Approaches toward Identification of Surrogates To Validate Antimicrobial Washes as Preventive Controls for Fresh-Cut Leafy Greens

A. SHAZER, 1 D. STEWART, 1* K. DENG, 2 AND M. TORTORELLO 1

1U.S. Food and Drug Administration, Division of Food Processing Science and Technology, and 2Institute for Food Safety and Health, Illinois Institute of Technology, 6502 South Archer Road, Bedford Park, Illinois 60501, USA

ABSTRACT

In fresh-cut produce production, antimicrobials may be used during washing to control the risk of cross-contamination by microbial hazards. Surrogate microorganisms have long been used to validate processes, but none have been identified for validating the efficacy of antimicrobial washing of fresh-cut produce. The objective of this study was to develop procedures by which surrogates may be identified for use in validating the control of cross-contamination for fresh-cut lettuce operations. Four microbial characteristics, which may be important factors in cross-contamination events, were quantitatively evaluated in potential surrogate microorganisms for comparison to a reasonably foreseeable hazard, Escherichia coli O157:H7: sensitivity to chlorine in solution, sensitivity to chlorine on lettuce leaf surfaces, shedding from contaminated lettuce leaves into the water during washing, and cross-contamination from inoculated to uninoculated lettuce leaves during chlorhexidine washing. A procedure of practical quantitative experiments for comparing the characteristics reduced the original pool of 80 potential strains, which consisted of lactic acid bacteria, probiotics, and isolates obtained from lettuce enrichment cultures, to five strains: Lactobacillus plantarum, Pediococcus pentosaceus, probiotic 22C, and two lettuce enrichment isolates. These strains may be evaluated in additional studies involving comparisons to other reasonably foreseeable hazards and including other potential process variables that should be understood and controlled to prevent cross-contamination in fresh-cut lettuce operations.

Key words: Antimicrobial washing; Fresh produce; Leafy greens; Preventive controls; Validation surrogates

The U.S. Food Safety Modernization Act (21 USC §350g) requires food producers to implement preventive controls for known or reasonably foreseeable microbial hazards during food production. The preventive controls may need to be validated, based on scientific and technical information (21 CFR 117.160) (19). Validation is performed to ensure that processes are adequate to control the hazards and produce microbiologically safe food; such validations are often performed using surrogate microorganisms. The characteristics that should be demonstrated by surrogates have been published (1, 14, 18). First, a surrogate must mimic the pathogen’s behavior in the process. Second, it must be nonpathogenic because it will be used on-site in the food facility. Finally, it must be easy to work with in the validation trials, i.e., easily cultured and enumerated, and readily differentiated from indigenous bacteria present in the food. In fresh produce production, microbial hazards may be controlled using antimicrobials during the washing processes. Contamination can easily spread throughout a production batch if antimicrobials are not used or if they are not present at sufficient levels in the wash water (5, 15). Although the use of antimicrobial washes for inactivating pathogens on the surface of the product can result in some reduction of the hazard, the mitigation is limited because the product surface is very difficult to decontaminate. The primary function of antimicrobials in the wash water, instead, is to prevent cross-contamination (5, 12, 13).

Approaches to validating antimicrobial washes have been developed and include the use of surrogate microorganisms in inoculated produce trials (6), which should demonstrate the ability of the antimicrobial agent to prevent cross-contamination. The characteristics of pathogen cross-contamination behavior are not entirely understood; thus, the identification of surrogates is difficult at this time. Factors that have been proposed for evaluating washing process surrogates include sensitivity to the antimicrobial agent, transfer between contaminated and uncontaminated produce during washing (3), attachment to leaves in the wash water (10), and transfer from contaminated leaves to the water (22). These traits seem to be the relevant features associated with a cross-contamination event; however, there may be others. A potential surrogate should exhibit these features in a manner that is similar to the target pathogen, so that the prevention of cross-contamination by the antimicrobial chemicals can be validated in the washing process. The
goal of this study was to develop a strategy to identify appropriate surrogates for antimicrobial washing processes involving fresh-cut lettuce, with respect to the following characteristics: (i) ease of use in the laboratory; (ii) sensitivity to the antimicrobial agent; and (iii) ability to cross-contaminate the lettuce, i.e., shedding of the surrogate from contaminated lettuce leaves into the wash water and its transfer to uncontaminated lettuce leaves. Chlorine was selected as a model antimicrobial agent because of its widespread use in the fresh-cut lettuce industry. We developed experimental procedures for comparing bacterial strains as potential surrogates for a known microbial hazard in fresh-cut lettuce, Escherichia coli O157:H7.

