



Effect of single and combined chemical and physical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 attached to Valencia oranges



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1. Introduction

Within the last three decades, foodborne outbreaks have been linked to the consumption of fresh orange juice contaminated with *Salmonella* spp. in many countries, such as United States, Canada, Netherlands and Australia (Callejón et al., 2015; Vojdani et al., 2008). Information about the presence of bacterial pathogens on the surface of oranges (Castillo et al., 2006) and in the freshly-squeezed orange juice prepared by street vendors in developing countries has been reported (Castillo et al., 2006; Diana et al., 2012; Lewis et al., 2006; Rai et al., 2014; Sharma, 2013). The presence of foodborne pathogens on the epicarp of oranges might be a prevention concern due to the fact that such pathogens can be transferred to the fruit juice during the extraction process, as well as to other contact surfaces (Martínez-González et al., 2003).

In order to address the problem of microbial contamination in fresh orange juice associated with pathogenic bacteria, certain studies have been conducted to evaluate the effectiveness of chlorine, hydrogen peroxide, lactic acid, hot water and ethanol, among others, in the inactivation of *Salmonella*, *Escherichia coli* O157:H7 and/or *Listeria monocytogenes* on the surface of oranges (Bhagat et al., 2011; Martínez-González et al., 2011; Pao and Davis, 1999, 2001; Venkitanarayanan

et al., 2002). A study in our laboratory (Martínez-González et al., 2011) showed that by washing pathogen-inoculated oranges with single treatments that included hot water, lactic acid, ethanol or chlorine resulted in reductions of total population of 1.9 to > 4.9 log CFU/cm² for *E. coli* O157:H7, 1.9 to > 4.6 log CFU/cm² for *S. Typhimurium*, and 1.4 to 3.1 log CFU/cm² for *L. monocytogenes*. Differences in the bacteria count reduction in oranges may be attributed to the application conditions of the antimicrobial agents, sensitivity of pathogens to agents, surface roughness, surface hydrophobicity, the number of attached pathogens to the surface, biofilm production, and to the accessibility of sanitizer to cells attached to rough surfaces of fruit, among others. Wang et al. (2009) found that an increase in fruit surface roughness would introduce protection to *E. coli* O157:H7 entrapped on the surface resulting in reduced washing efficiency. In their study, the remaining bacteria population was higher in cantaloupes > oranges > avocados > apples after a 5-min washing treatment with sterilized deionized water, acidic electrolyzed water, or peroxyacetic acid. They reported that surface roughness probably plays a more important role than hydrophobicity in attached bacteria removal when applying washing treatments, since the apples and avocados have similar surface hydrophobicity but different roughness and the residual populations of

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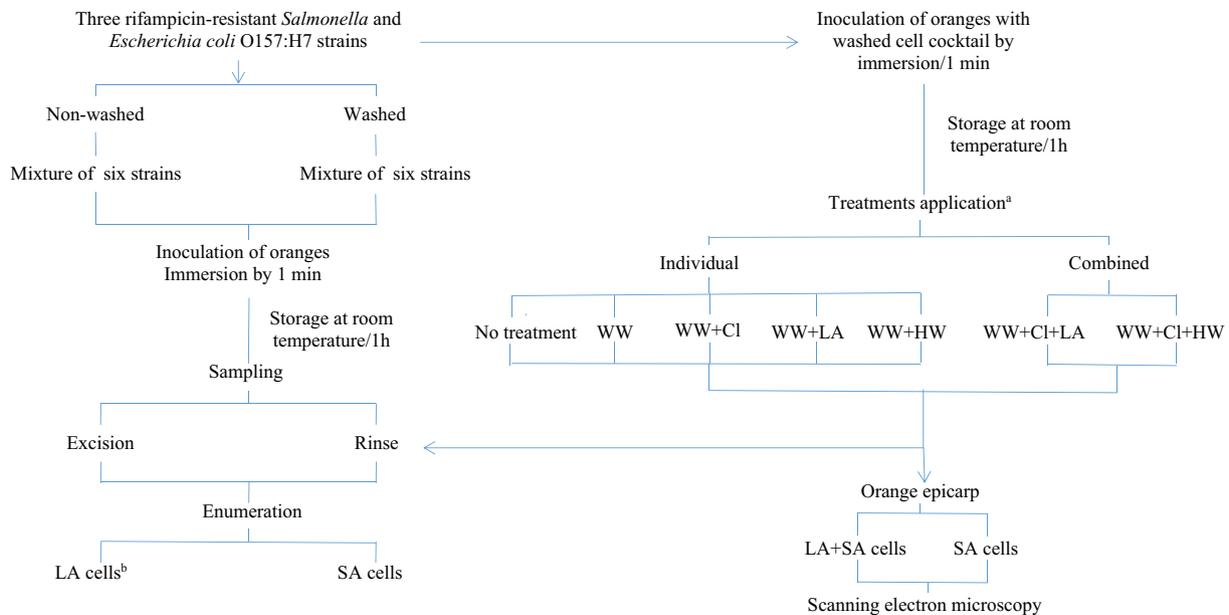


Fig. 1. Flow diagram for the effect of cell washing and sampling method on the recovery of *Salmonella* and *E. coli* O157:H7 attached to the surface of oranges and their relationship with the effect of single and combined chemical and physical treatments on the survival of loosely and strongly attached cells of both bacteria on the epicarp of Valencia oranges.

^a WW = spraying water for 15 s; WW + Cl = WW followed by spraying 200 mg/l chlorine at pH 6.5 for 15 s; WW + LA = WW followed by dipping in 2% lactic acid at 55 °C for 1 min; WW + HW = WW followed by dipping in hot water at 80 °C for 1 min; WW + Cl + LA = WW followed by spraying 200 mg/l chlorine at pH 6.5 for 15 s, followed by dipping in 2% lactic acid at 55 °C for 1 min; WW + Cl + HW = WW followed by spraying 200 mg/l chlorine at pH 6.5 for 15 s, followed by dipping in hot water at 80 °C for 1 min.

^b LA = loosely attached cells; SA = strongly attached cells.

E. coli O157:H7 were higher on avocado than apples. A rough surface provides greater area of contact between bacteria and the material, which facilitates a higher strongly bacterial attachment (Holah and Thorpe, 1990). Therefore, bacterial attachment is an important issue in developing decontamination treatments for food and processing equipment (Pao and Davis, 2001).

