

Antimicrobial effect of acidified sodium chlorite, sodium chlorite, sodium hypochlorite, and citric acid on *Escherichia coli* O157:H7 and natural microflora of fresh-cut cilantro

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ABSTRACT

Fresh-cut cilantro is particularly susceptible to microbial growth and, therefore, use of an effective sanitizer on this product is of great importance. The objective of this study was to evaluate the efficacy of different sanitizing treatments on reducing *Escherichia coli* O157:H7 populations, aerobic mesophilic bacterial, yeast and mould counts on fresh-cut cilantro. Cut cilantro was treated with sodium hypochlorite (SH) at 0.2 g L⁻¹ free chlorine and acidified sodium chlorite (ASC) at 0.1, 0.25, 0.5 and 1 g L⁻¹, along with the components of ASC, i.e., citric acid (CA) at 6 g L⁻¹ and sodium chlorite (SC) at 1 g L⁻¹. In the present study, it was found that SH inactivated, at maximum, 1–1.3 logcfu g⁻¹ of background or pathogenic microflora present on cut cilantro. However, reductions of more than 3 logcfu g⁻¹ were observed after washing with 1 g L⁻¹ of ASC. Moreover, when lower concentrations of ASC were used (0.25 and 0.5 g L⁻¹), microbial populations were reduced by about 2 logcfu g⁻¹. SC was as effective as ASC at 1 g L⁻¹ in reducing aerobic mesophilic bacteria and *E. coli* O157:H7 populations, although it was not as effective as ASC in reducing yeast and mould populations.

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1. Introduction

Food safety constitutes a growing concern for regulatory agencies, producers and the public due to the incidence of foodborne illness caused by enteric human pathogens in various foods at retail and commercial food service facilities (Bhagwat, Saftner, & Abbott, 2004; CAST, 1994; CDC, 2004). Fruits and vegetables are important components of a healthy diet. However, recent studies show that the occurrence of foodborne illness related to the consumption of fruit and vegetables has increased, such as the two outbreaks associated with the consumption of lettuce and spinach in the fall of 2006 (Behrsing, Winkler, Franz, & Premier, 2000; Beuchat et al., 2001; Erickson & Doyle, 2007; FDA, 2006). A wide variety of illnesses associated with fresh produce have involved herbs such as cilantro and parsley (Campbell et al., 2001). An analysis conducted by the FDA in 1999 and 2000, determined that cilantro was one of three imported produce items with a high incidence of pathogen contamination (FDA, 2001). Of significant concern are the human pathogens *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*.

Washing produce with sanitizing solutions is the only step in the fresh-cut produce production chain where a reduction in spoilage microorganisms and potential pathogens can be achieved (Allende, Aguayo, & Artés, 2004; Allende, Selma, López-Gálvez, Villares, & Gil, 2008; Beuchat, Nail, Adler, & Clavero, 1998; Wiley, 1994). However, limited scientific information is available on the efficacy of many disinfection methods for reducing the populations of pathogenic bacteria on fruits and vegetables (Lukasik et al., 2003).

Sodium hypochlorite (NaOCl; SH) is commonly used to sanitize fresh-cut cilantro. However, the antimicrobial effectiveness of this chlorinated water is limited and at the consumer level, residual chlorine and its reaction products in the commodity shall be reduced to a quantity that is technologically unavoidable, has no persisting technological effect in the product, and is harmless to health (Delaquis, Stewart, Toivonen, & Moyls, 1999; Klaiber, Baur, Wolf, Hammes, & Carle, 2005; Nguyen-the & Carlin, 1994; Simons & Sanguansri, 1997). It was reported that if a pathogen can persist on the phylloplane, then the chance of an infectious dose remaining at consumption is increased and this microbial attachment to the hydrophobic plant surface is believed to limit contact between chlorinated water and contaminating microorganisms (Beuchat, 1992; Delaquis et al., 1999; Heaton & Jones, 2007). Furthermore, fresh-cut processing can lead to faster microbial growth by break-

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ing protective surface structures and increasing the availability of nutrients and surface area (Brackett, 1994). Liao and Cooke (2001) reported that bacterial human pathogens bound to cut surfaces of green pepper were more difficult to kill with conventional sanitizers than those present on non-cut surfaces. Moreover, the reaction of active hypochlorite with nitrogen-containing compounds in foods resulting in the formation of toxic compounds, especially trihalomethanes, has incited research for alternative disinfection agents (Allende et al., 2008; Bower & Daeschel, 1999; Inatsu, Bari, Kawasaki, Isshiki, & Kawamoto, 2005).

