Microscopic Observation and Processing Validation of Fruit Sanitizing Treatments for the Enhanced Microbiological Safety of Fresh Orange Juice†

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ABSTRACT

Studies were conducted to evaluate the infiltration of dye and bacteria into the interior of orange fruit and the impact of possible infiltration on achieving a 5-log microbial reduction during fresh juice processing. Fresh orange fruit were treated at the stem end area with dye and either Salmonella Rubislaw or Escherichia coli strains expressing green fluorescent protein. Microscopic images showed that bacterial contaminants localized at the surface or near surface areas that may be sanitized by surface treatments. Dye infiltration was not a reliable indicator of bacterial penetration in citrus fruit. To quantify the reduction of bacterial contamination, orange fruit were inoculated with E. coli and processed with and without hot water treatments. Greater than 5-log reductions were achieved in juice extracted from fruit immersed in hot water for 1 or 2 min at 80°C, in comparison to the E. coli level detected in the control juice obtained by homogenization of inoculated fruit.

Fruit and vegetables are frequently in contact with soil, insects, and animals during growing and harvesting in the field. Consequently, their surfaces are not free from natural contaminants. In general, fresh produce retains populations of 4 to 6 log CFU/g when they arrive at the packinghouse (1). Although most of the natural microbial contaminants are not harmful to humans, an effective washing and sanitizing process is generally needed to reduce the risk of foodborne illness associated with the consumption of nonpasteurized fresh products.

The citrus fruit is a type of berry called a hesperidium that has a thick, leathery rind, with numerous oil glands, and a large flesh portion composed of several wedge-shaped sections (13). The physical strength of the citrus peel allows citrus fruit to be handled in a fruit packinghouse or juice processing plant with rigorous washing, brushing, and sanitizing. Water flume systems, that may lead to the infiltration of contaminated water into various fruits under certain processing circumstances (2, 12, 14) are generally not used by the fresh citrus juice industry.

Currently, both chemical and physical treatments are being utilized by the fresh citrus juice industry to minimize microbial populations on the surfaces of fruit before juice extraction. Several recent studies affirm that microbiological safety of fresh citrus juice may be enhanced through surface sanitizing. It is known that packinghouse procedures generally reduce surface microflora on citrus fruit (5). Alkaline washing applied with an adequate spray volume effectively reduces the surface contamination of fruit and decreases the microbial loads of fresh juice as well (8). Furthermore, mildly heated, high pH waxes may be utilized in the packinghouse to complement the overall sanitizing procedures (9). Pao and Davis (6) demonstrated that an estimated 5-log reduction of E. coli was achieved by immersing inoculated Valencia orange fruit in hot water at 80°C for 1 min or 70°C for 2 min. The rapid hot water treatments effectively reduced both fruit surface and initial juice microbial loads without altering the original sensory quality of fresh juice.

An important assumption behind the development of fruit sanitizing treatments is that the fruit peel serves as a natural protective barrier that prevents the internalization of human pathogens into the fruit interior under normal processing conditions. Thus, unadulterated fresh juice would be produced after adequate fruit surface sanitizing treatments. This assumption, however, was recently challenged by the findings on the infiltration of Escherichia coli O157:H7 via open channels leading from the blossom end into the core region of intact apple fruit (2). The uptake of a dye solution was subsequently utilized to indicate the frequency and extent of pathogen internalization in apples.

This current study was designed to examine the potential bacterial infiltration and the adequacy of using dye solution as an indicator for the infiltration in citrus fruit. Furthermore, the efficacy of thermal treatment was assessed to assure that sanitizing fruit can be a means to achieve a 5-log microbial reduction in fresh juice processing.

MATERIALS AND METHODS

Inoculum for microscopic observation. Strains of Salmonella enterica ser. Rubislaw or E. coli ATCC 25922 expressing green fluorescent protein (GFP) were obtained from Dr. Randy Worobo (Cornell University, Geneva, N.Y.) and were maintained on tryptic soy agar (Difco, Detroit, Mich.) amended with 100 ppm ampicillin (Sigma, St. Louis, Mo.). A 3-mm loopful of GFP-expressing bacteria from a 24- to 48-h culture was suspended in
sterile phosphate buffer and diluted to A₅₅₀nm = 0.25. Blue food dye erioglaucine (FD & C blue no. 1; Sigma) was then added at a concentration of 0.1% to obtain a bacteria-dye suspension for inoculation. Food dyes have been used to indicate infiltration in previous research (2).

Inoculation for microscopic observation. Valencia orange fruit (Citrus sinensis L.) were harvested on the day of the experiment and placed in an incubator for 2 h at 25°C. The button (attached stem and calyx) was manually removed from the fruit and 100 μl of bacteria-dye suspension was immediately placed at the debotted area. The fruit was incubated at 25°C for 4 h.