When bacteria are present on the surface of an orange and there are conditions that favor the attachment, they can be found as loosely (LA) or strongly attached (SA) cells. LA cells are considered those that can be removed from the tissue surface by gentle shaking, whereas SA cells remain attached to the surface after washing and can be removed by procedures that imply the use of higher force, such as blending (Firstenberg-Eden et al., 1978), stomaching or ultrasound. The presence and number of LA and SA cells of pathogenic bacteria on the surface of decontaminated Valencia oranges has not been reported. Estimating the count of attached cells of pathogens with differentiation between LA and SA cells in oranges is a matter of interest because such knowledge can be used for designing better decontamination treatments focused on the elimination of both types of cells. Oranges have a rough surface with few deep valleys (Wang et al., 2009), in which LA cells of pathogens are localized more superficially than SA cells, which could be trapped in the surface pores. Therefore, SA cells have advantages respect to LA cells during the application of decontamination treatments, since they may be less exposed to solutions and mechanical forces, making them more difficult to remove from the orange surface (Firstenberg-Eden, 1981). Also, the hydrophobicity of the orange surface may difficult the contact between solutions and bacteria during the application of decontamination treatments (Adams et al., 1989). However, sanitizing treatments applied to oranges also should exhibit the capability of eliminate high counts of LA in order to be considered effective.

Chlorine solutions are the sanitizing solutions most commonly used, yet, they have limited bactericidal effects, showing microbial reductions of no > 2 log units on fruit surfaces; furthermore, a gradual reduction of its antimicrobial activity is observed due to the organic

matter present on the food (Gil et al., 2009; Liao and Sapers, 2000). The antimicrobial effectiveness of chlorine solutions on the surface of oranges to remove attached pathogens could be improved if used in combination with other antimicrobial agents, such as hot water and lactic acid; however, the effects of this procedure has not yet been investigated.

The present study was designed to assess the effect of single decontamination treatments with hot water at 80 °C for 1 min, 2% lactic acid solution at 55 °C for 1 min, or 200 mg/l sodium hypochlorite solution at pH 6.5 for 15 s, on the survival of LA and SA cells of *Salmonella* and *E. coli* O157:H7 on Valencia oranges after decontamination, as well as to investigate the effect of combining a chlorinated solution followed by hot water or lactic acid to eliminate both pathogens attached on the surface of oranges. For the purpose of achieving these objectives, the effect of using washed and non-washed cells of *Salmonella* and *E. coli* O157:H7 to inoculate the surface of oranges was investigated, likewise the effect of different sampling methods to recover LA and SA cells of both pathogens from the surface of inoculated oranges was tested.

2. Materials and methods

2.1. Bacterial strains

Rifampicin-resistant (R+) mutants (Kaspar and Tamplin, 1993) derived from parent strains of *Salmonella* and *E. coli* O157:H7 were used in this study. *Salmonella* strains included *S. Typhimurium* isolated from human feces, *S. Agona* isolated from river water in avocado orchards, and *S. Newport* recovered from avocado epicarp. *Escherichia coli* O157:H7 ATCC 43895, an isolate from a human clinical case provided by Ms. Corina Hernández-Mireles of University of Guadalajara, and a strain obtained from the culture collection of the Food Microbiology and Safety Laboratory of the University of Guadalajara were also included. Stock cultures of each strain were stored in tryptic soy broth with 20% glycerol at -80 °C for long-term preservation. Working cultures were maintained on tryptic soy agar (TSA) (Bioxon, Becton

Dickinson, Mexico) supplemented with 0.6% yeast extract (YE) (Bioxon) (TSAYE) slants at 5 °C for no > 30 days prior to the experiments.

2.2. Inoculum preparation

Prior to each experiment, a mixture containing the three described strains of *Salmonella* and the three strains of *E. coli* O157:H7 was prepared and used to inoculate the surface of the Valencia oranges (Fig. 1). For the preparation of the mixture, each strain was individually cultured in 10 ml of tryptic soy broth (TSB; DIFCO Becton Dickinson and Company, Sparks, MD) at 35 °C for 18 h. An aliquot of 100 µL from each culture was individually transferred to 10 ml of fresh TSB and incubated at 35 °C for 4 h. Afterwards, an aliquot of 100 µL was transferred to 35 ml of TSB in duplicate and incubated at 35 °C for 18 h. For the experiments involving the use of non-washed cells, 35 ml of each strain culture was mixed into a sterile Whirl-Pak bag (Nasco, Wisconsin, USA) to obtain 210 ml of the inoculum cocktail. For the experiments involving the use of washed cells, two tubes each with 35 ml of the strain culture were washed three times with 35 ml of a sterile saline solution (SS, 0.85% NaCl) and centrifuged at 4507 × g for 10 min at 4 °C. After the final wash, each pellet was resuspended in 17.5 ml of SS, and equal volumes of each strain suspension were mixed into a sterile Whirl-Pak bag in order to obtain a final volume of 210 ml of the washed cells inoculum mixture. The bacterial concentration in both inoculum mixtures (non-washed and washed) was determined by plating appropriate serial decimal dilutions prepared in 0.1% peptone water (PW), on lactose-sulfite-phenol red-rifampicin agar (LSPR) (Castillo, 1998), which allowed a differential enumeration of *Salmonella* and *E. coli* O157:H7. The colonies were numbered after incubation at 37 °C for 24 h. Initial counts of 8.6 and 8.7 log CFU/ml with coefficients of variation (CV) of 2.5 and 1.6% were obtained for the non-washed cells cocktail of *Salmonella* and *E. coli* O157:H7, respectively, while the washed cells cocktail contained 9.2 log CFU/ml (CV 1.7%) of *Salmonella* and 9.3 log CFU/ml (CV 2.3%) of *E. coli* O157:H7.

2.3. Selection of oranges

Valencia oranges (*Citrus sinensis*) were purchased at a local market and stored at room temperature (25 °C) to be inoculated and treated on the next day. Fresh, unwaxed and without physical damage pieces were washed with tap water for 15 s and then allowed to dry over the lab counter at room temperature for 30 min before their inoculation for the experimental studies described in Sections 2.4 and 2.5.