Acidified sodium chlorite (ASC; Alcide Corp., Redmond, WA) is a highly effective antimicrobial that is produced by lowering the pH (2.5–3.2) of a solution of sodium chlorite (NaClO_2 ; SC) with any GRAS acid (Warf, 2001). The FDA has recently approved ASC ($0.5\text{--}1.2\text{ g L}^{-1}$) for spray or dip application on various food products, including fresh and fresh-cut produce (Code of Federal Regulations, 2000). Inatsu et al. (2005) demonstrated the same sanitation efficacy of different organic acid-activated acidified sodium chlorite solutions. Currently, ASC is commercially supplied as a kit containing citric acid (CA) and SC. These chemicals when combined produce active chlorine dioxide (ClO_2), which is more soluble than sodium hypochlorite (NaOCl) in water and has about 2.5 times greater oxidizing capacity than hypochlorous acid (HOCl) (Inatsu et al., 2005). A number of reports have described the strong efficacy of ASC in the FDA approved application concentration range of $0.5\text{--}1.2\text{ g L}^{-1}$ on inactivation of pathogens, including *E. coli* O157:H7 and *Salmonella* spp., (Gonzalez, Luo, Ruiz-Cruz, & McEvoy, 2004; Park & Beuchat, 1999; Ruiz-Cruz, Acedo-Félix, Díaz-Cinco, Islas-Osuna, & González-Aguilar, 2007). However, a negative impact on organoleptic quality of red meat and shredded carrots occurred when ASC was used within the approved concentration range (Bosilevac, Shackelford, Fahle, Biela, & Koohmaraie, 2004). Therefore, it is critical to find the concentration of ASC that will optimize microbial safety while maintaining quality of fresh-cut cilantro.

The main objectives of this research were to compare the efficacy of ASC at various concentrations to that of SH on reducing microbial populations (including the human pathogen *E. coli* O157:H7) on cut cilantro, and to examine the roles of the individual components of ASC, i.e., SC and CA, in this inactivation phenomenon.

2. Materials and methods

2.1. Preparation of cilantro

Fresh cilantro (*Coriandrum sativum* L.) was obtained from a local wholesale market in Jessup, MD (USA), on the day of its arrival from the grower. The product was transported (within 30 min) under refrigerated conditions to the Product Quality and Safety Laboratory (Beltsville, MD, USA). The product was physically inspected and stems and defective leaves were removed. The product was stored overnight at $5\text{ }^\circ\text{C}$. The next morning, 3 kg of cilantro were processed in a fresh-cut preparation room at $10\text{ }^\circ\text{C}$. Selected cilantro leaves were cut into approximately 1.0 cm segments using a sharp knife. Samples of 100 g of fresh-cut cilantro were placed in nylon mesh bags (Linens N' Things, Clifton, NJ). All samples were stored at $5\text{ }^\circ\text{C}$ for about 2 h before the inoculation process was carried out.

2.2. Natural microflora analyses of fresh-cut cilantro

Cut cilantro samples of 25 g each were homogenized in 225 mL sterile peptone water (8.5 g L^{-1} of NaCl [S9625, Sigma-Aldrich, Inc.] plus 1 g L^{-1} of neutralized bacteriological peptone [Difco, Detroit, Mich.]) using a stomacher 400 Biomaster (Seward Limited, London, UK). Sterile filter stomacher bags (Seward Limited, Lon-

don, UK) were used to eliminate solid particles from the cilantro homogenate. Ten fold dilution series were made in peptone saline solution as needed for plating. Samples ($100\text{ }\mu\text{L}$) of each cilantro filtrate or their corresponding dilutions were logarithmically spread on agar plates (Wasp II Spiral Plater, DW Scientific, West Yorkshire, UK). Aerobic mesophilic bacteria were enumerated on Tryptic Soy Agar (TSA, Difco) plates after incubation at $30\text{ }^\circ\text{C}$ for 48 h and yeast and moulds on Potato Dextrose Agar (PDA, Difco) supplemented with chloramphenicol (200 mg L^{-1} ; Sigma-Aldrich, St. Louis, MO, USA) after incubation at $30\text{ }^\circ\text{C}$ for 48–72 h. Microbial colonies were counted with an automated plate counter (ProtoCOL, Synoptics, Cambridge, UK).

