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Evaluation of chlorine, benzalkonium chloride and lactic acid as sanitizers for reducing *Escherichia coli* O157:H7 and *Yersinia enterocolitica* on fresh vegetables

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ABSTRACT

The effectiveness in the assurance of fresh vegetable microbiological quality of wash solutions containing 200 ppm free chlorine, 0.1 mg/ml benzalkonium chloride, 0.2% and 1% lactic acid was assessed on *Escherichia coli* O157:H7 and *Yersinia enterocolitica* contaminated lettuce and tomatoes. *Y. enterocolitica* reduction on tomatoes (5.08, 4.77 and 4.21 log after 0.2% lactic acid, 200 ppm chlorine and 0.1 mg/ml benzalkonium chloride treatments, respectively) were significantly higher than those for *Y. enterocolitica* on lettuce and *E. coli* O157:H7 on both vegetables. Antimicrobial treatment effects on bacterial counts and product quality after subsequent 7 day storage (4 °C and 22 °C) were determined. No pathogens were found in natural microflora of fresh vegetables.

1. Introduction

The number of foodborne illness outbreaks linked to the presence of pathogenic microorganisms on fresh produce has increased over the past years. Possible contamination sources are soil, faeces, manure, irrigation and washing water, animals (including insects and birds), product handling, harvesting and processing equipment, and transport (Johannessen, Loncarevic, & Kruse, 2002). Fresh vegetables have been identified as carriers of pathogenic bacteria that are highly relevant to public health, such as *Salmonella* (Gupta et al., 2007), *Shigella* (Reller et al., 2006), *Listeria monocytogenes* (Johnston, Jaykus, Moll, Inciso, Mora, & Moe, 2006), *Yersinia enterocolitica* (Madden, 1992), enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli*, and enterohemorrhagic *E. coli* O157:H7 (Frank & Takeushi, 1999; Solomon, Yaron, & Matthews, 2002).

The severity of illness caused by *E. coli* O157:H7 and the low infective doses (less than 100 cfu/g) characterizing both foodborne outbreaks and sporadic cases have contributed to consider this bacterium among pathogens of highest risk for human health. Outbreaks of *E. coli* O157:H7 infections associated with consumption of lettuce have been documented in the USA (Ackers et al., 1998 and Hilborn et al., 1999). In Argentina, the first reported case of a foodborne outbreak linked to ready-to-eat salads contaminated with *E. coli* O157:H7 and *S. aureus* subsp. *aureus* took place in March–April 2006 (Leotta et al., 2006).

The presence of *Y. enterocolitica* has been demonstrated in fresh produce in various countries. Cavazzani, Ceccherini, Prati, and Rausa (1982) performed the first *Y. enterocolitica* isolations from several horticultural products in Italy. Lee et al. (2004) reported 4% *Yersinia* spp contaminated ready-to-eat vegetables in Korea. In Norway, Johannessen et al. (2002) detected *Y. enterocolitica* in 3% of lettuce samples screened by PCR. In Australia, Szabo, Scurrah, and Burrows (2000) isolated *Y. enterocolitica* from minimally processed lettuce; although most of the *Y. enterocolitica* isolates lacked many of the phenotypic and genetic markers associated with virulence. In Japan, Sakai et al. (2005) reported an outbreak of food poisoning caused by salads contaminated with *Y. enterocolitica* O:8, one of the most pathogenic bioserotypes associated with human disease. In San Luis, Argentina, *Y. enterocolitica* strains have been isolated from several foods, samples of animal origin and stool specimens from symptomatic patients.

Different washing chemical agents have been studied to determine their efficacy in the inactivation of pathogenic bacteria on vegetables (Alvarado-Casillas, Ibarra-Sánchez, Rodríguez-García, Martínez-Gonzales, & Castillo, 2007; Nascimento, Silva, Catanozi, & Silva, 2003; Rodgers, Cash, Siddiq, & Ryser, 2004). Solutions of 50–200 ppm chlorine are widely used at commercial scale for washing fruits, vegetables and fresh-cut produce (Beuchat, Nail, Adler, & Clavero, 1998). However, since chlorine compounds can react with organic material on fresh produce to form carcinogenic organochlorine compounds, alternative sanitizers are being assayed for the reduction of bacterial pathogens (Rodgers et al., 2004).

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Quaternary ammonium compounds may provide effective treatment in bacterial removal without affecting the structural integrity of produce. The mode of action of these compounds against bacterial cells involves a general perturbation of lipid bilayer membranes. At low concentrations, they are bacteriostatic and at high concentrations, they are bactericidal (Houari & Di Martino, 2007). In this group, benzalkonium chloride is a synthetic antimicrobial agent widely used as a disinfectant in processing lines and surfaces in the food industry, as clinical disinfectant and antiseptic in health care facilities and domestic households and as antimicrobial preservative in drugs (Mangalappalli-Illathu & Korber, 2006). Its surfactant ability provides a great capacity to penetrate and adhere to porous surfaces.