2.4. Effect of cell washings and sampling method on the recovery of *Salmonella* and *Escherichia coli* O157:H7 attached to the surface of Valencia oranges

Prior to evaluate the effect of single and combined chemical and physical treatments on the survival of *Salmonella* and *E. coli* O157:H7 attached to Valencia oranges, we conducted a preliminary study to select a sampling method (rinsing versus excision) that was the most effective method for the recovery of the inoculated pathogens on oranges (Fig. 1). The oranges were randomly divided into two groups (A and B) for comparing the effect of cell washing and sampling method in the same experiment. For group A, the oranges were inoculated with a *Salmonella* and *E. coli* O157:H7 mixture of non-washed cells prepared as described in Section 2.2, while group B was inoculated with a mixture of washed cells. Each orange was immersed in 210 ml of the corresponding inoculum mixture for 1 min, gently rotating to ensure coverage of the complete surface in all oranges. Oranges were aseptically removed from the mixture and allowed to drain for 1 h at room temperature in a biosafety cabinet. From each group, three oranges were randomly selected to enumerate LA and SA cells of *Salmonella* and *E. coli* O157:H7 from the surface by rinsing each orange in 100 ml of PW,

or by excision of 30 cm² of the epicarp. The excision samples consisted of three 10-cm² areas excised from the rind of each orange (one from the area around the stem scar, one from the central area, and one from the blossom scar), using a sterile borer and sterile scalpel and forceps. The excision samples or a whole piece of the fruit were placed into a Whirl-Pak bag with 100 ml of PW and gently homogenized by manually rotating the bag at 90° (front wise) for 30 s. The LA cells were enumerated from this rinse by preparing the appropriate decimal dilutions in PW and spread plating on LSPR at 35 °C for 24 h. Afterwards, both sample types were then transferred separately to another Whirl-Pak bag containing 100 ml of PW and immersed in an ultrasonic bath at 192 W and 40 kHz for 5 min (Fisher Scientific, model 15337418, MX) for enumeration of the SA cells on LSPR. Typical colonies were counted in the plates and subjected to identification tests that included Gram stain, triple sugar iron agar, urea agar, and lysine iron agar, to confirm the presence of *Salmonella* and *E. coli* O157:H7 (Andrews et al., 2016; Feng et al., 2015). The experiment was repeated three times.

Based on the results of this preliminary study, all oranges in the subsequent experiments were immersed into 210 ml of the inoculum cocktail containing washed cells of *Salmonella* and *E. coli* O157:H7. In the same manner, the rinse method was selected to enumerate LA and SA cells of both pathogens. Under these conditions, the total (LA + SA) and LA cells counts were 5.2 and 5.3 log CFU/piece for *Salmonella* and *E. coli* O157:H7 (CV 5.3 and 5.0%), whereas the SA cell counts were 3.5 and 4.1 log CFU/piece (CV 17.7 and 19.7%), respectively.

2.5. Effect of single and combined chemical and physical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 attached to Valencia oranges

2.5.1. Sanitizing solutions preparation

The sanitizing solutions were applied within 30 min after its preparation. For the lactic acid solution, 80% (wt/vol) l-lactic acid (Almacen de Drogas La Paz, Guadalajara, Mexico) was used to prepare a 2% solution with pH 2.2. The resulting concentration was checked with 0.1 N NaOH and phenolphthalein. Before application on the inoculated oranges, this solution was heated to 55 °C. A concentrated bleach solution (Cloralex, Alen del Norte S. A. de C. V., Santa Catarina, Mexico) was used to prepare a hypochlorite solution with a final concentration of 200 mg/l and pH 6.5 (adjusted with hydrochloric acid). The free chlorine content was measured by the iodometric method. For the treatment with hot water, an appropriate volume of distilled water was heated to 80 °C and poured into a water bath (WNB7, Memmert, Germany). A type K thermocouple (model 45538, Thomas Scientific, Swedesboro, NJ) was inserted in the water bath at a depth of 2 to 3 cm to monitor the temperature of the hot water.

2.5.2. Effect of single and combined chemical and physical treatments on the survival of pathogens attached to Valencia oranges

The oranges were inoculated using the cocktail containing washed cells of *Salmonella* and *E. coli* O157:H7 strains as described in Section 2.4. (Fig. 1). After inoculation, the oranges were allowed to dry for 1 h at room temperature in a biosafety cabinet and then randomly divided into groups to be treated with one of the following treatments: i) control (no treatment), ii) water wash (WW), iii) WW + Chlorine spray (Cl) (200 mg/L NaClO, pH 6.5 for 15 s), iv) WW + lactic acid (2%) dip (55 °C for 1 min), v) WW + hot water dip (80 °C for 1 min) and vi) WW + Cl followed by lactic acid or hot water.

For all spraying treatments, the inoculated oranges were individually placed on a polyethylene crate, which was inside a Nalgene™ autoclavable polypropylene tub; afterwards they were sprayed one at time using a hand-held, noncorrosive polyethylene compressed-air sprayer (15.1 l; ECHO model MS-400, Chicago, Illinois, USA) at a 10 cm distance. The sprayer nozzle was adjusted to deliver 250 ml in 15 s. Separate sprayers were used to apply the chlorine solution and water treatments. The pump was pressurized by air compression to assure the

target flow rate before the solutions were sprayed. During the spraying, the oranges were rotated to fully cover the surface.

For all dip treatments, inoculated oranges were immersed into 6 L of water at 80 °C or 2% lactic acid (55 °C) in a water bath for 1 min. During each experiment, three oranges were immersed into each decontamination solution, one at a time; preliminary studies showed that both pathogenic bacteria were not recovered from the solutions after immersing the first orange. To assure that the oranges were fully submerged into the solutions, a vinyl-coated lead ring (VWR, West Chester, PA) was placed on the top the crate containing each orange. The crate was then subjected to gentle motion during treatment to make sure that the oranges had an even contact with the hot water or lactic acid. When each orange was immersed in the hot water or lactic acid solution, the temperature decreased by 2 °C and 0.1 °C, respectively, after 1 min of treatment. When it was necessary, the temperature was immediately adjusted before immersing the next orange into the same hot water or lactic acid solution. For the treatments that included WW + Cl followed by lactic acid or hot water, each orange treated with chlorine solution was placed in 100 ml of Dey/Engley broth (D/E; Becton Dickinson Difco, USA) by 30 s, prior to the application of the second decontamination treatment, to inactivate any further antimicrobial effect of chlorine.

After applying each treatment, the oranges were immediately sampled and analyzed as described in Section 2.4. Each experiment was repeated three times on different days and triplicate samples were collected for each treatment ($n = 9$).