2.3. *Escherichia coli* O157:H7

A cocktail of three nalidixic acid-resistant (Nal^R) strains of *E. coli* O157:H7, which were derived from the outbreak strains, F6460, F15110, H26696, were used in this study. F6460 was isolated from patient fecal samples during a 1999 Nebraska lettuce outbreak and a gift from Timothy Barrett, Centers for Disease Control, Atlanta, Ga. (Wachtel & Charkowski, 2002). The strains F15110 and H26696 were clinical samples from an outbreak associated with fresh-cut watermelon in Wisconsin in 2000, and a gift from Milwaukee Children's Hospital. Cultures were kept at $-80\text{ }^\circ\text{C}$ in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) containing 25% (vol/vol) glycerol. *E. coli* O157:H7 strains were grown at $37\text{ }^\circ\text{C}$, shaken in LB broth supplemented with nalidixic acid (Nal) ($50\text{ }\mu\text{g L}^{-1}$) until stationary phase (20 h growth) and cultured onto LB-Nal agar at $37\text{ }^\circ\text{C}$ for 24 h.

2.4. Inoculation

The inoculation process involved the use of a cocktail of three *E. coli* O157:H7 Nal^R strains. The *E. coli* O157:H7 Nal^R strains were consecutively subcultured twice in 100 mL of LB-Nal broth at $37\text{ }^\circ\text{C}$ for 24 h with constant agitation at 175 rpm to obtain a final OD_{600} reading of about 0.4. After cultures were transferred the second time, they were allowed to adapt to a final temperature of $12\text{ }^\circ\text{C}$ for 4 h. Cultures were washed twice by centrifugation (4000g , 15 min, $4\text{ }^\circ\text{C}$) with 0.1% peptone water. The final pellets were resuspended in 5–10 mL of 0.1% peptone water containing 5% horse serum according to the method of Beuchat et al. (2001) and Burnett, Iturriaga, Escartin, Pettigrew, and Beuchat (2004). Equal volumes of cell suspensions were combined to give approximately equal populations of each culture. The strain cocktail was proportionally diluted in deionized water at $12\text{ }^\circ\text{C}$ to achieve a final concentration of about 10^7 cfu mL^{-1} of *E. coli* O157:H7 Nal^R . Final concentrations of the inoculum solutions were confirmed by plating on Sorbitol MacConkey agar (SMAC) (Difco) supplemented with nalidixic acid ($50\text{ }\mu\text{g L}^{-1}$). The pathogenic suspension was maintained at room temperature and applied to cilantro within 10 min of preparation. The mesh bags of cut cilantro were completely immersed in the inoculum solution and kept under constant agitation for 30 min. After inoculation, the product was maintained at $4\text{ }^\circ\text{C}$ for approximately 60 min. to increase the number of cells attached to the product. Finally, excess inoculum was removed by centrifugation using a manually-operated enclosed spinner (OXO Good Grips, Elmira, NY) for approximately 20 s. The entire experiment was carried out in a Biosafety Level 2 Laboratory.

2.5. Wash treatments

Cut cilantro was washed with water solutions of sodium hypochlorite (NaOCl , SH, Aldrich Chemical Co., Inc., Milwaukee, Wis.) at 0.2 g L^{-1} of free chlorine (pH 6.5), acidified sodium chlorite (NaClO_2 , ASC, SANOVA[®], Alcide Corp., Redmond, Wash) at 0.1, 0.25, 0.5 and

1 g L⁻¹, citric acid (C₆H₈O₇, CA, Aldrich Chemical Co., Inc., Milwaukee, Wis.) at 6 g L⁻¹ and sodium chlorite (NaClO₂, SC, Aldrich Chemical Co., Inc., Milwaukee, Wis.) at 1 g L⁻¹. The initial free chlorine concentration present in the chlorinated solutions was determined using a Chlorine Photometer (CP-15, HF Scientific Inc., Ft. Myers, FL). Three liters of tap water at 5 °C were used for the preparation of each wash. Washing solutions were prepared immediately before application and used within 30 min. Approximately 1 h after the inoculation step, each mesh bag was dipped into one sanitizer solution for 1 min. The excess wash solution was removed by centrifugation with a hand operated enclosed spinner (OXO Good Grips, Elmira, NY) for 30 s. The washing treatments were carried out in a Biosafety Level 2 Laboratory.