The identification of surrogate strains for validating antimicrobial wash systems for fresh-cut leafy greens has been cited as a critical research need (6). Nevertheless, it is recognized that validating the prevention of cross-contamination using a microbial surrogate is without precedent; thus, the characteristics displayed by a surrogate in validating this control concept need thorough examination. Therefore, this study is not intended to provide a recommendation of a surrogate strain to be used in validating antimicrobial washing systems. Rather, it is an examination of the factors that seem to be the primary considerations for surrogate identification (3, 10, 22). As more is learned about the process of cross-contamination and its control, additional criteria may be developed for surrogate strain evaluation and selection.

MATERIALS AND METHODS

Bacterial strains and culture. A collection of 80 bacterial strains, which included strains obtained from the American Type Culture Collection (ATCC; Manassas, VA), lactic acid bacterial species from the U.S. Food and Drug Administration culture collection, isolates from commercial probiotic formulations, and isolates from enrichment cultures of romaine lettuce, was used in initial screening. Most of the bacterial strains were grown in brain heart infusion (BHI) broth (BD, Franklin Lakes, NJ) at 37°C. But all lactic acid bacteria and probiotics were cultured in deMan Rogosa Sharpe (MRS) broth (BD) at 25°C, and the strains from the lettuce enrichment cultures were cultured in either tryptic soy broth (TSB; BD) or BHI broth and incubated at 25 or 37°C, as appropriate. Lactobacillus plantarum (ATCC 10241), Pediococcus pentosaceus (ATCC 43200), probiotic 22C, and lettuce enrichment isolates 813-F1 and 813-F2 were adapted for nalidixic acid resistance for ease of selection in the cross-contamination experiments. The target strain E. coli O157:H7 H1827, previously isolated from the 1996 illness outbreak associated with lettuce (9), was adapted for chloramphenicol resistance. For experiments requiring selective enumeration, the agar media were supplemented with nalidixic acid (50 μg/mL) or chloramphenicol (30 μg/mL), as appropriate.

Isolation of bacterial strains from lettuce enrichment cultures. Approximately 60 strains were isolated from enrichment cultures of juiced farm-fresh romaine lettuce leaves after exposure to a 5- to 10-ppm sodium hypochlorite wash. Briefly, the enrichments were performed in either TSB or BHI broth and incubated for 24 h at 25 or 37°C. After streaking the enrichment cultures onto the respective agar media and applying the appropriate incubation, we picked morphologically distinct colonies for further characterization of growth and chlorine sensitivity.

Bacterial growth determinations. Growth was assessed using optical density measurements at 600 nm (OD600) after 18 ± 2 h of incubation. We generated the growth curves in a Bioscreen C automated microbiology growth curve system (Growth Curves, Piscataway, NJ).

Preparation of chlorine solutions. Sodium hypochlorite solution (Sigma-Aldrich, St. Louis, MO) was diluted appropriately, and the free chlorine concentrations were verified using Swift DP Free Chlorine Reagent and the Pocket Colorimeter II chlorine meter (Hach, Loveland, CO) or a Palintest ChloroSense meter (Palintest Ltd., Tyne & Wea, UK).

Lettuce inoculation. Packaged washed whole romaine lettuce hearts were purchased from local retail grocery stores. Leaf sections measuring approximately 2 by 4 cm were cut aseptically. Duplicate leaf sections were inoculated at appropriately 5 log CFU per leaf over 10 spots of 3 μL each across the leaf surface with 2× Butterfield’s phosphate buffer (PB)–washed culture and were air-dried at 25°C until visibly dry (~1.5 h) in a biosafety cabinet before use.