2.5.3. Sampling and microbiological examination of treated oranges

The sampling for LA and SA cells of both *Salmonella* and *E. coli* O157:H7 on oranges treated with disinfectant solutions was carried out by rinsing each entire orange as described in Section 2.4. To enumerate LA cells, each orange was placed in a bag with 100 ml of rinse liquid selected according to the treatment. For treatments that included WW, WW + hot water, WW + Cl followed by hot water, also on non-treated (control) fruits, oranges were individually immersed in 100 ml of PW. Those oranges treated with WW + Cl were immersed in 100 ml of D/E, while the oranges treated with WW + lactic acid and WW + Cl followed by lactic acid were immersed in 100 ml of buffered peptone water (Difco, Becton, Dickinson and Co., Sparks, MD, USA) (Martínez-González et al., 2013). The selected specimen was sampled for LA cells by manually rotating the bag at 90° (front wise) for 30 s. Each orange then was separated from the rinse liquid and sampled for SA cells by individually placing in a new bag containing 100 ml of PW and afterwards immersing in an ultrasonic bath at 192 W and 40 kHz for 5 min to detach the SA cells. The enumeration of the LA and SA cells was conducted by spread plating onto LSPR agar plates, or by the membrane-filtration method, according to the treatment applied and the reductions expected. The spread plating technique was used to number and tag both pathogens from the control, WW and WW + Cl treatments; whereas the membrane-filtration method was used for oranges treated with WW + hot water and WW + Cl followed by hot water. Both methods were used to enumerate the LA and SA cells of both pathogens on oranges treated with WW + lactic acid and WW + Cl followed by lactic acid. For the membrane-filtration method, the total rinsing volume obtained from each sample was divided into three portions and each one was filtered through 0.45 µm membranes (Millipore, USA), afterwards placed on LSPR agar plates and incubated at 35 °C for 24 h. After numbering the colonies of each pathogen, pure cultures of the presumptive-positive colonies were transferred to biochemical tests for confirmation purposes, as previously described in Section 2.4.

2.5.4. Temperature and pH measurement on oranges

The temperatures of orange rind and pulp of six uninoculated oranges were measured during and after hot water and lactic acid treatments. A type K thermocouple was inserted into the orange rind and the pulp at a depth of 0.3 mm and 2 to 3 cm, respectively. Temperature was

recorded every 30 s. Likewise, the pH on the oranges surface was measured before and after treating with lactic acid, using a flat surface electrode (Sensorex, S450CD Garden Grove, CA).

2.6. Scanning electron microscopy

A group of inoculated oranges were subjected to the decontamination treatments described in Section 2.5.2. Two portions (8 × 5 mm) of the epicarp were cut from non-inoculated oranges, from inoculated oranges without treatment (negative controls) and from inoculated and treated oranges. One of the two portions was placed into a Whirl-Pak bag with 100 ml of PW and gently rinsed by manually rotating the bag at 90° (front wise) for 30 s. The second portion was not rinsed. The samples were individually deposited in 3-ml centrifuge tubes containing 1.5 ml of 2% glutaraldehyde solution and kept refrigerated at 5 °C before shipping to the High Resolution Microscopy Laboratory of the Universidad Autónoma de Aguascalientes. The samples were fixed in glutaraldehyde at 2% for 24 h, rinsed with distilled water in order to remove the excess of the fixative, and dehydrated in a gradient of ethyl alcohol solutions (60, 70, 80, 90, 96 and 100%) for 15 min, followed by drying in a critical point desiccator (Polaron, model CPD7501; UK) for 1.5 h to remove excess moisture. The critical point was reached at a 31.1 °C temperature and at a 1072 psi for 4 min. The samples were then placed in a cylinder with graphite tape and covered with gold (Denton Vacuum, model Desk II, USA) for 230 s. The samples were observed under a scanning electron microscope (SEM, JEOL model JSM-5900LV, USA) at different magnifications.

2.7. Data analysis

Prior to data analysis, the total count (LA + SA), as well as the LA and SA cell counts for *Salmonella* and *E. coli* O157:H7 obtained in experiments described in Section 2.4. were transformed to log CFU/cm². For quantifying the variability associated with bacteria, cell washing and sampling methods, the CV was calculated. When the samples were obtained by excision, each CFU count was divided by 30. For the specimen where the whole orange was sampled by immersion in PW, each CFU count was divided by 173 cm², because this was the mean surface area calculated per orange ($A = 4 \times \pi \times r^2$) after measuring 67 oranges used for this purpose. The total *Salmonella* and *E. coli* O157:H7 counts were computed as the sum of LA and SA counts, which are expressed as log CFU/cm². The significance of differences in populations was assessed by conducting an analysis of variance (ANOVA) using Statgraphics Centurion XV ver.17.01.06 (Statpoint Technologies, Inc., Warrenton, VA, USA).

The total count (LA + SA), as well as the LA and SA cell counts for each pathogen, were transformed into log CFU/orange in those experiments described in Section 2.5.2, where decontamination treatments were applied. Also, the variability associated with bacteria and treatments was expressed with the CV. Comparison of counts for *Salmonella* and *E. coli* O157:H7 was performed as per the ANOVA. The LSD's multiple ranges test was performed to determine statistical differences ($P < 0.05$) between treatments and type of attached cells.

3. Results and discussion

3.1. Effect of cell washing and sampling method on the recovery of *Salmonella* and *Escherichia coli* O157:H7 attached to the surface of Valencia oranges

The use of an inoculum cocktail prepared with either non-washed or washed cells did not have a significant effect ($P > 0.05$) on the recovery of the total population (LA + SA) of *Salmonella* and *E. coli* O157:H7 from the surface of inoculated oranges (Table 1). Similarly, the mean LA counts observed were not different ($P > 0.05$) for both pathogens, and only the mean SA counts of *E. coli* O157:H7 were higher

Table 1Effect of cell washing and sampling method on the recovery of *Salmonella* and *Escherichia coli* O157:H7 attached to the surface of Valencia oranges (*Citrus sinensis*, var. Valencia).

