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EFFECT OF PREHARVEST APPLICATION OF CHITOSAN AND TEA TREE ESSENTIAL OIL ON POSTHARVEST EVOLUTION OF LETTUCE NATIVE MICROFLORA AND EXOGENOUS *ESCHERICHIA COLI* O157:H7

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ABSTRACT

The main objective was to assess the effectiveness of preharvest application of chitosan (CH) and tea tree essential oil (TT) in butterhead lettuce in controlling native microflora growth and counteract exogenous *Escherichia coli* O157:H7 contamination. TT and CH were applied to lettuce plants at 14, 10, 7, 3 and 0 days before harvest. CH showed significant reductions at harvest on mesophilic (−2.0 log cfu/g), psychrotrophic (−1.0 log cfu/g) and yeast and molds (−1.8 log cfu/g), compared with control samples. CH also reduced total coliform bacteria (−2.0 log cfu/g) compared with inoculated plants. Furthermore, CH exerted a bacteriostatic effect on *E. coli*. TT was only able to reduce total coliforms compared with inoculated plants. Preharvest application of CH exerted an inhibitory effect on lettuce native microflora and a bacteriostatic effect on exogenous *E. coli* during postharvest storage. Therefore, CH appears to be a promising method for enhancing the safety of lettuce, exposed to an inadequate postharvest handling.

PRACTICAL APPLICATIONS

Consumers demand products with less use of chemicals; therefore, there has been an increasing research for natural antimicrobials. Chitosan (CH) and essential oils, such as tea tree (TT), have become promising alternatives to chemical decontamination because of its natural character, antimicrobial activity and elicitation of defense responses in plant tissue. The objectives of this work were to examine the residual effectiveness of CH and TT solutions, applied during preharvest, to control *E. coli* O157:H7 inoculated in lettuce heads, simulating an inadequate manipulation of the vegetable at postharvest. CH solution was a good alternative for controlling not only the native microflora in lettuce during storage, but also reducing the survival of *E. coli* inoculated in the vegetable at harvest. Preharvest application of CH in the late stages of lettuce head development could easily be introduce in the good agricultural practice routine, without consequences on the environment because CH is a generally recognized as safe substance.

INTRODUCTION

Lettuce is one of the most popular vegetables in the world and it is preferably consumed fresh and in salads dishes (Wieβner *et al.* 2009). Because its consumption has increased significantly, unintended consequences have also developed (Huang and Chen 2011). Lettuce consumption has been implicated in a large number of *Escherichia coli* O157:H7 outbreaks (Moreira *et al.* 2007). Therefore, this pathogen is clearly of public health concern because it is the second most important causal agent of outbreaks from fresh produce (Gomes *et al.* 2009).

The mechanisms by which *E. coli* O157:H7 is introduced into lettuce plants are not fully understood. Fresh produce can become contaminated during harvest and at postharvest stages because of workers' poor hygiene and low sanitation in the processing plant (Goldberg *et al.* 2011). Therefore, it is very important to consider the possibility of lettuce contamination with *E. coli* O157:H7 during postharvest processing by inadequate manipulation and poor sanitation.

In general, the earliest the contamination by the pathogen, the more difficult is its decontamination. It is important to decontaminate fresh produce as soon as possible, as pathogens could be firmly attached or internalized in leaf portions unreachable for the decontamination agent (Delaquis *et al.* 2007; Goldberg *et al.* 2011). The low effectiveness of surface sanitizing agents is likely dependent on whether the target microorganisms are readily accessible or not (Gomes *et al.* 2009). *E. coli* O157:H7 cells have shown ability to penetrate into the stomata and junction zones of cut lettuce, becoming trapped between 20 and 100 μm below the cut edge surface. Research suggests that surface sanitizing of lettuce after harvest is not an effective method to completely eliminate *E. coli* O157:H7 cells (Beuchat 1999; Gleeson and O'Beirne 2005). Under those circumstances, even a low level of contamination could present a health risk, as *E. coli* O157:H7 infective dose is less than 10^3 cells (Taormina and Beuchat 1999; Alegre *et al.* 2010).