The potential of organic acids to reduce populations of microorganisms on fresh vegetables has been previously emphasized. Their inhibiting effect is based on their pK_a, the antimicrobial activity of their non-dissociated form, and the specific effects of each acid. Less direct antibacterial activities include interference with nutrient transport, cytoplasm membrane damage resulting in leakage, disruption of outer membrane permeability, and influence on macromolecular synthesis (Ricke, 2003). Different concentrations of lactic acid alone or in combination with other chemicals have been shown to be effective in the elimination of bacterial pathogens (Akbas & Olmez, 2007).

This study was aimed at comparing the effectiveness of various sanitizing agents (sodium hypochlorite, benzalkonium chloride and lactic acid) in the reduction of *E. coli* O157:H7 and *Y. enterocolitica* from surface inoculated lettuce and tomatoes and the effects of these antimicrobial treatments on microbial counts and product quality after subsequent storage of produce. Natural microflora of uncontaminated and untreated vegetables was analysed for counts of mesophilic aerobes and molds and yeasts, estimation of total and fecal coliforms and detection of spoilage and pathogenic bacteria.

2. Materials and methods

2.1. Bacterial strains

The following bacterial strains were used to inoculate fresh vegetables: (i) *Escherichia coli* O157:H7 Sor-/beta glu-/E-Hly+/*eae*+, biotype C, producer of Stx1 and Stx2, from the collection of the National Institute of Infectious Diseases "Dr Carlos G. Malbrán", Buenos Aires, Argentina, and (ii) a two-strain *Y. enterocolitica* cocktail composed by *Y. enterocolitica* O:9 W1024 pYV+, kindly provided by Dr. G. Cornelis (Louvain, Belgium) and *Y. enterocolitica* B2 O:9 pYV+ isolated from eggshell in our laboratory. Organisms were maintained at 4 °C on trypticase soy agar slants (TSA, Merck Laboratories, Darmstadt, Germany). Prior to each experiment, loop inocula were transferred to trypticase soy broth (TSB, Merck) and incubated for 24 h at 37 °C for *E. coli* O157:H7, and at 25 °C for *Y. enterocolitica*. Consecutive loop transfers of 24 h TSB cultures were made before use as inocula in experiments. Aliquots of *E. coli* O157:H7 and *Y. enterocolitica* cultures were plated onto Sorbitol Mac Conkey agar (SMC, Merck) and Mac Conkey agar (MC, Merck) respectively. Culture purity was confirmed by Gram staining and classical biochemicals tests.

2.2. Inocula preparation

One colony of each strain was used to inoculate 100 ml TSB and each broth was incubated 24 h at temperatures specified above. The inoculum concentration was standardized at OD_{600} 0.2 (Metrolab VD 40 Spectrophotometer, Lab. Rodriguez Corswant, Bernal, Argentina) and assessed by surface plating 0.1 ml of appropiate dilutions of each suspension on duplicate TSA plates. To prepare the two-strain *Y. enterocolitica* cocktail, equal portions of TSB cultures of both *Y. enterocolitica* strains were combined to give a final concentration of 8.78 log ± 0.27 log cfu/ml. The final concentration of the *E. coli* O157:H7 inoculum was 8.56 ± 0.55 log cfu/ml.

2.3. Sample collection

Fresh whole tomatoes of a variety locally known as "pear tomatoes" (*Lycopersicum esculentum Mill*) weighing 150–190 g each and free of external defects and wounds, and recently harvested lettuce plants (*Lactuca sativa)* were purchased in local retail groceries. They were not washed or sanitized before being used in experiments. Fresh lettuce leaves weighing between 11 and 14 g each were selected for washing procedures after discarding three or four outer leaves of plants. Uninoculated tomatoes and lettuce leaves were separated for estimation of total aerobic bacteria, total and fecal coliforms and pathogens in natural microflora. A total of one hundred and eighty tomatoes and forty lettuce plants were used in the present study. Produce were purchased the same day of experiment, stored at 4 °C for a short time and brought to room temperature (22 ± 2 °C) before inoculation.

2.4. Sample inoculation

Ten lettuce leaves or whole tomatoes per microorganism were used in each experiment. Groups of three or four samples (whole lettuce leaves or tomatoes) were submerged in a container with 2 l of standardized inocula of each microorganism and gently agitated for 2 min at 22 °C. Samples were removed and placed in a sterile container for 12 h for drying at 25 °C to facilitate bacterial adhesion.

2.5. Chemical agents added to the wash water

Fresh solutions of chemicals in sterile distilled water were prepared the same day of each experiment. The following washing solutions were assayed: sterile distilled water (W, pH 7.03), sodium hypochlorite at 200 mg/L of free available chlorine determined by titration with sodium thiosulfate (C, pH 7.87, vol/vol, Clorox S.A., Buenos Aires, Argentina), 0.1 mg/ml benzalkonium chloride (BzC, pH 7.07, 99% w/w, Parafarm, Buenos Aires, Argentina) and 0.2 or 1% lactic acid (LA, pH 2.57, 99% vol/vol, Parafarm, Buenos Aires, Argentina).