Pathogen	Inoculum type	Count ^a	Counts expressed as log CFU/cm ² (n = 9)					
			Rinse			Excision		
			Mean	CV ^b	Min–Max	Mean	CV	Min–Max
<i>Salmonella</i>	Non-washed cells	Total	5.3Bx ^c	2.8	5.0–5.4	4.4Ax	10.5	3.5–4.7
	Washed cells		5.2Bx	5.4	4.8–5.6	4.1Ax	15.8	3.2–4.8
	Non-washed cells	LA	5.3Bx	2.8	5.0–5.4	4.3Ax	11.8	3.4–4.7
	Washed cells		5.2Bx	5.3	4.8–5.6	3.9Ax	17.7	3.1–4.8
	Non-washed cells	SA	3.3Ax	11.0	2.6–3.7	3.2Ax	12.2	2.7–3.7
	Washed cells		3.5Ax	17.7	2.7–4.3	3.4Ax	16.4	2.4–4.0
<i>E. coli</i> O157:H7	Non-washed cells	Total	5.2Bx	2.0	5.1–5.4	3.7Ax	15.1	3.0–4.4
	Washed cells		5.3Bx	5.0	4.8–5.7	4.1Ax	21.6	2.8–5.2
	Non-washed cells	LA	5.2Bx	2.0	5.1–5.4	3.6Ax	17.7	2.8–4.4
	Washed cells		5.3Bx	4.8	4.8–5.6	3.8Ax	24.6	2.8–5.1
	Non-washed cells	SA	3.3Bx	13.6	2.5–4.0	2.9Ax	17.9	2.0–3.6
	Washed cells		4.1By	19.7	2.5–5.5	3.5Ay	26.2	2.0–4.4

^a LA = loosely attached cells; SA = strongly attached cells; Total count = LA + SA cells.

^b CV = coefficient of variation.

^c Values within rows for each inoculum type with the different letter (A, B) are significantly different ($P < 0.05$). Values within columns for each sampling method with different letter (x, y) are significantly different ($P < 0.05$).

when using washed cells ($P < 0.05$) (Table 1). These results suggest that the total population of pathogens inoculated on the orange epicarp is mainly constituted by LA cells. The inoculum mixture prepared with non-washed cells consisted of six strains cultured in TSB and contains the nutrient components of the broth and the metabolic (waste) products resulting from the bacterial growth. On the contrary, for the inoculum mixture prepared with washed cells, these materials were removed. For the LA cells recovery, the presence or the absence of nutrients and waste materials did not interfere, perhaps because the LA cells established a weak union with the fruit surface. In particular, the higher SA mean counts of *E. coli* O157:H7 on oranges inoculated with mixtures of washed cells in comparison with the use of non-washed cells, may be related to the ability of strains to respond to the effects of washing stages. During washing, the environment of cells was modified by exposing them to centrifugation at 4 °C and removing the components of the broth. Under these environmental conditions, strains might develop a stress response that favors a strong attachment; however our study was not designed to evaluate this response. Wesche et al. (2009) conclude in their review that the conditions of induced-stress on bacteria such as, starvation stress induce transformations in their cellular morphology and the components of the surface which contribute to improve the adherence and formation of biofilms. Meanwhile the behavior observed between non-washed and washed *Salmonella* cells could be the result of the use of a mixture of strains that showed a better capacity of adaptation to the conditions during the preparation of the two types of inoculum, in comparison to the strains of *E. coli* O157:H7.

In general, the sampling method (rinsing the whole fruit versus excising 30 cm² from the epicarp) had a significant effect ($P < 0.05$) on the recovery of both pathogen populations (Table 1). Rinsing the whole surface of the fruit led to higher total and LA mean counts of *Salmonella* ($P < 0.05$). Likewise, total, LA and SA mean counts of *E. coli* O157:H7 were higher ($P < 0.05$) when the rinsing method was used. A minor dispersion in counts of both pathogens was observed after using rinsing method than excision method, except to SA cells (Table 1). High dispersion in the counts of cells by the excision method can be a result of factors inherent to this method and to the characteristics of the surface of the orange, such as potential release of juice from the pulp, or oils from the rind, which may have additional antimicrobial, or a washing effect. Further research evaluating these effects should provide a better explanation of these results. Although the units of data obtained by the excision and rinsing methods were adjusted to cm² to compare the results, it is important to consider that by excision it was

sampled 30 cm² vs. 173 cm² in the sampling by rinsing, which influence the recovery of *Salmonella* and *E. coli* O157:H7 from the oranges surface.

The results of this part of our research cannot be compared due to the lack of published studies on methodologies to detach LA and SA cells of pathogenic bacteria from the epicarp of oranges. The results of our study showed that the rinsing method allowed higher counts of attached cell of *E. coli* O157:H7 than excision. In the same manner, rinsing the surface of orange resulted in higher counts of LA cells, but it was not significantly different than the excision for the SA mean counts of *Salmonella*. Furthermore, this evaluation demonstrates that the use of washed or non-washed cells was equally effective for quantifying attached cells of *Salmonella*, independently of attachment type, whereas to recover the SA cells of *E. coli* O157:H7 it is better to use washed cells than non-washed cells. Therefore, the use of washed cells and rinsing sampling were selected to evaluate the effectiveness of decontamination treatments based on the recovery of attached cells of *Salmonella* and *E. coli* O157:H7 on the surface of oranges. Under the conditions of our trials, the SA counts were lower than the LA cells counts for both pathogens, it is necessary to develop further research to evaluate factors that influence the attachment of cells to the surface of the oranges.

3.2. Effect of single and combined chemical and physical treatments on the survival of pathogens attached to Valencia oranges

Survival was defined in this study as the ability of LA and SA cells of each bacterium to outlive the decontamination treatments conditions. The mean populations of the LA and the SA cells of *Salmonella* and *E. coli* O157:H7 were evaluated on the surface of oranges after the single chemicals application (chlorine or lactic acid) and physical (hot water) treatments or combined (Table 2). In our study, the total mean counts (LA + SA) observed for *Salmonella* and *E. coli* O157:H7 on oranges that did not receive treatment were 7.6 and 7.7 log CFU/piece, respectively, while mean counts for LA were 7.5 and 7.7 log CFU/piece, and for SA were 5.5 and 5.4 log CFU/piece. These data demonstrated that the oranges surface had more LA cells than SA cells prior to the application treatments. The counts of attached cells of both pathogens were not different ($P > 0.05$) on oranges after applying the treatments that included chlorine, lactic acid and hot water. But the levels of pathogens surviving populations varied according to the applied treatment ($P < 0.05$) (Table 2). The application of decontamination treatments led to mean counts for LA cells of *Salmonella* and *E. coli* O157:H7

Table 2

Surviving populations of attached cells of *Salmonella* and *Escherichia coli* O157:H7 on the surface of Valencia oranges after applying alone or combined treatments with different wash solutions.