2.6. Antimicrobial activity of wash solutions

Cilantro samples of 25 g were collected from each disinfection treatment immediately after washing and homogenized in 225 mL sterile peptone water and plated on agar plates as previously indicated in Section 2.2. Sorbitol MacConkey agar (SMAC) (Difco) supplemented with NaI (50 µg L⁻¹) and sodium pyruvate (0.1%) was used to determine the survival of *E. coli* O157:H7 incubated at 37 °C for 24 h (Strockbine, Wells, Bopp, & Barrett, 1998). The inclusion of sodium pyruvate (0.1%) was to aid in the recovering of injured *E. coli* O157:H7 cells (Mizunoe, Wai, Takade, & Yoshida, 1999). Aerobic mesophilic bacteria, yeasts and moulds were enumerated as indicated in Section 2.2. Microbial colonies were counted with an automated plate counter (ProtoCOL, Synoptics, Cambridge, UK).

2.7. Experimental design

The described experiment was repeated three times separately in time, each with duplicate samples. Statistical analysis of the data was carried out using the SAS general linear models procedure (SAS version 8.2, SAS Institute Inc., Cary, NC, USA) to determine significant differences in microbial counts for treatments.

3. Results and discussion

Unwashed and uninoculated cilantro showed typically high initial microbial loads. Aerobic mesophilic bacterial counts on unwashed cut cilantro were very similar in all the replications, with an average value of 7.00 ± 0.12 logcfu g⁻¹, while the average value of yeasts and moulds was 4.57 ± 0.37 logcfu g⁻¹. The obtained bacterial values agree with the initial aerobic mesophilic bacterial counts reported by Wang, Feng, and Luo (2004) (6.7 logcfu g⁻¹), and is only slightly higher than counts reported by Fan, Niemira, and Sokorai (2003) (5.9 logcfu g⁻¹). Babic and Watada (1996) attributed this elevated contamination to the fact that cilantro is a low-growing crop. The cilantro leaf pattern also contributes to its susceptibility to microbial growth by providing a large exposed surface area for microbial attachment and growth. Therefore, a sanitizing procedure is often used in the production of fresh-cut cilantro for improved quality and safety.

Washing cut cilantro in a SH solution resulted in reductions of aerobic mesophilic bacterial and *E. coli* O157:H7 counts of about 1 logcfu g⁻¹ (Figs. 1 and 2). This value agrees with previous reports in fresh-cut vegetable products (Beuchat et al., 1998; Foley, Euper, Caporaso, & Prakash, 2004). However, yeast and mould counts were not significantly ($P < 0.01$) reduced in fresh-cut cilantro after washing with SH, when compared to unwashed produce (Fig. 3). It can be concluded that SH inactivated, at maximum, 1–1.3 logcfu g⁻¹ of background microflora present in fresh-cut products. Therefore, according to Beuchat (1992), chlorine dips

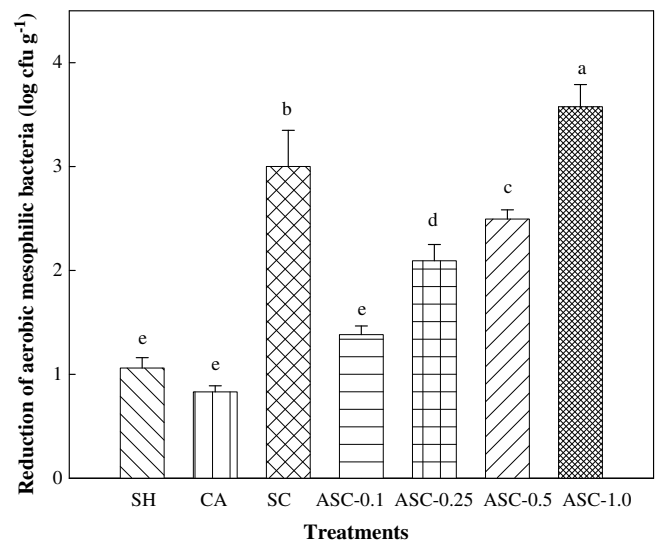


Fig. 1. Reduction of aerobic mesophilic bacterial populations on fresh-cut cilantro after washing with sodium hypochlorite (SH, 0.2 g L⁻¹), citric acid (CA, 6 g L⁻¹), sodium chlorite (SC, 1 g L⁻¹) and acidified sodium chlorite (ASC 0.1–1.0 g L⁻¹), relative to an unwashed control. Vertical bars represent means of three replications ± SE. Bars labeled with different letters indicate significant difference at $P < 0.05$.