2.6. Washing procedures

After drying, samples were individually placed inside sterile plastic bags and given one of four 50 ml washing treatments. Two samples per treatment were assayed. Two untreated samples contaminated with *E. coli* O157:H7 or *Y. enterocolitica* were used to determine the initial pathogen population on the sample surface. Samples were hand agitated for 1 min to facilitate wetting by the washing solution. A single operator performed rubbing of samples in all experiments to reduce variability in operations.

After washing, each sample was transferred to a new sterile bag and subjected to a 100 ml sterile distilled water rinse. Each bag was massaged by hand for 1 min to remove chemical residues which might interfere with subsequent microbiological analysis. Then, each sample was placed in a new sterile bag containing two sterile paper towels to remove remaining liquid before bacterial counting. Wash liquids containing chlorine, benzalkonium chloride and lactic acid were stored at 4 °C until microbiological analysis.

2.7. Bacterial counts on contaminated samples

In order to perform counts of remaining pathogen populations on washed and rinsed vegetables, each sample was placed in a sterile plastic bag with 20 ml of Dey-Engley neutralizing broth (DE, Merck) and agitated for 1 min to detach bacteria. This suspension was serially diluted in DE broth and spread plated on TSA and on SMC or MC agar. Counts were reported as log cfu/tomato or log cfu/lettuce leaf. Counts of remaining pathogen cells in wash liquids were performed as described. Organism confirmation was carried out at random for one or two typical colonies from one plate per experiment using Gram stain and standard biochemical tests. For stored samples, produce were contaminated and treated as described above. The bags were sealed and stored in refrigerator (4 °C) or in a culture chamber (22 °C) for 7 days. After storage, counts of bacterial populations on produce were performed as described below. Quality of vegetables was assessed by visual examination, taste and odour.

2.8. Natural microflora of vegetables

The microbial quality of vegetables was assessed by performing counts of total mesophilic aerobes and molds and yeasts, estimation of total and fecal coliforms, and investigation of indigenous *Salmonella, E. coli* O157:H7, *Y. enterocolitica, Pseudomonas aeruginosa* and *S. aureus*. Twenty five grams of shredded lettuce leaves were submerged into 225 ml of 0.1% peptone water pH 7.2 (PW, Merck) and homogenized in a stomacher (IUL Masticator, Koningswinter, Germany) for 1 min. Tomatoes were individually placed in sterile plastic bags containing 225 ml PW and hand rubbed for 2 min. Serial decimal dilutions were prepared in PW and 0.1 ml of each dilution were spread in duplicate on plate count agar (PCA, Merck) and oxytetracyclin-glucose-yeast extract agar (OGY, Merck) for counts of mesophilic aerobes and molds and yeasts, respectively. Total and fecal coliforms were investigated by the three-tube Most Probable Number (MPN) procedure in Mac Conkey broth at 37 °C for 48 h. Presumptive results of total coliforms were confirmed in brilliant green lactose broth (BGLB, Merck) at 35 °C for 24 h. Results of fecal coliforms were confirmed in EC broth at 44.5 °C for 24 h, and isolations were performed on Eosin Methylene Blue agar (EMB, Merck). Suspect *E. coli* colonies were studied by Gram staining and biochemical tests.

Presence of pathogens was investigated by submerging 25 g of shredded lettuce leaves or whole tomatoes in 225 ml of enrichment broth according to the following protocols. *Salmonella* spp was detected by seeding samples in peptone buffered water (pH 7.1, PBW) and incubation for 24 h at 37 °C. One-ml aliquots were transferred into two tubes with 9 ml of tetrathionate broth and two tubes with 9 ml of Rappaport broth. One tube of each selective broth was incubated 24 h at 37 °C and the other one was incubated 24 h at 42.5 °C. Loopfulls were streaked on bismuth sulfite agar and suspect colonies were examined by Gram staining and tested by biochemical and serological tests. *E. coli* O157:H7 was detected by incubating samples in EC broth for 2 h at 25 °C. After supplementing with 20 mg/L sodium novobiocin (Sigma Chemicals, St. Louis, MO) and 1.12 g/L bile salts (Merck), enrichment continued at 37 °C for 18 h. After isolation on Sorbitol Mac Conkey agar (SMC, Merck) at 37 °C for 24 h, sorbitol non-fermenting colonies were studied. Challenge against O157 antiserum and PCR for examination of the *stx1, stx2* and *rfbO* genes were available for confirmation. *Y. enterocolitica* was detected by enrichment of samples in phosphate buffered saline pH 7.6 (PBS) added with 1% sorbitol and 0.15% bile salts and stored 21 days at 4 °C. After isolating on MC agar for 48 h at 22–25 °C, presumptive *Yersinia* colonies were subjected to Gram staining and biochemical tests. *S. aureus* was detected by placing vegetables in brain heart infusion broth for 24 h at 37 °C. After enrichment, samples were surface spread on Baird Parker agar. Black colonies surrounded by a circular transparent zone were selected for Gram staining and catalase and coagulase tests. *P. aeruginosa* was detected by enriching samples in asparagine broth for 5 days at 22 °C and plating on cetrimide and milk agar media. Bacterial growth with fluorescent green pigment was indicative of *P. aeruginosa* presence.