Treatments ^a	Surviving populations ^b mean log CFU/piece											
	<i>Salmonella</i> count						<i>E. coli</i> O157:H7 count					
	Total	CV	LA	CV	SA	CV	Total	CV	LA	CV	SA	CV
No treatment	7.6E	4.6	7.5Fy ^c	4.7	5.5Cx	6.7	7.7D	5.5	7.7Ey	5.6	5.4Dx	7.8
WW	5.5D	9.6	5.1Ex	9.6	5.3Cx	10.6	5.3C	13.3	4.7Dx	14.2	5.1Cx	14.2
WW + Cl	5.2D	7.4	4.1Dx	12.2	5.2Cy	7.5	5.0C	9.6	3.8Cx	15.0	4.9Cy	9.7
WW + LA	3.6C	10.4	2.3Cx	19.5	3.6By	10.5	3.2B	16.0	1.7Bx	51.9	3.2By	16.1
WW + HW	1.0B	106.0	0.8Bx	120.6	0.4Ax	181.0	0.7A	107.4	0.5Ax	133.6	0.2Ax	176.0
WW + Cl + LA	3.3C	20.2	2.2Cx	18.6	3.2By	24.8	3.0B	23.3	1.4Bx	46.6	2.9By	24.2
WW + Cl + HW	0.3A	300.0	0.2Ax	300.0	0.3Ax	300.0	0.2A	236.0	0.1Ax	300.0	0.2Ax	233.0

^a WW = spraying water for 15 s; WW + Cl = WW followed by spraying 200 mg/l chlorine at pH 6.5 for 15 s; WW + LA = WW followed by dipping in 2% lactic acid at 55 °C for 1 min; WW + HW = WW followed by dipping in hot water at 80 °C for 1 min; WW + Cl + LA = WW followed by spraying 200 mg/l chlorine at pH 6.5 for 15 s, followed by dipping in 2% lactic acid at 55 °C for 1 min; WW + Cl + HW = WW followed by spraying 200 mg/l chlorine at pH 6.5 for 15 s, followed by dipping in hot water at 80 °C for 1 min.

^b LA = loosely attached cells; SA = strongly attached cells; Total count = LA + SA cells; CV = coefficient of variation.

^c Values within columns for each count with different letter (A, B) are significantly different ($P < 0.05$). Values within rows for each LA and SA count with the different letter (x, y) are significantly different ($P < 0.05$).

between 0.2 and 4.1 log CFU/piece and 0.1 to 3.8 log CFU/piece, respectively, while for SA cells were between 0.3 and 5.2 log CFU/piece and 0.2 to 4.9 log CFU/piece, respectively. The treatments that included chlorine and/or lactic acid led to higher surviving populations of both pathogens than hot water treatments, regardless of whether they were applied individually or combined. In regards to the type of attachment (LA vs. SA cells), we found to have a lower recovery of LA cells on the surface of treated oranges than of SA cells, an exception was hot water treatment. The use of hot water alone or the combination of chlorine with hot water showed similar mean counts for both types of attached cells of the pathogens. This evidenced an effect of the type of treatment ($P < 0.05$) in the detachment of LA and SA cells of *Salmonella* and *E. coli* O157:H7 from oranges.

The washing treatment only with water led to mean counts of 5.1 and 4.7 log CFU/piece for the LA cells and 5.3 and 5.1 log CFU/piece for SA cells of *Salmonella* and *E. coli* O157:H7, respectively (Table 2). Comparison of the counts of untreated oranges in regards to those of only washed oranges with water showed an effect of washing by decreasing counts of both pathogens, which was different ($P < 0.05$) only for LA cells of *Salmonella* and both types of attached cells for *E. coli* O157:H7 ($P < 0.05$). Data showed that washing mainly detaches LA cells of both pathogens. Other data show that washing oranges alone with water reduces 0.96 to 2.1 log CFU/cm² of the total *E. coli* counts (Bagci and Temiz, 2011; Pao and Davis, 1999; Pao et al., 2000), or 0.8, 1.4 and 1.6 log CFU/cm² for *L. monocytogenes*, *S. Typhimurium* and *E. coli* O157:H7, respectively (Martínez-González et al., 2011).

Treatment of the surface of oranges with chlorine led to lower counts ($P < 0.05$) of LA cells and similar counts ($P > 0.05$) of SA cells for both pathogens than when they were washed only with water. This suggests that chlorine treatment removes the LA cells but not the SA cells. Published data indicated that at 200 ppm chlorine, the effects of treatment on the reduction of total bacterial population on oranges were within the range of 2.1 to 2.5 log units counts (Bagci and Temiz, 2011; Martínez-González et al., 2011; Pao and Davis, 1999; Pao et al., 2000). This limited effectiveness is due in part to the inactivation of chlorine by organic matter (Gil et al., 2009; Liao and Sapers, 2000). Likewise, may result from the inability of aqueous solution to contact with attached cells of pathogens present on the surface of the orange. The hydrophobic nature of the waxy cuticle of fruit could protect the bacteria cells from the action of chlorine. However, the use of other compounds such as chlorine dioxide show better results than 200 ppm chlorine, Bhagat et al. (2011) reported a > 5 log reduction of attached *Salmonella* cells per sample of Navel orange surface after using 0.1 and

0.3 mg/L. A higher decontamination ($P < 0.05$) of LA and SA cells was observed when chlorine and lactic acid were combined than with the sole application of chlorine (Table 2). However, the effect of this combined treatment was not different ($P > 0.05$) to the one shown with the single use of lactic acid for the attached cells. The two treatments that included 2% lactic acid solution at 55 °C had a lower surviving population of LA cells for both bacteria than SA cells. A higher CV for the count of LA and SA cells was obtained in the single and combined treatments with lactic acid than those after applying only chlorine. For *Salmonella*, the wider CV was 24.8% for SA cells with the treatment combined of chlorine and lactic acid was applied, whereas for *E. coli* O157:H7 was obtained a higher CV for LA cells after applying single or combined treatments with lactic acid (Table 2). Increased variability regarding LA and SA cells can be attributed to the inclusion of the membrane filtration method, which improved the sensitivity in counts as well as to a greater efficiency of employed chemical treatment. Likewise, this fact may be explained by the location of LA cells in sites that are reached by the lactic acid solution during dip treatment. The use of these two chemical agents in a combined treatment did not result in a higher removal ($P > 0.05$) of LA and SA cells comparing against single lactic acid treatment. Although, the lactic acid solution detaches SA cells, its effect is limited for this type of attached cell due to the fact that the population levels were 3.6 and 3.2 log CFU/piece for *Salmonella* and 3.2 and 2.9 log CFU/piece for *E. coli* O157:H7 after applying lactic acid alone or combined, respectively.