Although current techniques used by fresh vegetable processing industry have improved the overall quality and extended the shelf life of these products, safety is still an issue of concern (Cliffre-Byrnes and O'Beirne 2005; Gleeson and O'Beirne 2005; Francis *et al.* 2012). On the other hand, consumers demand less use of chemicals on vegetables; therefore, more attention has been paid to the search for natural antimicrobials (Roller 2004; Moreira *et al.* 2009).

In this context, chitosan (CH) has become a promising alternative treatment for fruits and vegetables decontamination because of its natural character, antimicrobial activity and elicitation of defense responses in plant tissue. CH, as a natural biopolymer, has several advantages, such as safety, availability from renewable resources, biocompatibility and biodegradability, leading to ecological safety (Moreira *et al.* 2011a,b). As a postharvest biopreservative, CH extends the shelf life of treated fruits and vegetables. Specifically in relation to their use in agriculture, CH has been proven to control numerous preharvest and postharvest diseases on various horticultural commodities (Bautista-Baños *et al.* 2006).

Another alternative as natural antimicrobial agents are essential oils. They have been used for various purposes for many years. Several studies have confirmed the antimicrobial activity of phytochemicals from herbs and spices such as clove, tea tree (TT), cinnamon and oregano (Burt 2004; Moreira *et al.* 2005, 2007). Among them, TT essential oil has been proven to control microbial growth *in vitro* and during postharvest storage in several studies (Moreira *et al.* 2007; Ponce *et al.* 2011; Alvarez *et al.* 2013).

Early intervention strategies have focused on preventing the pathogen contamination of the lettuce plant instead of attempting to remove them once it is already contaminated. Although CH and TT have been previously used as a postharvest preservation technology, scarce studies have reported the bactericidal or bacteriostatic effects of CH and TT applied as a preharvest intervention strategy to improve the microbiological quality during postharvest processing and storage.

The aims of the present work were to assess the effectiveness of two biopreservatives (CH and TT essential oil) applied before harvest in: (1) controlling native microflora growth in butterhead lettuce plants during the postharvest refrigerated storage; and (2) counteracting *E. coli* O157:H7 contamination during the inadequate postharvest management of the leafy vegetable by assessing the survival and growth of the pathogen.

MATERIALS AND METHODS

CH and TT Solutions

Medium molecular weight CH, with a deacetylation degree of 98%, was supplied by ACOFAR (Mar del Plata, Argentina). The solution was prepared by dispersing CH (10 g/L) in lactic acid solution (0.7% w/v), adding of Tween 80 (0.5% w/v) and glycerol maintaining glycerol/CH ratio at 0.28 (Moreira *et al.* 2011b). The pH solution was adjusted to 5.6 with 1M NaOH. To achieve complete CH dispersion, the solution was stirred for 2 h at 100 rpm and 23C in an orbital shaker (TS-1000, CHINCAN, Zhejiang, China) (Beverlya *et al.* 2008).

TT (*Melaleuca alternifolia*) essential oil was provided by Nelson and Russell (London, England). TT concentration used was three times the minimal inhibitory concentration (MIC), determined in a previous work by Moreira *et al.* (2005). MIC represents the minimal concentration that reduces *in vitro* microbial growth by 90%. The solution was prepared immediately before application to prevent the vaporization of volatile compounds responsible for its antimicrobial activity. Three MICs of TT (2.7/100 mL) were diluted in sterile distilled water and thoroughly shaken at 30C for 30 min to obtain reasonable stable dispersion (Ponce *et al.* 2011). Higher concentrations than those required at *in vitro* assays were used during preharvest application to avoid possible dilution of the antimicrobial activity of the essential oils.