2.9. Statistical analysis

Three replications of each experiment were performed on different days. Means of plate counts were analysed for differences in response to sanitizing treatments by analysis of variance with Statistix version 3.5 software and Student's *t* test. Statistical calculations were based on confidence level equal or higher than 95% ($p \leqslant 0.05$ was considered statistically significant). The reductions of the initial counts for each treated group were calculated as the difference: [log cfu per vegetable$_{(\text{non treated group})}$ – log cfu per vegetable$_{(\text{treated group})}$] on TSA. For each treated group, bacterial counts obtained after 7 day storage at 4 °C and 22 °C were compared to corresponding counts obtained on day 0 immediately after sanitizing. The variations of bacterial populations on produce were estimated as

$$[\log \text{cfu per vegetable}_{(\text{day } 7)} - \log \text{cfu per vegetable}_{(\text{day } 0)}]_{\text{treatment, temperature}}$$

3. Results and discussion

3.1. Natural microflora

Table 1 shows the results of microbiological analysis of uncontaminated and untreated lettuce and tomatoes. Although aerobic plate counts reported in fresh produce can range between 10^4 and 10^6 cfu/g (Lopez, Romero, & Duarte, 2003; Nascimento et al., 2003), in the present study mesophilic aerobic counts from lettuce exceeded 7 log cfu/g, with high levels of total coliforms also observed. In contrast, low levels of fecal coliforms were detected on lettuce and negative results were obtained on tomatoes. Our results were similar to values for total coliforms (3.25 log cfu/g) and *E. coli* (1.64 log cfu/g) reported for lettuce by Nascimento et al. (2003) and higher than values reported by Johnston et al. (2006), who observed 1.2–1.3 log cfu/g for total coliforms and 0.7 log cfu/g for *E. coli* in samples obtained during the packaging of leafy greens of domestic and Mexican origin. Among pathogens, no *Salmonella* spp were detected by Nascimento et al. (2003) and only 1% of *L. monocytogenes* positive produce was reported by Johnston et al. (2006). Pathogens were not detected in the natural microflora of vegetables analysed in our study.

Table 1
Investigation of natural microflora on uncontaminated and untreated lettuce leaves and tomatoes

	Lettuce log cfu/g	Tomatoes log cfu/tomato
Mesophilic aerobes	7.30 ± 0.25	5.87 ± 0.10
Molds and yeasts	4.60 ± 0.17	4.90 ± 0.21
Total coliforms	>3.04	>3.04
Fecal coliforms	1.06	ND
Salmonella spp	ND	ND
E. coli O157:H7	ND	ND
Y. enterocolitica	ND	ND
S. aureus	ND	ND
P. aeruginosa	ND	ND

$n = 6$ (number of samples).
ND: not detected.

3.2 Inoculated samples

As known, the initial attachment sites for plant pathogens are often protected areas on produce such as the stomata, broken trichomes, and wounds or cracks in the cuticle layer. The role of factors such as hydrophobic interactions, bacterial surface charge, the presence/absence of fimbriae and exocellular polysaccharides as well as duration of exposure and inoculum level in bacterial attachment to the vegetable surface has been investigated with contradictory results (Solomon & Matthews, 2006). Curli fibers, a type of extracellular protein, are produced by some *E. coli* cells and it is not known whether their expression influences the cell's ability to attach to produce surfaces (Boyer et al., 2007). The role of YadA, an adhesin expressed by virulence plasmid bearing *Y. enterocolitica* strains, and other surface moeities in the attachment of this pathogen to plant tissue has not been reported. Moreover, growth of microorganisms can result in the formation of biofilms (Burnett & Beuchat, 2001). Although a low level of pathogens such as *Y. enterocolitica* and *E. coli* O157:H7 may be initially found on naturally contaminated vegetables, sufficient time and appropriate environmental conditions may allow pathogens to grow to populations exceeding 10^7 CFU/g of vegetable, which is similar to the initial *Y. enterocolitica* and *E. coli* O157:H7 inocula used in this work.