Stereomicroscopy. After incubation, the debotted area of the fruit was excised with a sterile knife. About a 1.5-cm² area of tissue surrounding the button area was then removed with a sterile razor blade. The excised tissue was immediately cut into 1- to 2-mm-thick sections with a sterile sharp razor blade starting from the distal end or mounted on a microscope stage with prechilled Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, N.C.) followed by freezing in liquid nitrogen. From the frozen tissue, thin sections (80 to 100 μm thickness) were made with a microtome (American Optical Co., Buffalo, N.Y.). The sections were viewed immediately on slides with a stereomicroscope (Stemi SVII; Zeiss, Thornwood, N.Y.) equipped with a filter cube to provide excitation and emission wavelengths of 480 and 515 nm, respectively. Twelve samples were evaluated for each organism, and at least four sections per sample were examined.

Scanning electron microscopy. After incubation, the debotted area of the fruit was excised with a sterile knife. About a 1.5-cm² area of tissue was removed surrounding the button area with a sterile razor blade and fixed overnight at 4°C in phosphate buffer containing 3% glutaraldehyde (Ted Pella Inc., Redding, Calif.). Fixed samples were cut into 0.5-cm pieces and washed three times with phosphate buffer followed by a final rinse with double distilled water. Sample dehydration was carried out with ascending concentrations of ethanol: 10, 25, 50, 75, 90 (15 min each), and 100% (15 min; three times). Samples were dried with CO₂ in a critical-point dryer (model 2800; Ladd Research Industries Inc., Burlington, Vt.). Samples were mounted on stubs and coated with gold-palladium for 90 s in a coating unit (model 30800; Ladd Research Industries). Samples were then viewed with a scanning electron microscope (S-530; Hitachi, Tokyo, Japan) at 20 kV.

Inoculum for processing evaluation. E. coli ATCC 25922 (Difco) and ATCC 11229 (Remel, Lenexa, Kans.) cultures were maintained on tryptic soy agar. After consecutive daily transfers, cultures were individually cultivated in 8 liters of nutrient broth (Difco) at 37°C for 24 h. The plates were then covered with a layer of violet red bile agar with 4-methylumbelliferyl-β-d-glucuronide (Difco) and incubated for approximately 20 h at 35°C. Purple-red colonies that were ≥0.5 mm diameter and surrounded by a zone of precipitated bile acids were counted after confirmation by their fluorescent appearance under longwave UV light (4). The population of E. coli for each juice was estimated by multiplying the observed plate count with the respective dilution factor of the sample.

Statistical analysis. Microbial results were based on a minimum of three replications per treatment. The Duncan’s new multiple range test was performed by PlotIt (Scientific Programming Enterprises, Haslett, Mich.) software to determine differences (P < 0.05) between fruit treatments.

RESULTS AND DISCUSSION

Microscopic observation. Figure 1A is a low magnification view of the nontreated debottoned stem end area of an orange fruit. The infiltration of blue dye in the same area was readily observed in the fruit that were treated with bacteria-dye suspensions at stem scar areas (Fig. 1B). With a higher magnification, microscopic images further show the distinction between the infiltration patterns of bacteria and dye in the fruit. While the infiltration of blue dye (erioglaucine solution) into the fruit was evident under regular light (Fig. 1C and 1E), the GFP-expressing Salmonella Rubislaw localized at the surface or near surface areas as reflected by fluorescence (Fig. 1D and 1F). In general, dye penetrated deeper than bacteria, dye spread laterally and bacteria did not, and bacteria were not observed in every vascular bundle that was infiltrated with dye. In a similar experiment, E. coli showed similar infiltration patterns (data not shown).

Scanning electron microscopy revealed that xylem tissue of vascular bundles of an orange is constituted with multiple vessels having perforated walls and plates (Fig. 2A). These xylem vessels are the natural channels that allow proper transportation of water and nutrients in fruit. Although perforated walls and plates are common among different kinds of plants, their shape and size varies (3).
FIGURE 1. Differences between the infiltration of blue dye and Salmonella Rubislaw at the stem end area of orange fruit. (A) Low magnification of nontreated debuttoned stem end area showing vascular bundle area, and (B) similar area sectioned and viewed after dye and bacterial treatment; (C, E) high magnification of stem end area showing dye infiltration in and around vascular bundles, and (D, F) respective areas viewed under ultrahigh pressure mercury lamp with a filter cube; arrows pointing to GFP bacteria confined at and near surfaces.

Some variation also exists within the same fruit. Figure 2B exhibits a typical perforation plate and Figure 2C shows an atypical perforation plate in the same orange fruit tissue.