Chlorine MIC. Stationary-phase cultures of the potential surrogate strains and the E. coli O157:H7 target were centrifuged at 4200 × g for 5 min at 4°C, washed twice in PB (pH 7.2), and adjusted to an OD600 of 0.8 in PB. Sodium hypochlorite was diluted to 10 ppm of free chlorine, and adjusted to pH 6.5 with 0.1 M citric acid. We then made serial twofold dilutions in 4 ± 1°C pH-adjusted water in 96-well cluster tubes (300 μL per tube; Corning, Corning, NY) to result in range of ~0.01 to 10 ppm of chlorine. We added the washed and diluted cells (5 log CFU/mL, 60 μL of the buffer solution) to each of the tubes and mixed. After a 30-s exposure, 90 μL of 1 M sodium thiosulfate was added to each tube to neutralize the chlorine. After we added 450 μL of double-strength BHI broth, TSB, or MRS, as appropriate, to each tube, the cluster tubes were incubated for 24 to 48 h at the appropriate growth temperature and the growth was assessed by measuring the OD600. The lowest concentration of chlorine that prevented growth was considered to be the strain’s MIC (17).

Chlorine inactivation kinetics in solution. We adjusted each of the stationary-phase cultures of potential surrogates and the E. coli O157:H7 target strain to approximately 5 log CFU/mL. We added 100 μL of each strain to 0.25 ppm of prechilled (4 ± 1°C) sodium hypochlorite in 60 mL of 0.1 M potassium phosphate buffer (pH 6.5) on a stir plate set so that the liquid agitated without splashing. At 10-s intervals up to 50 s, 2.2-mL samples were removed and diluted 1:10 in PB containing sodium thiosulfate neutralizer, followed by serial dilution in PB and plating onto BHI agar with the appropriate antibiotic supplement. Membrane filters (0.2-μm pore size) were used to enumerate the samples suspected of having low counts. Two independent trials were conducted for each strain. The percentage of inactivation at each time was calculated as in equation 1, using average values for the initial CFU per milliliter (before hypochlorite exposure) and final CFU per milliliter (after hypochlorite exposure).

% Inactivation = \[ \frac{\text{Initial CFU/mL} - \text{Final CFU/mL}}{\text{Initial CFU/mL}} \times 100 \]
Chlorine inactivation on lettuce leaves during washing. An inoculated leaf section (2 by 4 cm) was added to 30 mL of prechilled (4 ± 1°C) 0, 0.25, 0.5, or 1.0 ppm of sodium hypochlorite, washed for 1 min with agitation in an end-over-end mixer at 60 rpm, and then transferred to 5 mL of BPB containing 1 M sodium thiosulfate neutralizer and five sterile glass beads and vortexed (Vortex Genie 2, Scientific Industries, Bohemia, NY) for 1 min at maximum speed to extract the bacteria remaining on the leaf (3). These bacteria were enumerated on the appropriate selective agar plates after the appropriate incubation. Duplicate samples were tested for each hypochlorite concentration, and at least three independent trials were conducted. The percentage of inactivation for each hypochlorite concentration was calculated as in equation 1.

Shedding from contaminated lettuce leaves into water during washing. An inoculated leaf section was added to 30 mL of prechilled (4 ± 1°C) water and washed for 1 min in an end-over-end mixer at 60 rpm. After washing, the water was plated onto the appropriate agar, and the bacteria that had been shed into the water were enumerated after the appropriate incubation. The percentage of shed was calculated as in equation 2, using the average values on the leaves and in the water.

\[
\text{% Shed} = \frac{\text{Total CFU in water}}{\text{Total CFU on leaf}} \times 100
\]

Cross-contamination from inoculated to uninoculated lettuce in chlorine solution. An inoculated leaf section (2 by 4 cm) and three uninoculated leaf strips (approximately 0.5 by 4 cm each) were added to 30 mL of prechilled (4 ± 1°C) aqueous solution containing 0, 0.25, 0.5, or 1.0 ppm of sodium hypochlorite and washed for 1 min in an end-over-end mixer at 60 rpm. The leaf strips were removed, gently shaken to remove the excess wash water, transferred to 5 mL of BPB containing 1 M sodium thiosulfate neutralizer and five sterile glass beads, and then vortexed at maximum speed for 1 min to extract the bacteria remaining on the strips. The transferred bacteria were enumerated by plating or membrane filtration count. In addition, the remaining solution in the tubes was mixed with an equal volume of double-strength MRS or BHI broth. After incubating the tubes for 18 to 24 h at 25 or 37°C, we assessed the presence of the bacterial strain from the leaf strip enrichment by streaking the solution onto appropriate selective agar plates. Duplicate samples were tested for each hypochlorite concentration, and at least three independent trials were conducted. Cross-contamination to the leaf strips for each hypochlorite concentration was indicated by the number of positive enrichments.