The efficacy of the wash treatments seemed to be related to surface characteristics of vegetables and the antimicrobial susceptibility of each species. The *Y. enterocolitica* and *E. coli* reduction range obtained with water washes after treatment of both vegetables was 0.18–2.29 log on TSA. This range includes values reported by Wright, Sumner, Hackney, Pierson, and Zuecklein (2000), who found that water treatment of *E. coli* O157:H7 inoculated apples decreased bacterial populations by 1.1 log, and by Sapers and Jones (2006), who reached population reductions of <1 log after immersion of *E. coli* NRRL B-766 inoculated tomatoes in water after vigorous agitation.

The exposure of fresh fruits and vegetables to 100–2000 ppm chlorine has been shown to decrease microbial populations by approximately 2–4 log. Rodgers et al. (2004) reported that 200 ppm chlorine after 5 min exposure reduced *E. coli* O157:H7 and *L. monocytogenes* to undetectable levels on apples, lettuce, strawberries and whole melons. In this study, the best reduction produced by this chemical was 4.77 log for *Y. enterocolitica* on tomatoes (Table 2).

A multilayer hydrophobic cuticle composed of cutin and amorphous wax molecules which covers the epidermis of fruits and vegetables is considered responsible for the highly water-repellent nature of plant surfaces. It has been suggested that hydrophobic interactions between this epidermal layer and bacteria play a major role in bacterial attachment to plant tissue (Burnett & Beuchat, 2001). Detergents may offer a way to disrupt such interactions and rinse pathogens off surfaces more readily from vegetable surfaces (Raiden, Sumner, Eifert, & Pierson, 2003). The sanitizing activity of benzalkonium chloride on planktonic cells and biofilms has been previously studied (Houari & Di Martino, 2007; Romanova, Gawande, Brovko, & Griffiths, 2007). In this study, pathogens likely unprotected by an exopolymeric matrix were susceptible to effects by this chemical. *Y. enterocolitica* and *E. coli* O157:H7 populations on 0.1 mg/ml benzalkonium chloride treated tomatoes were reduced by 4.21 and 2.06 log, respectively, as compared to controls (Tables 2 and 3). The first value was better than 2 log reductions in total aerobic counts estimated by Lopez et al. (2003) from water washed and 100 ppm benzalkonium chloride sanitized pre-cut Chilean vegetables. The Argentine Food Code (http://www.anmat.gov.ar/codigoa/caa1.htm) includes no regulations related to the use of this sanitizer on foods.

Lactic acid at concentrations between 0.3% and 4% has demonstrated to be effective in the in vitro reduction of *Y. enterocolitica* (Virto, Sanz, Alvarez, Condon, & Raso, 2005). Akbas and Olmez (2007) obtained maximal reductions of about 2 log cfu/g and 1.5 log cfu/g for *E. coli* and *L. monocytogenes* respectively, after treatment of fresh-cut iceberg lettuce dipped in 0.5% lactic acid. In our work, a lactic acid concentration as low as 0.2% was assayed to avoid alterations of vegetables appearance, smell or odour. This concentration gave 5.08 log reductions of *Y. enterocolitica* on tomatoes (Table 2). In contrast, 1% lactic acid produced only 1.71 log cfu reductions of *E. coli* O157:H7 on lettuce (Table 5).

Selective plating media inhibit the recovery of stressed pathogenic cells. Therefore, higher bacterial counts observed on TSA than on MC or SMC could be attributed to the recovery of injured bacterial cells by TSA but not by these selective media.

The analysis of wash liquids revealed the presence of remaining viable *Y. enterocolitica* and *E. coli* O157:H7 cells at levels ranging between 1.35 and 4.87 log cfu/ml. The reduction of microorganisms in wash and rinse water is important in the prevention of cross-contamination from other produce items, washing surfaces, and the hands of the washer (Parnell & Harris, 2003).

The treated and contaminated produce were subsequently stored for 7 days at 4 °C and 22 °C in order to study the effects of sanitizing treatments on microbial counts and product quality. Variable bacterial regrowth was observed on treated and pathogen contaminated vegetables depending on the sanitizing treatment. *Y. enterocolitica* counts on treated and contaminated lettuce leaves significantly increased by 1 to 3 log cfu per leaf after storage at 4 °C and 22 °C, respectively, when compared to values obtained immediately after sanitizing (Table 4). Under the same storage

Table 2
Recovery of *Yersinia enterocolitica* from contaminated tomato surfaces immediately after sanitizing treatments (day 0) and after 7 day storage at 4 °C and 22 °C (day 7)