Preharvest Application of CH and TT Solutions

Butterhead lettuce heads (*Lactuca sativa var. Lores*) were grown in Sierra de los Padres, Mar del Plata, Argentina. Lettuce heads were cultivated in a greenhouse with mulch technology (a black plastic film separating each plant from the soil). The assays were performed in the fall-early winter (April to August). At the greenhouse, CH and TT solutions were applied in five successive applications (14, 10, 7, 3 days previous to harvest and 0) to each plant by spray (7–10 mL/plant), taking care not to spread it to other plants. To avoid the biopreservative dissemination, a plastic cone with an upper hole was placed over each plant and the spray was applied through the cone. Lettuce plants from the same greenhouse, sprayed with distilled water but without biopreservatives, were used as control. After harvest, lettuce heads were transported to the laboratory within the hour.

Culture Maintenance and Inoculum Preparation

Approximately 2 h after harvest, lettuce heads were inoculated with *E. coli* O157:H7 strain ATCC 43895 (American Type Culture Collection: Manassas, Virginia, USA) to simulate handling contamination. Before it was used, the strain was cultured in brain–heart infusion broth (BHI, Britania, Buenos Aires, Argentina) for 24 h at 37C. Stock culture was maintained on tryptic soy broth (Britania) containing 40% glycerol (w/v) at 25C. Approximately 0.1 mL of culture was transferred to 9.9 mL of BHI at two consecutive 24-h intervals prior to each experiment to obtain optimal populations of *E. coli* O157:H7. Active *E. coli* O157:H7, ATCC 43895 cultures were centrifuged at 8,000 *g* for 12 min. The pellet was washed twice with 25 mL of 1% peptonated water and then resuspended in another 25 mL of peptonated water. The resuspended culture was added to 100 mL of peptonated water.

For inoculation, 100 μL of *E. coli* O157:H7 bacterial suspension in the form of spray (final concentration of approximately 10^4 cfu/mL) was placed on lettuce heads. Bacterial populations in the inoculum were determined by surface plating duplicate samples on eosin methylene blue (EMB) and incubation at 37C for 24 h. Only those colonies with greenish metallic brightness (*E. coli* typical colonies in EMB) were counted. Two control treatments were used: untreated and noninoculated lettuce plants (referred as control) and untreated and *E. coli* inoculated lettuce plants (referred as inoculated). CH solution was prepared with lactic acid, glycerol and Tween 80, which could affect directly microbial growth and survival. Therefore, a blank treatment was added, in which lettuce plants were treated with lactic acid (0.7% w/v), glycerol and Tween 80 (0.5% w/v).

Finally, treated lettuce heads were placed in low density polyethylene bags (40 μm) and stored at 7–8C and 98% relative humidity for 7 days to assess the biopreservative effectiveness (CH and TT) in controlling the native microflora growth and inoculated *E. coli* survival. Three independent experimental runs were performed for each treatment proposed.

Microbiological Studies

For the microbiological analysis, three heads from each treatment were sampled at different times of refrigerated storage (0, 3, 5 and 7 days), in each independent run. About 10 g of lettuce leaves from each head were macerated in 90 mL phosphate buffer solution (0.1 mol/L) and were homogenized with a stomacher 400 circulator homogenizer: Seward Medical, London, UK (pH 7.2). Serial dilutions (1:10) of each homogenized sample were made in the same diluents and surface spread in duplicate. The enumeration of the microbial populations was performed according to Ponce *et al.* (2011) using the following culture media and culture conditions: mesophilic aerobic bacteria on plate count agar incubated at 30–32C for 48–72 h; psychrotrophic bacteria on the same medium incubated at 5–7C for 5–7 days; Enterobacteriaceae and total coliforms in MacConkey agar incubated at 30–32C for 24 h; molds and yeast on yeast-glucose-chloramphenicol medium incubated at 25C for 5 days. The viable *E. coli* counts were monitored as follows: 0.1 mL sample of each treatment was spread on the surface plating on EMB and the colonies were counted after incubation at 37C for 24–48 h. All culture mediums were from Britania. Microbial counts were performed in duplicate, in three independent experimental runs.

