmond, WA, USA). Differences were considered statistically significant at \( P < 0.05 \). Specifically, each treatment involving a labeling step was compared to the appropriate control at each time point.

3. Results and discussion

The conceivable contamination of the edible portion of citrus fruits and citrus juices by the penetration and growth of \( \text{Salmonella} \) into the fruit endocarp through natural-light-etched labels on citrus peels was addressed in a series of experiments aimed at reproducing a large number of possible scenarios that may be encountered at the packinghouse and afterward. The belief that the superficial disruption to the cuticle created by the natural-light-etched label could become a potential entryway for pathogenic microorganisms fueled the original apprehension. These concerns have persisted despite earlier demonstrations indicating the inability of decay organisms to colonize citrus fruits through the labeled areas (Soood et al., 2008, 2009), by the lack of \( \text{Salmonella} \) growth on tomato surfaces on natural-light-etched labeled mature green tomatoes, (even in the presence of soft-rot spoilage...
organisms; Danyluk et al., 2010), and by the lack of infiltration into etched or smooth mature red tomato surfaces (Yuk et al., 2007).

To further address this issue, a series of experiments, including worst case scenarios that might result in the enhanced persistence of Salmonella on the natural-light-etched label area of citrus peel and/or penetration into the juice area, were formulated (Fig. 1). The different combinations covered the range of conceivable (but highly unlikely) events that may take place at the packinghouse or shortly afterward along the commercial chain. Evaluations of highly unlikely events, assumed to be the greatest risk for Salmonella contamination, were evaluated to ensure that under normal conditions contamination risks would be slight. Treatments included: Salmonella inoculated on unwaxed natural-light-etched labels; Salmonella inoculated on waxed natural-light-etched labels; Salmonella inoculated on unwaxed natural-light-etched labels, followed by wax reapplication; and Salmonella inoculated prior to labeling. Control samples consisted of Salmonella inoculated on unlabeled citrus epidermis, in combination with various wax applications, and a puncture surface with wax reaplication (Fig. 1).

At no time during these experiments was Salmonella recovered from juices of sound fruits despite favorable conditions of temperature and humidity (data not shown). Only in 4 highly decomposed fruit following storage, primarily due to mold growth, was Salmonella isolated from juice at concentrations close to the limit of detection (1 CFU/mL). These 4 fruit, out of 672, would have been discarded prior to reaching the consumer. These 4 isolations included fruit from treatments C (the unlabeled control; 26 °C storage on day 10), G (the punctured control; 10 °C storage on day 3), K (26 °C storage on day 10) and I (26 °C storage on day 7). When fruit peel was analyzed, the overall behavior of Salmonella in all treatments was not statistically different (P > 0.05) from those of control fruit at both temperatures (Tables 1 and 2), although there were few significantly different individual data points. In general, Salmonella populations declined with time of incubation in all treatments regardless of the storage temperature. Beyond the third week of the experiment, especially with storage at 10 °C, etched fruit samples were close to or at the limit of detection. A faster decline in Salmonella numbers was observed for fruit stored at 10 °C (Table 1). The decline in Salmonella populations was not due to a detrimental effect caused by the carnauba wax. When mixed with carnauba wax at different ratios and concentrations, Salmonella growth on culture media was not affected compared to controls (data not shown).

The lower initial inoculation values at time zero obtained for some treatments were the result of the partial removal of inoculum by the mechanical application of wax using the sterile swabs. The unintended removal of some inoculum was corroborated by a separate set of control experiments. When swabs used to apply wax on top of the inoculum were cultured, populations between 2.6 and 3.1 log CFU/swab were recovered (data not shown).

Although differences between treatments were not statistically different (Tables 1 and 2), the increase in populations during the first 7 days and the large standard deviations prompted us to repeat these initial, but critical, 7 days as a cautionary measure. Figs. 3–5 confirm the lack of growth in any particular treatment and the statistical similarity between experimental treatments and controls.

Worst case scenarios under recommended citrus storage conditions were devised to validate the ability of Salmonella to survive or grow on orange peels over time periods of actual storage under three possible commercial situations. As in the previous set of experiments at static temperatures, there was no infiltration of Salmonella into the juice area in any treatment (data not shown). The fluctuating temperatures and lengths of storage time had no effect on Salmonella populations at the natural-light label area as compared to individual controls (Tables 3–5). The lower Salmonella population values for the natural-light labeled treatments were likely the result of some mortality induced during the initial light-etching event as demonstrated by in situ labeling plated lawns of the experimental Salmonella cocktail (Fig. 6).

Results from the present study are consistent with earlier reports by Sood et al. (2008, 2009) in which Penicillium, an aggressive spoilage organism of citrus, was unable to grow or infiltrate mature grapefruit or tangerines, respectively, through natural-light labeled areas over storage for 14 days at 25 °C. It is noteworthy that Penicillium only penetrates citrus fruits through surface openings (Sood et al., 2009), yet its inability to grow and colonize through natural-light label openings in citrus suggests a self-cauterizing process, a contention supported by the data from the present study and by previous anatomical studies (Eseberria et al., 2009). Similarly, no growth of the soft rot pathogen Pectobacterium carotovorum or Salmonella was observed on natural-light labeled mature green tomatoes, further indicating the inability of decay organisms or Salmonella to colonize fruits through labeling wounds (Danyluk et al., 2010; Yuk et al., 2007).
The consistent similarities between treatments at all temperatures and the congruencies between treatments and controls are a solid indication that the superficial perforations created by the natural-light labeling did not in any way modify the survival ability of *Salmonella*. The lack of juice contamination and *Salmonella* growth was demonstrated in spite of applying an unattainable worst case scenario where *Salmonella* was inoculated onto the open peel disruptions between labeling and waxing (treatments J and I), a procedure that commercially takes place within 110 ms. Furthermore, it is highly unlikely that *Salmonella* would occur at such high levels on citrus surfaces after normal harvesting and rigorous washing expected at the packinghouse prior to labeling. The fact that even sound fruit inoculated on physically created punctures (treatment G) did not have contaminated juice suggests that the citrus peel itself may constitute a physical barrier to *Salmonella*. Similarly, Yuk et al. (2007) describe results where *Salmonella* is unable to infiltrate into natural-light labeled tomatoes.

4. Conclusion

Based on the data obtained from the many combinations of treatments and under extreme conditions, it is concluded that natural-light labeling citrus peels and subsequent waxing in any order do not influence the fate of *Salmonella* populations on citrus fruits.

Acknowledgments

This study was supported by Sunkist, Inc. and Durand-Wayland, Inc. The authors are grateful for the input of Drs. Arthur J. Miller and Richard Whiting and the staff of Exponent, Inc. for their assistance in experimental design and interpretation of data. The insight provided by Greg Drouillard is much appreciated. The authors are also thankful for the technical support of Pedro Gonzalez, Gwen Lundy, Luis Martinez, Vanessa Moosavifazel, Lisseth Proaño and Denise Dunn.

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