Treatment	*Y. enterocolitica* populations (log cfu/tomato) (mean ± SD)					
	Day 0*		Day 7**			
			4 °C		22 °C	
	TSA	MC	TSA	MC	TSA	MC
Non treated	8.28 ± 0.23^{Ab}	8.09 ± 0.17^{Ab}	8.13 ± 0.14^{Ab}	7.53 ± 0.09^{Ac}	8.91 ± 0.18^{Aa}	8.20 ± 0.14^{Ab}
Water	5.99 ± 0.67^{Bd}	5.71 ± 0.73^{Bd}	7.87 ± 0.07^{Bb}	6.53 ± 0.33^{Bc}	8.36 ± 0.60^{Aa}	7.72 ± 0.17^{Ab}
200 ppm chl	3.51 ± 0.97^{Db}	2.96 ± 0.84^{Dd}	7.15 ± 0.30^{Ca}	6.22 ± 0.89^{Bb}	7.32 ± 0.60^{Ba}	7.15 ± 0.30^{Ba}
0.1 mg/ml BzC	4.07 ± 0.95^{Cde}	3.46 ± 1.18^{Ce}	6.24 ± 0.20^{Db}	4.83 ± 0.12^{Cd}	8.14 ± 0.80^{Aa}	5.36 ± 0.40^{Cd}
0.2% LA	3.20 ± 0.39^{Dc}	2.89 ± 0.29^{Dd}	4.71 ± 0.14^{Eb}	2.80 ± 0.14^{Dd}	7.13 ± 0.20^{Ba}	4.65 ± 0.21^{Db}

TSA: trypticase soy agar, MC: Mac Conkey agar, chl: chlorine, BzC: benzalkonium chloride, LA: lactic acid.
*Day 0: counts ($\log_{10}$ cfu/tomato) of *Y. enterocolitica* populations on untreated and treated tomatoes immediately after sanitizing.
**Day 7: counts ($\log_{10}$ cfu/tomato) of *Y. enterocolitica* populations on untreated and treated tomatoes after 7 day storage at 4 °C and 22 °C.
$^{A–E}$Values with different capital letters within the same column differ significantly ($p \leqslant 0.05$).
$^{a–e}$Values with different lower letters within the same row differ significantly ($p \leqslant 0.05$).
The statistical analysis were performed for a 95% confidence level.

Table 3
Recovery of *E. coli* O157:H7 from contaminated tomato surfaces immediately after sanitizing treatments (day 0) and after 7 day storage at 4 °C and 22 °C (day 7)

Treatment	*E. coli* O157:H7 populations (log cfu/tomato) (mean ± SD)					
	Day 0*		Day 7**			
			4 °C		22 °C	
	TSA	SMC	TSA	SMC	TSA	SMC
Non treated	5.84 ± 1.73^{Ac}	5.87 ± 1.72^{Ac}	7.98 ± 0.50^{Ab}	8.04 ± 0.13^{Ab}	9.24 ± 0.14^{Aa}	8.11 ± 0.40^{Ab}
Water	4.52 ± 0.76^{Bd}	4.33 ± 0.91^{Bde}	7.50 ± 0.30^{Bb}	5.06 ± 0.16^{Cd}	8.10 ± 0.28^{Ba}	5.98 ± 0.50^{Cc}
200 ppm chl	3.28 ± 0.61^{Db}	3.22 ± 0.66^{Cb}	3.91 ± 0.17^{Da}	3.87 ± 0.16^{Da}	3.97 ± 0.53^{Da}	3.90 ± 0.17^{Da}
0.1 mg/ml BzC	3.78 ± 0.67^{Cd}	3.41 ± 0.50^{Cd}	6.73 ± 0.50^{Cb}	5.70 ± 0.58^{Bc}	7.67 ± 0.77^{Ba}	6.80 ± 0.14^{Bb}
0.2% LA	3.65 ± 0.65^{Cb}	3.33 ± 0.77^{Cb}	6.35 ± 0.20^{Ca}	3.95 ± 0.70^{Db}	6.73 ± 0.50^{Ca}	6.77 ± 0.90^{Ba}

TSA: trypticase soy agar, SMC: Sorbitol Mac Conkey agar, chl: chlorine, BzC: benzalkonium chloride, LA: lactic acid.
*Day 0: counts ($\log_{10}$ cfu/tomato) of *E. coli* O157:H7 populations on untreated and treated tomatoes immediately after sanitizing.
**Day 7: counts ($\log_{10}$ cfu/tomato) of *E. coli* O157:H7 populations on untreated and treated tomatoes after 7 day storage at 4 °C and 22 °C.
$^{A-D}$ Values with different capital letters within the same column differ significantly ($p \leqslant 0.05$).
$^{a-e}$ Values with different lower letters within the same row differ significantly ($p \leqslant 0.05$).
The statistical analysis were performed for a 95% confidence level.