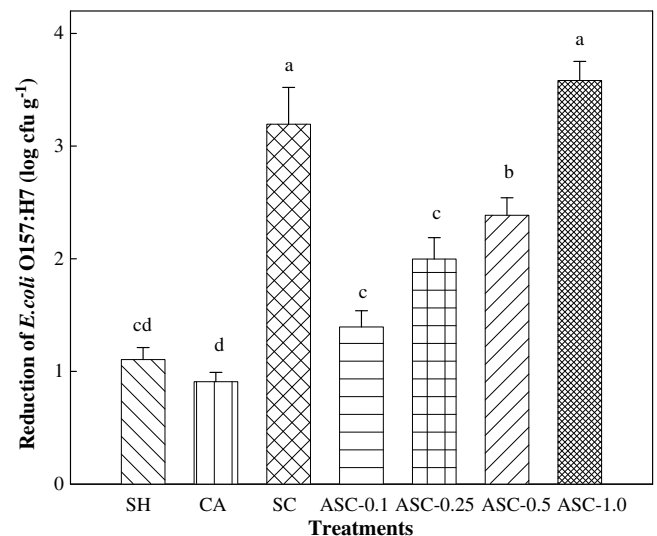


Fig. 2. Reduction of *Escherichia coli* O157:H7 (F6460, F15110N, H26696N) populations on fresh-cut cilantro after washing with sodium hypochlorite (SH, 0.2 g L⁻¹), citric acid (CA, 6 g L⁻¹), sodium chlorite (SC, 1 g L⁻¹) and acidified sodium chlorite (ASC 0.1–1.0 g L⁻¹), relative to an unwashed control. Vertical bars represent means of three replications ± SE. Bars labeled with different letters indicate significant difference at $P < 0.05$.

should not be relied on to kill pathogens on produce and they should be used to reduce viable microorganisms rather than eliminate them.

ASC at 1 g L⁻¹ has been found to effectively reduce aerobic bacterial growth in shredded carrots (Gonzalez et al., 2004; Ruiz-Cruz, Luo, Gonzalez, Tao, & González, 2006; Ruiz-Cruz et al., 2007). In the present study, the antimicrobial activity of ASC in cut cilantro was significantly ($P < 0.01$) influenced by the applied concentration. Thus, the reduction in microbial counts increased with the increase in ASC concentration (Figs. 1–3). Maximum bacterial reductions, of more than 3 logcfu g⁻¹, were observed after washing with 1 g L⁻¹ of ASC and SC (Figs. 1 and 2). Moreover, when lower concentrations of ASC were used (0.25 and 0.5 g L⁻¹), the obtained reductions in

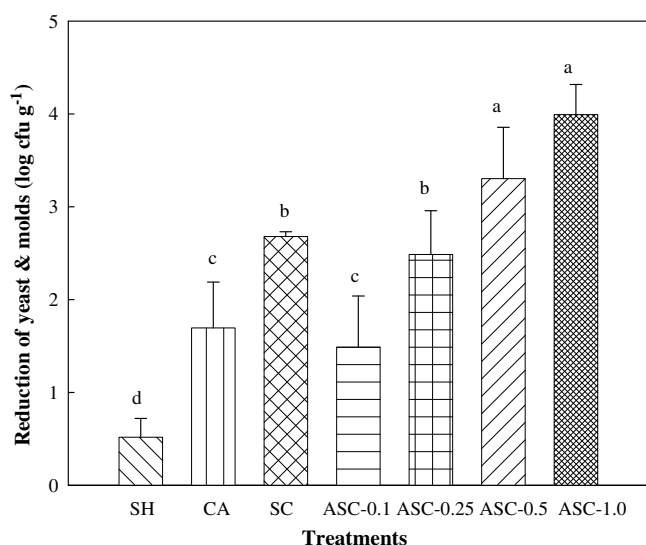


Fig. 3. Reduction of yeast and mould populations on fresh-cut cilantro after washing with sodium hypochlorite (SH, 0.2 g L⁻¹), citric acid (CA, 6 g L⁻¹), sodium chlorite (SC, 1 g L⁻¹) and acidified sodium chlorite (ASC 0.1–1.0 g L⁻¹), relative to an unwashed control. Vertical bars represent means of three replications ±SE. Bars labeled with different letters indicate significant difference at $P < 0.05$.

aerobic mesophilic bacterial and yeasts and moulds were still significantly ($P < 0.01$) higher than those obtained in SH treated samples (Figs. 1–3). Similar results were obtained in shredded carrots by Ruiz-Cruz et al., 2006, 2007. On the other hand, CA treatment achieved the lowest reduction in bacterial counts (Figs. 1 and 2).