Statistical Analysis

A completely randomized design with repeated measures was used. Time (storage time) and treatment (CH and TT solutions, along with inoculated and noninoculated controls) were defined as factors and variation source for the analysis of variance (ANOVA). Three independent experiments were performed and data were analyzed with SAS 9.0 (SAS Inc., 2001). Results presented in the present work are lsmean values and their standard deviations (Khuel 2001). Differences among treated samples were tested by ANOVA. PROC GLM (General linear model procedure) with "repeated" statement was used for the ANOVA. When significant differences were found between treatments, the Tukey–Kramer multiple comparison test was performed ($P < 0.05$).

RESULTS AND DISCUSSION

Technologies that substantially reduce or inhibit gram-negative bacteria by food-grade compounds are of

considerable interest to the food industry, as there are both public health and economic concerns. In food protection, gram-negative spoilage organisms, and pathogens such as *E. coli* O157:H7, are especially problematic because of their inherent resistance to some antimicrobials (applied preharvest or postharvest) (Belfiore *et al.* 2007). Consequently, the objectives of the present study were to examine the residual effectiveness of CH and TT solutions, applied during preharvest, to control *E. coli* O157:H7 inoculated in lettuce heads, simulating an inadequate manipulation of the vegetable at postharvest.

Figure 1 shows the postharvest evolution of mesophilic (MES), psychrotrophic (PSC) and yeast and molds (YM) during refrigerated storage of lettuce heads treated with TT and CH (preharvest application) and inoculated with *E. coli* O157:H7. CH treatment produced significant reductions on MES, PSC and YM populations compared with respective populations in control samples. No significant interactions were found for MES and YM ($P = 0.5679$ and $P = 0.2963$, respectively) while both factors (treatment and time) resulted highly significant ($P < 0.0001$). On the other hand, a significant interaction treatment–time was found for PSC evolution during postharvest storage ($P = 0.0194$). No significant differences were found for MES, PSC or YM among control and inoculated lettuce head throughout storage. Blank treatment did not produce a significant effect in any of the microbial populations studied.

Initially, CH treatment of lettuce heads resulted in significantly lower microbial counts than control, especially reducing MES (2.0–2.5 log cfu/g), which was maintained during the course of the storage (7 days) (Fig. 1a). On the other hand, the inhibitory effect of CH exerted on PSC and YM was only observed until day 3. After that, no significant differences were found in the populations of CH-treated and control plants. At day 3, PSC and YM counts in CH-treated plants were approximately 2.0 log cfu/g lower than those in control plants (Fig. 1b,c).

The antifungal and antibacterial activities of CH are related to its polycationic nature (Durango *et al.* 2006; Kim *et al.* 2011). One hypothesis states that there is an electrostatic interaction between NH_3^+ groups in CH and the phosphoryl groups of the phospholipid components of the cell membrane (Beverlya *et al.* 2008). CH also induces the expression of a variety of genes involved in plant defense responses that result in increased synthesis of secondary plant metabolites (Bitteli *et al.* 2001). Foliar application of CH also reduces stomata aperture, reducing respiration and transpiration rates (Bitteli *et al.* 2001; Farouk *et al.* 2011). Moreover, CH induces structural barriers, e.g. inducing the synthesis of lignin-like material. CH is commonly used as component of antimicrobial edible films (Devlieghere *et al.* 2004; Dutta *et al.* 2009; Gonzalez-Aguilar *et al.* 2009; Ansorena *et al.* 2011; Moreira *et al.* 2011a,b).