The scanning electron microscopy images also show that these reticulated vessel walls and perforation plates in orange fruit may confine and entrap the test organism Salmonella Rubislaw at surface or near surface areas (Fig. 2D and 2E). Note the absence of bacteria in the lower vessel (Fig. 2F). Thus, the difference in infiltration may be simply explained by the natural sieving effect that limited the lateral and downward spread of bacteria while permitting the penetration of the dye solution. The adhering properties of bacteria cells may also affect their movement in fruit tissues, although it is not investigated in this study.

Unlike pome fruit, such as the apple, the citrus fruit is derived from a superior ovary, an ovary completely separate from the calyx (13). The development of citrus fruit does not create a cavity at the blossom end of the fruit at any maturation stage. These anatomical distinctions allow citrus fruit to be sanitized more effectively for fresh juice processing in contrast to washing and sanitizing fruit with a cavity at the blossom end. The difficulties associated with sanitizing fruit having a blossom end cavity and open channels into the inner core region were described in a recent report on apples (2).

**Processing validation.** The level of *E. coli* on noninoculated fruit surfaces was <1 CFU/cm², and the juice obtained by homogenization of the noninoculated fruit had <1 CFU/ml of *E. coli*. After inoculation, fruit were contaminated with 5.4 log CFU/cm² of *E. coli*. This surface contamination was effectively reduced by the combination of hot water treatments and juice extraction that produced fresh juice with an estimated <1 CFU/ml of *E. coli* (Table 1). In addition, fresh juice extracted from the fruit that was immersed in hot water at 80°C for 1 or 2 min had >5.4 log reduction of the inoculated *E. coli* in comparison to the *E. coli* level (5.4 log CFU/ml) detected in the juice of homogenized fruit without hot water immersion (treatment controls). Juice extraction alone reduced the inoculated levels of *E. coli* by approximately 2.0 log, which is consistent with our former observation (7). In general, *E. coli* was effectively reduced by hot water immersion treatments, thereby verifying previously reported results for citrus (6).
FIGURE 2. Scanning electron micrographs of longitudinal sections showing vascular bundles at the stem end area of an orange fruit. (A) Xylem tissue showing helical structure and multiple perforation plates “pp.” (B) Structure of a typical perforation plate “tp.” (C) Structure of an atypical perforation plate “ap.” (D) Top edge of a debotted orange showing large number of inoculated Salmonella Rubislaw cells in the xylem vessels near the fruit surface. (E) Lower end of a vessel (~100 µm below fruit surface) showing Salmonella Rubislaw cells confined within the reticulated vessel wall. (F) Connecting xylem vessels showing a sieving effect that entrapped bacteria at the lower end of the preceding vessel. Bars represent 10 µm.

This current thermal sanitizing study confirmed that hot water immersion is an effective technique to destroy undesirable microorganisms on the fruit surface to improve fresh juice quality and safety. Because the treatment may be applied to other varieties of citrus fruit with different peel properties, an optimized treatment for each type of fruit should be established on an individual experimental basis.

In conclusion, studies were conducted to confirm that sanitizing fruit can be used as a means to achieve a 5-log microbial reduction during fresh juice processing. Microscopic observations indicated that bacterial contaminants localized at or near the surface where they may be reached by surface sanitizing treatments. Dye infiltration was not a reliable indicator of bacterial penetration in citrus fruit due to its greater capacity for lateral and longitudinal movement in fruit tissues. Nevertheless, dye may be used in research to indicate the penetration capability of sanitizers in surface sanitizing treatments. It has also been confirmed that a surface sanitizing treatment, such as hot water immersion, can be used on fresh citrus fruit to achieve a 5-log reduction during fresh juice production. The treatment may be integrated into existing good manufacturing practices and hazard analysis and critical control point programs (10, 11) to protect the integrity of fresh citrus products.
TABLE 1. Effect of fruit sanitizing treatments on the population of test organism E. coli in juice

<table>
<thead>
<tr>
<th>Treatment for surface-inoculated fruit</th>
<th>E. coli in juice (log CFU/ml)</th>
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<tbody>
<tr>
<td>Homogenization in a juice blender</td>
<td>5.4 ± 0.1 a^b</td>
</tr>
<tr>
<td>Extraction by a juice extractor</td>
<td>3.4 ± 0.5 b</td>
</tr>
<tr>
<td>Hot water immersion (80°C, 1 min) + extraction</td>
<td>&lt;0.0 ± 0.0 c</td>
</tr>
<tr>
<td>Hot water immersion (80°C, 2 min) + extraction</td>
<td>&lt;0.0 ± 0.0 c</td>
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^a Thirty Hamlin oranges were treated per treatment and triplicate experiments were conducted. Inoculated fruit surfaces had 5.4 ± 0.2 log CFU/cm^2 of E. coli.

^b Means (n = 3; ± SD) followed by the same letter are not significantly different (P > 0.05).

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REFERENCES