Conner (2001) and Caldwell, Adler, Anderson, Williams, and Beuchat (2003) affirmed that ASC applied to inoculated fresh fruits and vegetables at 1.2 g L⁻¹ for 1 min, killed at least 99.9% of *Salmonella* serotypes, *E. coli* O157:H7, and *L. monocytogenes* on carrots, strawberries, tomatoes, cucumbers, lettuce, cantaloupe and apples. Gonzalez et al. (2004), Inatsu et al. (2005) and Ruiz-Cruz et al. (2007) found a strong *E. coli* O157:H7 reduction, even under process water conditions, when using 0.5 and 1 g L⁻¹ ASC on shredded carrots and Chinese cabbage. Lukasik et al. (2003) found that ASC at 0.1 and 0.2 g L⁻¹ was more effective at reducing *E. coli* O157:H7 and *Salmonella* Montevideo populations on strawberries than stabilized chlorine dioxide or free chlorine disinfectants at comparable concentrations. However, they did not recommend concentrations greater than 0.2 g L⁻¹ because of observed deleterious effects on the strawberries. In the present study, all tested washing solutions significantly ($P < 0.01$) reduced *E. coli* O157:H7 populations on fresh-cut cilantro (Fig. 2) when compared to unwashed produce. However, clear differences ($P < 0.01$) were observed among treatments. Thus, ASC at 1 g L⁻¹ achieved the greatest reduction (3.58 ± 0.17 log cfu g⁻¹), followed by 1 g L⁻¹ SC (3.19 ± 0.62 log cfu g⁻¹) and ASC at 0.5 g L⁻¹ (2.38 ± 0.15 log cfu g⁻¹). A reduction of less than 2 log units was obtained by using CA at 6 g L⁻¹ and ASC at 0.1 and 0.25 g L⁻¹. However, similar to the viable aerobic mesophilic bacterial and yeast and mould counts, ASC at 0.25 g L⁻¹, still achieved a significantly ($P < 0.01$) higher reduction than SH.

Warf (2001) hypothesized that the mode of action of ASC derives from the uncharged chlorous acid, which is formed by the acidification of chlorite. Chlorous acid gradually decomposes to form chlorate ions, chlorine dioxide, and chloride ions. These reactive intermediates are highly oxidative with broad-spectrum germicidal activity (FDA, 2007). Chlorous acid is also able to penetrate bacterial cell walls. This ability is thought to facilitate proton leakage into cells, which increases energy use by the cells

in order to maintain homeostasis (Warf, 2001). To determine whether or not the combination of SC with CA is needed for the effectiveness of the treatment, both ingredients were separately tested. It was observed that despite the low pH of the CA solution (pH 2.2 ± 0.1), this treatment did not reduce growth of *E. coli*, aerobic mesophilic bacterial and yeasts and moulds to the same degree as either SC (pH 9.4 ± 0.3) or ASC (pH range 2.6 ± 0.4 – 2.5 ± 0.2). In fact, SC alone reduced microbial populations nearly as much as ASC (Figs. 1–3). This suggests that SC (and not CA or the pH of the treatment) was the major antimicrobial factor.

4. Conclusions

In summary, the commercial ASC product exhibited strong efficacy on reduction of microorganisms, including *E. coli* O157:H7. Both ASC and SC significantly reduced aerobic mesophilic bacteria, yeast and moulds and *E. coli* O157:H7 populations, even when ASC applied at low concentrations. Since ASC at the current FDA approved range (0.5–1.2 g L⁻¹) is known to cause tissue damage to some food products, our findings that ASC or SC at concentrations below the FDA approved range achieved better efficacy on microbial inhibition than SH, provide valuable insight regarding the optimization of ASC and SC applications to maintaining both food safety and quality.

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