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Citric acid as alternative to sodium hypochlorite for washing and disinfection of experimentally-infected spinach leaves

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ABSTRACT

This research aims to investigate citric acid (CA) 0.5% as alternative to sodium hypochlorite (SH) 200 ppm for washing and disinfecting spinach leaves (*Spinacia oleracea* L.). The initial disinfection achieved in leaves spot-inoculated with *Escherichia coli* and *Listeria innocua*, pathogen surrogates, was investigated along with the effects of time and temperature conditions before processing on the performance of CA and SH. Next, the effectiveness of CA and SH was evaluated throughout refrigerated storage (6.5 °C, 9 days) at a low and high contamination load, 5–6 and 8–9 log CFU.g^{-1}, respectively. And lastly, sensory impact was assessed through a trained panel and instrumental color. Results indicated that there were not significant differences between the initial disinfection achieved by CA and SH. Storing infected spinach under refrigeration, between harvest and processing, played a key role not only in reducing their deterioration but also in assuring their safety by maintaining CA and SH effectiveness against the inoculated surrogates. Citric acid performance was better in controlling surrogates' regrowth along refrigerated storage. And there were not significant differences between CA and SH treated samples with respect to their sensory quality. Therefore, CA could constitute an alternative washing and disinfection method for spinach leaves.

1. Introduction

Leafy greens are constantly demanding feasible technologies and optimized preservation methods to minimize safety risks in fresh and fresh-cut products. Minimally processed vegetables such as bagged leafy greens have increased greatly in the last years (Matthews, 2014), and in association with this pattern numerous foodborne outbreaks occurred worldwide. In 2006 contaminated fresh spinach with *Escherichia coli* O157:H7 was linked to a multi-state outbreak in North America, where the number of cases reached 206 (Lynch, Tauxe, & Hedberg, 2009). Furthermore, the lack of Good Agricultural Practices (GAP's) and improper post-harvest handling represent a safety risk on their consumption (Matthews, 2014).

Alternative washing and disinfection methods for leafy greens have been extensively studied, but most of the time they are not easy to apply or not effective enough to replace the existing ones (Matthews, 2014). Sodium hypochlorite solutions (chlorinated water), the most widespread disinfection agent, is criticized because it has limited efficacy against pathogens and it is believed that its use generates carcinogenic compounds that might reach consumers (Gómez-López, Lannoo, Gil, & Allende, 2014). Therefore, several alternative approaches to increase the microbial safety of fresh produce arose in the last years. Among them, organic acids have demonstrated their bactericidal activity through multiple mechanisms: pH reduction, disruption of cell membrane transport system and permeability, accumulation of ions, and a reduction in the internal cellular pH which affects their homeostasis (Neal et al., 2012). However, their effectiveness varies greatly depending on the type of organic acid, application method, microorganism tested, produce, and processing parameters. Several authors (Akbas & Ölmez, 2007; Almasoud, Hettiarachchy, Rayaprolu, Horax, & Eswaranandam, 2015; Bermúdez-Aguirre and Barbosa-Cánovas, 2013; Dikici, Koluman, & Calicioglu, 2015; Choi et al., 2012;

Abbreviations: CA, Citric acid; SH, Sodium hypochlorite; FT, Fresh tissue; C, Control.

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Ganesh et al., 2010, Ganesh, Hettiarachchy, Griffis, Martin, & Ricke, 2012; Ho, Rodde, Tang, & Phan, 2011; Huang, Ye, & Chen, 2012; Neal et al., 2012; Ölmez & Temur, 2010; Park et al., 2011) have studied the efficacy of organic acids (acetic, ascorbic, citric, lactic, malic, propionic, and tartaric acids) for washing and disinfecting leafy greens; nevertheless, only Almasoud et al., 2015 and Ölmez & Temur, 2010 considered biofilm formation and/or time before processing which mimics the lapse between microbial contaminations occur and the washing and disinfecting step is carried out. The lapse in-between contamination and processing along with temperature conditions during that period are factors that have been barely studied and might reduce the effectiveness of organic acids as leafy greens' sanitizers.

In our previous study (Finten, Finten, Agüero, & Jagus, 2015), it was demonstrated that a dip washing with citric acid (CA) 0.5% was effective against native microbiota present in spinach leaves without affecting their sensory quality, and it could constitute an alternative sanitizer to the traditional disinfection agent against foodborne pathogens. Therefore, the aim of the present research was to broaden the study of spinach leaves' washing and disinfection with citric acid 0.5%. Firstly, the initial disinfection achieved in spot-inoculated *Escherichia coli* and *Listeria innocua*, pathogen surrogates, was investigated along with the effects of time and temperature conditions before processing on the performance of sodium hypochlorite (SH) and CA. Next, the effectiveness of CA and SH was evaluated throughout 9 days of refrigerated storage (6.5 ± 1 °C) at a low and high contamination load, 5–6 and 8–9 log CFU.g^{-1}, respectively. And lastly, sensory impact of the selected disinfection treatment on spinach leaves was assessed through a trained panel and instrumental color.