Lactic acid solutions at concentration ranges from 0.3% to 4% have proved to effectively eliminate from 1.4 to < 4.9 log CFU of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* on different fruits and vegetables (Akbas and Ölmez, 2007; Ganesh et al., 2012; Huang and Chen, 2011; Martínez-González et al., 2011; Sagong et al., 2011; Samara and Koutsoumanis, 2009). Washing with deionized water containing 1.5% lactic acid plus 1.5% hydrogen peroxide for 15 min at 40 °C decreased the populations of *E. coli* O157:H7, *Salmonella* Enteritidis and *L. monocytogenes* by > 5 log CFU on apples, oranges and tomatoes (Venkitanarayanan et al., 2002). In order to compare the efficacy in the use of lactic acid alone or combined, it is important to consider that the majority of studies have reported total counts or counts of attached cells of pathogens without differentiating between LA and the SA cells, and that lactic acid solutions have been applied at concentrations under different timings and temperatures. Furthermore, the pH of 2.2 and temperature of 55 °C of the 2% lactic acid solution applied may be one of the reasons why lower surviving populations were observed in our experiments with the lactic acid comparing

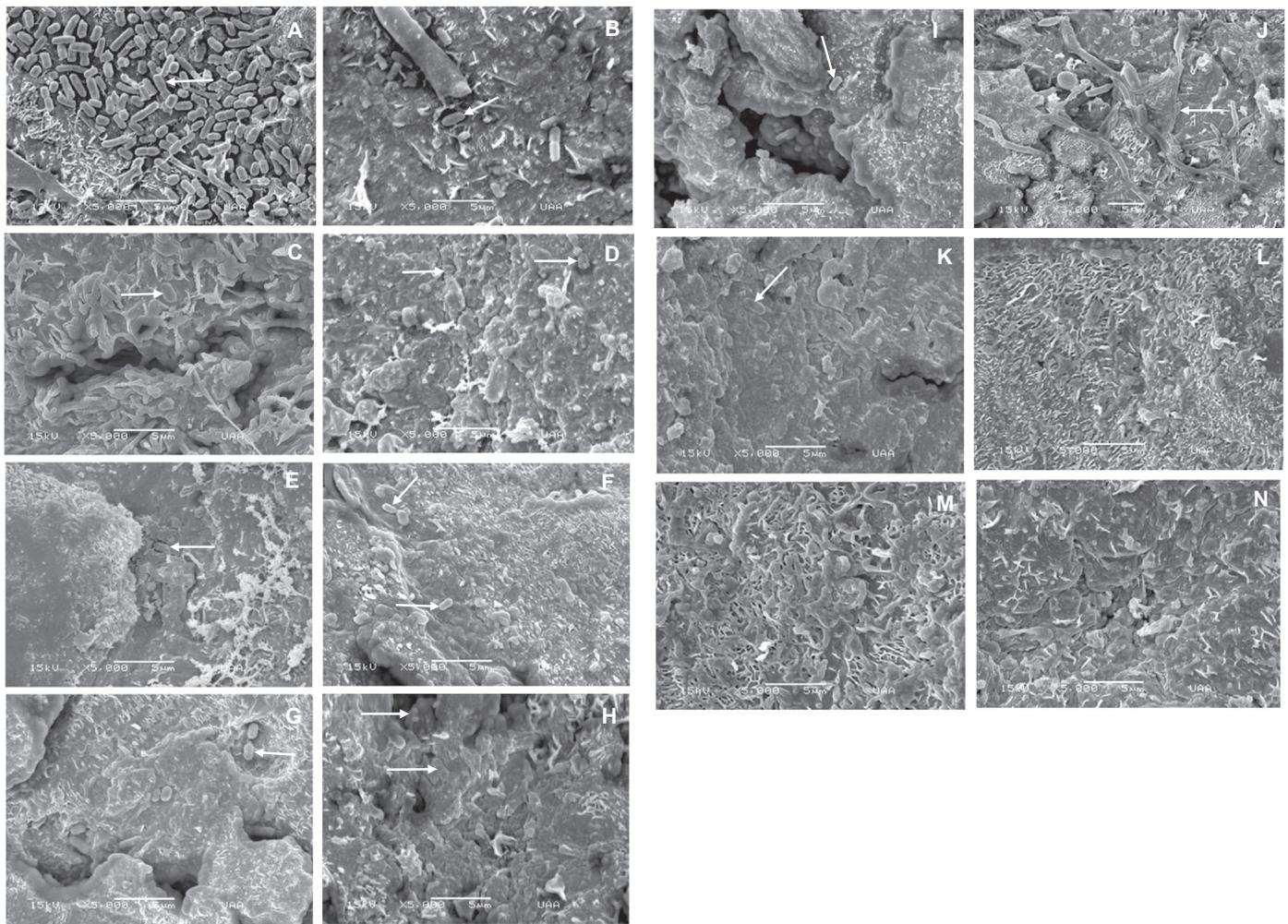


Fig. 2. Scanning electron micrographs (SEM, 5000 or 3000 ×) of the total population (LA + SA) and the strongly attached (SA) cells of *Salmonella* and *Escherichia coli* O157:H7 on the surface of Valencia oranges: A) LA + SA cells, without treatment, B) SA cells, without treatment, C) LA + SA cells, spraying water for 15 s (WW), D) SA cells, WW, E) LA + SA cells, WW followed by spraying 200 ppm sodium hypochlorite at pH 6.5 for 15 s (WW + Cl), F) SA cells, WW + Cl, G) LA + SA cells, WW followed by immersion in 2% lactic acid at 55 °C for 1 min (WW + LA), H) SA cells, WW + LA, I) LA + SA cells, WW followed by immersion in hot water at 80 °C for 1 min (WW + HW), J) SA cells, WW + HW, K) LA + SA cells, WW followed by spraying 200 ppm sodium hypochlorite at pH 6.5 for 15 s, followed by dipping in 2% lactic acid at 55 °C for 1 min (WW + Cl + LA), L) SA cells, WW + Cl + LA, M) LA + SA cells, WW followed by spraying 200 ppm sodium hypochlorite at pH 6.5 for 15 s, followed by dipping in hot water at 80 °C for 1 min (WW + Cl + HW), N) SA cells, WW + Cl + HW. Arrows indicate the presence of bacterial cells on the surface of Valencia oranges.

against other studies. In our research, the mean pH values (\pm standard deviation) on the oranges surface with no treatment and after treatment with 2% lactic acid were 5.3 ± 0.2 and 2.9 ± 0.2 , respectively. Moreover, the temperature on the oranges surface increased as the contact with 55 °C lactic acid solution increased, showing mean values from 24.8 ± 2.5 °C to 38.5 ± 2.4 °C. Despite the temperature increase observed on the surface of oranges, the temperature inside the pulp showed no change, as the rind may function as an insulator.