Table 4
Recovery of *Yersinia enterocolitica* from contaminated lettuce leaves immediately after sanitizing treatments (day 0) and after 7 day storage at 4 °C and 22 °C (day 7)

Treatment	*Y. enterocolitica* populations (log cfu/leaf) (mean ± SD)					
	Day 0*		Day 7**			
			4 °C		22 °C	
	TSA	MC	TSA	MC	TSA	MC
Non treated	9.92 ± 0.24^{Aa}	9.71 ± 0.28^{Aa}	9.64 ± 0.50^{Aa}	9.31 ± 0.10^{Ab}	10.37 ± 0.20^{Aa}	10.05 ± 0.17^{Aa}
Water	8.76 ± 0.46^{Bc}	8.45 ± 0.74^{Bc}	8.88 ± 0.58^{ABc}	8.62 ± 0.21^{Bc}	10.62 ± 0.30^{Aa}	9.77 ± 0.70^{ABb}
200 ppm chl	7.82 ± 0.59^{Dd}	7.58 ± 0.73^{Cd}	8.89 ± 0.09^{Bc}	8.34 ± 0.19^{Bc}	10.69 ± 0.32^{Aa}	9.31 ± 0.60^{Bb}
0.1 mg/ml BzC	8.38 ± 0.28^{Cc}	8.10 ± 0.31^{Bc}	9.39 ± 0.30^{Ab}	8.76 ± 0.20^{Bc}	10.59 ± 0.14^{Aa}	9.91 ± 0.50^{Ab}
0.2% LA	7.48 ± 0.17^{Dc}	7.17 ± 0.20^{Cc}	8.33 ± 0.77^{Bb}	7.12 ± 0.22^{Cd}	10.61 ± 0.56^{Aa}	10.28 ± 0.12^{Aa}

TSA: trypticase soy agar, MC: Mac Conkey agar, chl: chlorine, BzC: benzalkonium chloride, LA: lactic acid.
*Day 0: counts ($\log_{10}$ cfu/tomato) of *Y. enterocolitica* populations on untreated and treated tomatoes immediately after sanitizing.
**Day 7: counts ($\log_{10}$ cfu/tomato) of *Y. enterocolitica* populations on untreated and treated tomatoes after 7 day storage at 4 °C and 22 °C.
$^{A-D}$Values with different capital letters within the same column differ significantly ($p \leqslant 0.05$).
$^{a-d}$Values with different lower letters within the same row differ significantly ($p \leqslant 0.05$).
The statistical analysis were performed for a 95% confidence level.

conditions, bacterial loads on sanitized *Y. enterocolitica* contaminated tomatoes showed increases by 3.6 to 4 log cfu at 4 °C and 22 °C, respectively. The lowest bacterial regrowth corresponded to 0.2% LA treated tomatoes stored for 7 days at 4 °C (Table 2).

On the other hand, bacterial counts on sanitized and *E. coli* O157:H7 contaminated lettuce and tomatoes grew up to 3 and 4 log cfu at 4 °C and 22 °C, respectively, after 7 day-storage depending on the sanitizing treatment. On lettuce, the lowest bacterial regrowth was observed for benzalkonium chloride treated group at 4 °C (Table 5). Interestingly, *E. coli* O157:H7 levels on chlorine sanitized tomatoes remained without significant changes (<1 log cfu) after 7 day-storage at both temperatures (Table 3). The inability of sanitizers to totally remove or eliminate the pathogens from vegetable surfaces evidenced by the subsequent microbial growth after storage, as observed in the present work, suggests the possibility that any pathogenic surviving cells might be lodged in protected sites on vegetable surface or within pre-existing biofilms.

Nonpathogenic bacteria can constitute biofilm communities on common salad vegetables (including tomatoes and lettuce) and

Table 5
Recovery of *E. coli* O157:H7 from contaminated lettuce leaves immediately after sanitizing treatments (day 0) and after 7 day storage at 4 °C and 22 °C (day 7)

Treatment	*E. coli* O157:H7 populations (log cfu/leaf) (mean ± SD)					
	Day 0*		Day 7**			
			4 °C		22 °C	
	TSA	SMC	TSA	SMC	TSA	SMC
Non treated	8.07 ± 0.99^{Ac}	8.06 ± 0.83^{Ac}	9.45 ± 0.21^{Bb}	8.90 ± 0.14^{Ac}	10.02 ± 0.10^{Aa}	9.90 ± 0.45^{Aa}
Water	7.89 ± 0.25^{Ac}	7.65 ± 0.52^{Ac}	10.00 ± 0.24^{Aa}	9.10 ± 0.09^{Ab}	10.62 ± 0.35^{Aa}	9.53 ± 0.85^{Aab}
200 ppm chl	6.76 ± 0.54^{Bc}	6.66 ± 0.53^{Bd}	8.92 ± 0.10^{Bb}	8.67 ± 0.15^{Ab}	9.73 ± 0.49^{Ba}	8.33 ± 0.11^{Bc}
0.1 mg/ml BzC	7.68 ± 0.57^{Ac}	7.67 ± 0.52^{Ac}	9.21 ± 0.42^{Bb}	7.58 ± 0.16^{Bc}	10.46 ± 0.67^{Aa}	9.44 ± 0.10^{Ab}
1% LA	6.36 ± 0.92^{Bd}	6.55 ± 0.72^{Bd}	9.30 ± 0.05^{Bb}	7.62 ± 0.22^{Bc}	10.33 ± 0.09^{Aa}	9.66 ± 0.06^{Aab}