Data analysis. The data were analyzed using one-way analysis of variance and Student’s \( t \) test. Strains with differences at \( P < 0.05 \) were considered significantly different from the target \( E. coli \) O157:H7.

RESULTS AND DISCUSSION

Potential surrogate strains for validating the efficacy of antimicrobial lettuce washing processes were compared with a lettuce outbreak–associated strain of \( E. coli \) O157:H7 using several criteria: robustness of laboratory growth, sensitivity to chlorine as measured by MIC determination and inactivation kinetics in hypochlorite solution, inactivation on lettuce after exposure to hypochlorite, percentage of shed from contaminated lettuce into the wash water, and cross-contamination during the washing of lettuce in hypochlorite.

A surrogate should “have similar or more robust survival capabilities under the conditions being studied” (14, p. 147) compared with the target hazard. For fresh-cut lettuce washing, a surrogate should mimic the survival of the target but also demonstrate somewhat better survival in the process than the target hazard; i.e., it should be more resistant to chlorine in solution, show less inactivation on chlorine-treated lettuce, have greater shedding from contaminated lettuce into wash water, and demonstrate a greater ability to cross-contaminate untreated samples during lettuce washing in chlorine solution. The extent to which the surrogate should demonstrate these better survival characteristics are not understood, but with more studies, a better understanding of the criteria demonstrated by an optimal surrogate may be revealed.

Preliminary screening. Strains that produced flocculant or pellicle growth, or those that grew to ≤ 0.4 OD\(_{600}\) after 18 ± 2 h of incubation, were eliminated from further consideration as potential surrogate strains. We assessed the recommended growth kinetics (4), and the strains that did not exhibit robust growth were eliminated. Also the strains for which the MICs were lower than or equal to that of the \( E. coli \) O157:H7 target strain (0.3 to 0.6 ppm of chlorine) were eliminated. Through the growth and MIC determinations, the original collection of strains was reduced to five potential surrogate strains for further testing: \( L. plantarum \) (ATCC 10241), \( P. pentosaceus \) (ATCC 43200), probiotic 22C, and two lettuce enrichment isolates, 813-F1 and 813-F2.
TABLE 2. Comparison of strains for inactivation on lettuce leaves after exposure to various concentrations of hypochloritea

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean % inactivation (SE) after exposure to hypochlorite concn (ppm) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>E. coli O157:H7</strong></td>
<td>98.36 (0.19)</td>
</tr>
<tr>
<td>n = 60</td>
<td>n = 6</td>
</tr>
<tr>
<td><strong>L. plantarum</strong></td>
<td>93.50 (1.62)</td>
</tr>
<tr>
<td>n = 14</td>
<td>n = 6</td>
</tr>
<tr>
<td><strong>P. pentosaceus</strong></td>
<td>93.68a (1.22)</td>
</tr>
<tr>
<td>n = 14</td>
<td>n = 6</td>
</tr>
<tr>
<td><strong>Probiotic 22C</strong></td>
<td>93.04a (0.82)</td>
</tr>
<tr>
<td>n = 14</td>
<td>n = 8</td>
</tr>
<tr>
<td><strong>LI 813-F1</strong></td>
<td>99.03 (0.34)</td>
</tr>
<tr>
<td>n = 14</td>
<td>n = 8</td>
</tr>
<tr>
<td><strong>LI 813-F2</strong></td>
<td>97.36 (0.62)</td>
</tr>
<tr>
<td>n = 14</td>
<td>n = 8</td>
</tr>
</tbody>
</table>

a Mean % inactivation = [(initial CFU/mL – final CFU/mL)/initial CFU/mL] × 100.
b Significant difference found between the strain and the E. coli O157:H7 target at the specified hypochlorite concentration (P < 0.05).