2. Materials and methods

In the present study three experiments were carried out sequentially. The first and the second were microbial studies where the effectiveness of citric acid against *E. coli* and *Listeria monocytogenes*' pathogen surrogates inoculated on spinach disks was compared to that achieved by the traditional disinfection agent, sodium hypochlorite. Finally, the third experiment was a sensory study, carried out in order to establish whether or not citric acid could constitute a feasible alternative in the washing and disinfection step of spinach leaves minimally processed.

2.1. Microbial studies

2.1.1. Raw material, sample preparation, and initial disinfection

Spinach leaves (*Spinacia oleracea* L.) were directly obtained from local markets in Buenos Aires city, Argentina. Leaves without visual defects were sampled with a circular cutting edge (d = 60 mm) and weighed, each disc constituted an experimental unit (1.06 ± 0.03 g). With the purpose of reducing native microbiota each disc was disinfected with sodium hypochlorite 200 ppm at 25 °C. The procedure was the following: experimental units were placed in falcon tubes (50 mL) with a weight:volume of solution (w:v) ratio of 1:40, then gently shaken for 2.5 min, and finally air dried in tissue paper and placed in sterile Petri dishes.

2.1.2. Bacterial strains and culture conditions

Two strains of *Escherichia coli* (ATCC® CRM-8739™ and ATCC® PTA-3526™) and one of *Listeria innocua* (ATCC® 33090™) were grown in 100 mL of Tryptic Soy broth supplemented with 0.6% yeast extract (TSYE, Biokar Diagnostics, France), in a temperature-controlled shaker at 28 °C overnight. An aliquot (2 mL) of this preculture was transferred to a fresh TSYE broth and incubated under agitation until it reached the desired cells concentration (roughly 10^7 or 10^{10} CFU mL^{-1}). Cells concentration was determined by optical density using an UV-VIS spectrophotometer UV-1800 (Shimadzu, Japan) at 630 and 540 nm, respectively for *E. coli* and *L. innocua*; then microbial load was confirmed by plate count.

2.1.3. Spot inoculation

In the first experiment, experimental units were inoculated separately on the adaxial side with 100 μL of 10^{10} CFU mL^{-1} of each strain. Inoculum was spread on the surface of the disc by randomly depositing 20 droplets. Incubation for 3 h at 22 °C was performed in order to allow bacterial attachment. For the second experiment, the concentration of each strain was 10^7 and 10^{10} CFU mL^{-1} to reach a final count of 5–6 or 8–9 log cycles CFU per gram of fresh tissue (FT), respectively.

2.1.4. Washing/disinfection solutions

Citric acid 0.5% at 20–25 °C was prepared with anhydrous citric acid and distilled water; solutions presented a pH equal to 2.3. Sodium hypochlorite solution 200 ppm at 20–25 °C was prepared by adding commercial bleach into distilled water, its pH was adjusted to 6.5–7.0 with a solution of hydrochloric acid. Chlorine Test Papers and Free Chlorine High Range Test Strips (LaMotte®, USA) were used to check total chlorine and free chlorine, respectively. Solutions' pH was measured using a digital pH meter (Thermo Scientific Orion 3 Star, USA). Conditions tested are results of unpublished preliminary studies where processing parameters time and temperature of the washing/disinfecting solution were optimized.

2.1.5. Procedure for washing/disinfecting produce

For the first experiment, the effects of time and temperature conditions before processing on the effectiveness of citric acid (CA) and sodium hypochlorite (SH) to reduce pathogen surrogates were compared; inoculated experimental units were incubated for 24 and 48 h at 5 and 25 °C. Treatments were carried out as follows: inoculated leaves were aseptically placed in falcon tubes with a w:v ratio of 1:40, dip-washing (with CA and SH) was performed for 2.5 min with gentle shaking, and finally air dried on tissue paper. Inoculated samples that did not receive disinfection treatments were kept and analyzed as controls (C).

In the second experiment, units inoculated at two contamination loads were dip-washed by following the same procedure described previously. Both *E. coli* strains presented similar resistance to CA and SH, hence it was decided to continue the research with only one of them. Counts of *E. coli* ATCC 8739 and *L. innocua* were evaluated immediately after treatments and throughout refrigerated storage (6.5 ± 1 °C) for 9 days.

2.1.6. Microbial analysis

Each disk was aseptically homogenized for 2 min with 20 mL of sterile 0.1% peptone water (Biokar Diagnostics, France) using a vortex shaker. Next, serial dilutions were carried out using sterile 0.1% peptone water. *Escherichia coli* and *L. innocua* counts were performed using MacConkey Agar and Oxford Agar added with Oxford Selective Supplement (Biokar Diagnostics, France), respectively. Petri dishes were incubated at 37 °C for 24 h. Detection limit of the method was 2.3 log CFU.g^{-1} of FT.

2.2. Sensory study

2.2.1. Raw material, sample preparation, and treatments procedure

Raw material (*Spinacia oleracea* L.) for sensory analyses was prepared as follows: spinach leaves obtained from local markets in Buenos Aires city were transported to the laboratory in refrigerated