In this study, lower mean counts of LA (log CFU/piece) and SA (log CFU/piece) cells of both pathogens were achieved with a single antimicrobial agent such as hot water at 80 °C by 1 min or the treatment combined with chlorine and hot water, compared to the remaining decontamination treatments ($P < 0.05$) (Table 2). After applying a single hot water treatment more LA cells were counted than the SA cells of *Salmonella* and *E. coli* O157:H7, however this difference was not significant ($P > 0.05$) for each bacterium. During the application of the hot-water treatment, changes in the temperature of the oranges epicarp and pulp were observed. The temperature of the oranges surface increased rapidly from 24.2 ± 0.8 to 61.8 ± 1.8 °C, while the temperature of the pulp slightly raised from 23.7 to 24.5 °C.

Unlike the combination of two chemical agents (chlorine and lactic

acid), which affected more LA cells than SA cells ($P < 0.05$), the combined treatment of chlorine followed by hot water showed similar surviving populations ($P > 0.05$) for both types attached cells of the two pathogens. This suggests that the chlorine application followed by hot water may be needed to assure a higher reduction of LA cells, whereas in order to reduce high levels of SA cells of both pathogens a treatment may be used which only includes hot water. These findings indicated that LA and SA cells can be effectively detached from the surface of oranges with treatments alone or combined which include hot water. The application of hot water in combined or single treatments on the surface of oranges has better effectiveness than the chemical treatments, due to the conditions of applying of the agent (80 °C, 1 min, immersion) and to its mechanism of antimicrobial action. During immersion in hot water, the naturally occurring waxy components present on the orange surface may be removed, which could favor the wettability of the surface and contact with bacterial cells. The above could conduce to a breakdown of the union points between the adhered cells and the surface of the fruit. In addition, as a result of air displacement trapped in the irregular surface of the orange, the capacity of penetration of hot water may enhance the contact with attached bacteria entrapped inside the orange pores. When hot water contacts with

bacterial cells may cause damage to cytoplasmic membranes and nucleic acids (Mackey et al., 1991). Otherwise, its combination with 200 ppm chlorine results in a higher reduction for LA cells due to chlorine reduced initially almost 2 cycles, that followed by immersion in hot water allowed that the effect of this agent was superior when acting on a smaller number of bacterial cells compared to the individual application of hot water. The values of CV were higher for attached cells' counts in treated oranges with hot water alone or combined, in comparison with the rest of the treatments (Table 2). As with treatments that included lactic acid, this finding can be the result of a better estimation of counts by the use of membrane filtration method, since attached cells of both bacteria were enumerated in 4/9 samples for individual treatment with hot water and 1/9 samples for combined treatment of chlorine with hot water. It also is influenced by the greater efficiency of hot water as an antibacterial agent compared with lactic acid and chlorine.

Previously, we reported similar effectiveness of chlorine and lactic acid treatments to reduce the total populations of *Salmonella* and *E. coli* O157:H7 on Valencia oranges, and a lower effectiveness of the hot water treatments when they were individually applied (Martínez-González et al., 2011) when comparing to the results of this investigation. The current findings can be the result of use of a higher inoculum level, the sensitivity of six strains of the two bacteria in the inoculum, nature of treatments and the sampling and analysis methods used, which in particular allowed a better estimation of the effectiveness of hot water treatment.

Using scanning electron microscopy (SEM), the status of cells of *Salmonella* and *E. coli* O157:H7 on the surface of orange without treatment was observed. The absence of bacteria cells was observed in the samples used as a negative control (micrograph not shown). High populations of total cells (Fig. 2A) appear isolated and a lesser number of the SA cells (Fig. 2B) on the surface of the inoculated orange were visualized. These populations of total attached and SA cells were gradually reduced after the treatments of water washing and single or combined application of chlorine, lactic acid and hot water (Figs. 2C–2N). No relationship was observed between the surviving populations of the attached pathogens on the treated oranges and the number of cells in the SEM images, the cells of pathogenic bacteria were hardly observed in the micrographs. It is possible that the lower number of attached cells observed in SEM images is the result of analysis of a lower superficial area in comparison to the rinse of the whole orange. The SEM images do not exhibit SA cells on the surface after applying the treatments combining hypochlorite and lactic acid (Fig. 2L). Likewise, there were no cells of the total population and SA cells observed in the micrographs of the surface of the orange after applying the combined treatment with chlorine and hot water (Fig. 2M and N). The SEM micrographs demonstrated that the individual application of hot water and the combination of chlorine and hot water or lactic acid resulted in better bactericidal effects for the SA cells of pathogens. These results are in accordance to previous reports that indicate that bacterial contaminants are located on or near the surface of Valencia orange where they may be reached by surface sanitizing treatments (Pao et al., 2000). The development of technologies to deliver disinfectants into the sites where the organisms are located, particularly of SA cells on fresh produce is required. A hot water treatment could physically detach higher counts of SA cells of both pathogens from the surface of oranges and also can enhance the action of chlorine on the elimination of LA cells when applied combined. Additional studies probably are needed to assess the effect of higher temperature at 80 °C in the decrease of attached cells on the oranges surface. Ukuku et al. (2016) reported that wet steam processing killed *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* bacteria inoculated on cantaloupes rind surfaces.

The present study demonstrates that when the cells of *Salmonella* and *E. coli* O157:H7 are strongly attached to surface of orange, they are more resistant to chemical (chlorine and lactic acid) and physical agents (hot water) than LA cells. As it relates to the combination de

physical and chemical treatments, the combination of chlorine and lactic acid or hot water resulted more effective than the single use of chlorine. However, the antimicrobial activity of combined treatments depends on the agent with which chlorine is combined, as it was evidenced in the results of single treatments. These outcomes reinforce the need for good agricultural practices, hygienic handling of fruit, of processing equipment and other surfaces, since if cells of pathogenic bacteria are allowed to contaminate and attach the surface of the orange, it is necessary to apply treatments and develop technologies to detach them, particularly for the SA cells.

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