Chlorine inactivation kinetics in solution. The five chosen strains were compared for inactivation kinetics in sublethal levels of chlorine in solution at 10-s intervals up to 50 s. Table 1 compares the five strains to the target, E. coli O157:H7, L. plantarum, P. pentosaceus, and LI 813-F2 were statistically similar to the target; however, probiotic 22C and LI 813-F1 showed a significantly different rate of inactivation than the target. The inactivation of E. coli O157:H7 after 30 s in 0.25 ppm of hypochlorite is in agreement with published work (23). The lettuce enrichment isolate LI 813-F1 consistently showed the most resistance of all the strains to 0.25 ppm of hypochlorite.

The chlorine concentrations chosen for this and the other experiments were limited to some extent by the speed at which manual laboratory manipulations could be performed. A microfluidic device (21) for assaying inactivation kinetics in very short times of exposure (i.e., subseconds) could be useful for conducting studies at higher concentrations of hypochlorite.

Chlorine inactivation on lettuce. Although it is well recognized that antimicrobials are not entirely effective for eliminating microbial hazards on contaminated produce (5, 7, 16), there is a level of risk reduction that can be achieved from the antimicrobial treatment of the product. Therefore, the strains were compared for the inactivation of the target and potential surrogate strains inoculated onto the product. After a 1-min wash of the inoculated lettuce in several concentrations of chlorine, up to 1 ppm, the inactivation characteristics were compared (Table 2). Probiotic 22C showed a greater resistance to all the hypochlorite concentrations tested than did the target E. coli O157:H7.

Cross-contamination of lettuce leaves during washing in chlorine solutions. Cross-contamination to uninoculated leaves was assessed after inoculated leaves were washed in water (0 ppm hypochlorite) or hypochlorite solutions at 0.25, 0.5, and 1.0 ppm. In the absence of chlorine (0 ppm), nearly all the leaves were shown to be cross-contaminated with the various strains (Table 4). The presence of chlorine at various concentrations reduced the cross-contamination rate, and as expected, cross-contamination decreased in general as the chlorine concentration increased. However, two of the strains, probiotic 22C and lettuce enrichment isolate LI 813-F1, showed the ability to survive and cross-contaminate across the gradient of hypochlorite concentrations; they transferred to uncontaminated lettuce pieces irrespective of the hypochlorite concentration.

To conclude, the bacterial strains were examined for their similarity to a reasonably foreseeable hazard, E. coli O157:H7, in the lettuce washing processes, and we

TABLE 3. Comparison of strains for shedding into water from contaminated lettuce leavesa

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean % shed (SE) from leaf to water</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli O157:H7</strong></td>
<td>3.02 (0.73)</td>
</tr>
<tr>
<td>n = 60</td>
<td></td>
</tr>
<tr>
<td><strong>L. plantarum</strong></td>
<td>4.55 (1.11)</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
</tr>
<tr>
<td><strong>P. pentosaceus</strong></td>
<td>5.13 (0.93)</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
</tr>
<tr>
<td><strong>Probiotic 22C</strong></td>
<td>24.46b (2.97)</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
</tr>
<tr>
<td><strong>LI 813-F1</strong></td>
<td>2.92 (0.65)</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
</tr>
<tr>
<td><strong>LI 813-F2</strong></td>
<td>3.70 (1.16)</td>
</tr>
<tr>
<td>n = 14</td>
<td></td>
</tr>
</tbody>
</table>

a Mean % shed = (total CFU in water/total CFU on leaf) × 100.
b Significant difference found between the strain and the E. coli O157:H7 target (P < 0.05).
performed practical experiments to evaluate them. A Wash Water Validation Group (6) published multiple process variables that need to be understood and controlled to prevent cross-contamination, e.g., the product-to-water ratio, water pH, and solids levels. It may be useful to include some of these variable process parameters when evaluating surrogate strains, following the experimental procedures described in this study. Also, more work should be done to evaluate other target strains, e.g., *E. coli* strains showing varying degrees of resistance to chlorine (3, 23), *Salmonella, Listeria monocytogenes*, and other reasonably foreseeable hazards. Finally, the safety of all potential surrogate strains must be assessed. Molecular techniques have been recommended and used for developing genetically well-characterized avirulent strains (2, 8, 11, 20); however, such strains must be demonstrated to have not only the characteristics of safe use but also the required behavioral similarities to the target hazards.

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**REFERENCES**