TSA: trypticase soy agar, SMC: Sorbitol Mac Conkey agar, chl: chlorine, BzC: benzalkonium chloride, LA: lactic acid.
*Day 0: counts ($\log_{10}$ cfu/tomato) of *E. coli* O157:H7 populations on untreated and treated tomatoes immediately after sanitizing.
**Day 7: counts ($\log_{10}$ cfu/tomato) of *E. coli* O157:H7 populations on untreated and treated tomatoes after 7 day storage at 4 °C and 22 °C.
$^{A-B}$Values with different capital letters within the same column differ significantly ($p \leqslant 0.05$).
$^{a-d}$Values with different lower letters within the same row differ significantly ($p \leqslant 0.05$).
The statistical analysis were performed for a 95% confidence level.

then, potentially pathogenic microorganisms might become sequestered and protected within these biofilms (Donlan, 2002; Rayner, Veeh, & Flood, 2004). A number of reports have been published on the persistence of several foodborne pathogens such as *Y. enterocolitica* and *E. coli* O157:H7 in biofilms (Ganesh Kumar & Anand, 1998). In contrast to planktonic cells, bacterial biofilms on plant surfaces exhibit an increased resistance to sanitizers and detergents. This resistance has been attributed to various properties associated with the biofilm such as reduced diffusion, physiological changes due to reduced growth rates and the production of enzymes degrading antimicrobial substances (Ganesh Kumar & Anand, 1998). Given adequate environmental conditions, sub-lethally injured cells can grow if provided with nutrients (Romanova et al., 2007).

Alterations of appearance of produce were evident after 7 day storage. Lettuce leaves and tomatoes stored at 4 °C showed acceptable organoleptic characteristics except for benzalkonium chloride and lactic acid treated groups, which presented small spots of yellowish appearance on surface. Slightly modified flavour was detected from chlorine and lactic acid treated produce. Instead, marked decay with blackening on edges, fungal growth and disagreeable odour on sanitized lettuce leaves stored at 22 °C was observed for all the sanitizing treatments. Benzalkonium chloride treated leaves seemed more damaged than those belonging to the remaining groups, probably due to the fact that this agent has active surfacting properties and can potentially cause more evident surface damage than the other compounds.

While benzalkonium chloride and lactic acid sanitized and 22 °C stored tomatoes showed weakness and few fungal growth spots, the chlorine treated group exhibited good appearance without signals of decay. These results were observed on non-contaminated treated vegetables. In contrast, bacterial contaminated produce sanitized and stored for 7 days showed, especially at 22 °C, significant damage signals with unacceptable appearance.

In conclusion, 0.2% lactic acid followed by 200 ppm chlorine and 0.1 mg/ml benzalkonium chloride were the most effective sanitizing treatments, with 5.08, 4.7 and 4.21 log cfu reductions on *Y. enterocolitica* contaminated tomatoes. Lactic acid alone or in combination with other sanitizers has demonstrated good performance to inactivate or substantially reducing pathogenic microorganisms on fruits and vegetables (Venkitanarayanan, Lin, Bailey, & Doyle, 2002). Regarding benzalkonium chloride, although it has been documented to cause a moderate genotoxic effect in mammalian and plant cells at environmentally relevant concentrations (1 mg/l) as well as damages in epithelial cells when used in nasal sprays and ophthalmic solutions in concentrations up to 1 g/l (Ferk et al., 2007), its application as a sanitizer against foodborne pathogens is reported by the literature as well. Results obtained from *Y. enterocolitica* contaminated tomatoes may be promissory, although its efficacy on bacterial reductions without altering product sensory quality should be further assessed.

The Food and Drug Administration has proposed that sanitizing treatments of fresh vegetables should be capable of reducing pathogen loads by a minimum of 5 log cfu without affecting the sensory characteristics of the treated produce (Venkitanarayanan et al., 2002). In this work, bacterial reductions <5 log cfu obtained on lettuce immediately after sanitizing could be mainly attributed to topological characteristics of its surface that provide bacterial protection against the action of sanitizers. As Venkitanarayanan et al. (2002) observed in previous studies, the short time of exposure to chemicals could also result in low pathogen reductions on produce.

Storage of treated and contaminated produce showed that regrowth of pathogens after sanitizing is possible and depends on the sanitizer used and the storage temperature. Lettuce leaves were more susceptible than tomatoes to the challenge of sanitizing and subsequent storage at 4 °C and 22 °C. Low temperature contributed to avoid significant alterations in the product quality. Sanitizing treatments assayed in this study might be effective in the reduction of *E. coli* O157:H7 and *Y. enterocolitica* populations on vegetables depending on the inoculum size, type of vegetable and the time elapsed between sanitizing and consumption.

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