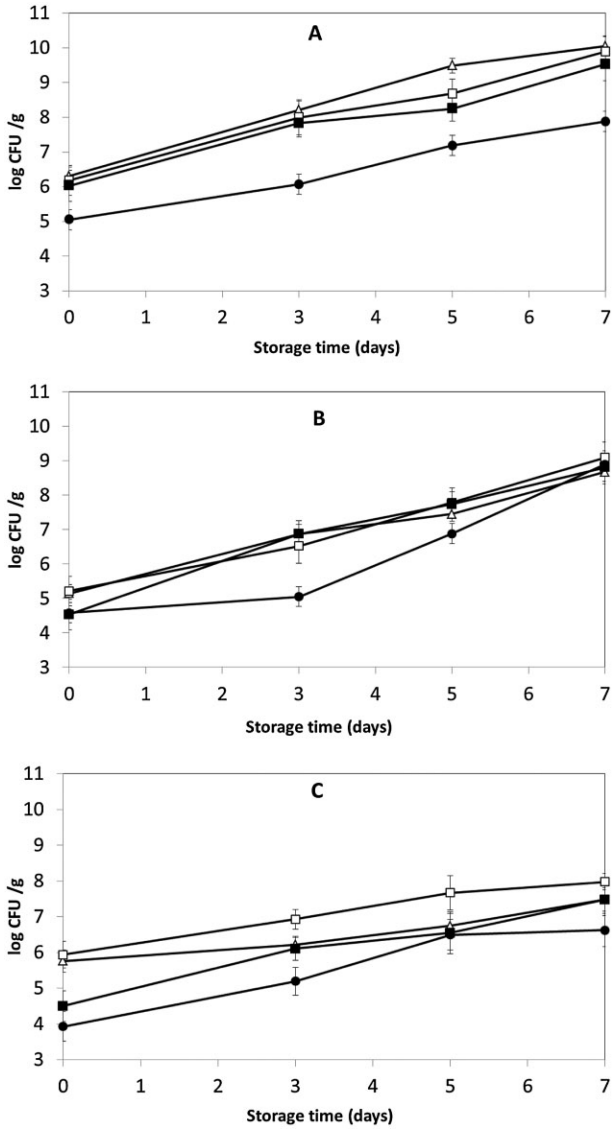

FIG. 1. POSTHARVEST MICROBIAL COUNTS EVOLUTION OF MESOPHILIC (A), PSYCHROTROPHIC (B) AND YEAST AND MOLDS (C) DURING REFRIGERATED STORAGE OF LETTUCE HEADS INOCULATED WITH *E. COLI* O157:H7 (□), PREHARVEST TREATED WITH CHITOSAN AND INOCULATED (●), PREHARVEST TREATED WITH TEA TREE AND INOCULATED (■) AND CONTROL (△)

Lettuce plant treated with TT resulted in different postharvest microbial growth, where no significant reductions in MES, PSC or YM were found, compared with control lettuce heads (Fig. 1). Even though TT reduced YM counts at harvest, that reduction was not maintained throughout the storage.

TT has gained popularity and acceptance and it has been used in a wide range of products, from pharmaceutical to food products (Cox *et al.* 2001). The chemical composition

of TT is well known and terpenoids were identified as the main active compounds (Carson and Riley 1993). TT can reduce or inhibit a wide variety of gram-negative and gram-positive bacteria, as well as yeast (Carson and Riley 1993; Cox *et al.* 2001; Moreira *et al.* 2007; Ponce *et al.* 2011). TT is a membrane-active biocide, it denatures proteins and disrupts membrane structure, leading to cytoplasmic leakage, interfering with the microbial enzyme systems and ultimately leading to cell death (Cox *et al.* 1998; Moreira *et al.* 2005).

Figure 2 shows the total coliforms (TC) and *E. coli* count (EC) evolution in lettuce heads treated and untreated with TT and CH solutions during preharvest, inoculated and noninoculated with *E. coli* O157:H7, simulating an accidental postharvest contamination. Significant treatment–time interactions were found for both, with $P = 0.0051$ and $P = 0.0007$ for TC and EC, respectively.

It was observed that CH exerted a highly significant reduction ($P < 0.05$) on TC compared with inoculated control lettuce heads at harvest (4.2 and 6.4 log cfu/g, respectively). CH preharvest application also produced a bacteriostatic effect on TC throughout storage, with reductions of approximately 2.0 log cfu/g with respect to inoculated lettuce heads. It is worth noting that, by the end of the postharvest storage, TC were significantly lower on CH-treated plants, even compared with control lettuce plants, indicating a reduction of the endogenous TC in addition to the inoculated lettuce plants (9.3, 8.5 and 7.5 log cfu/g for inoculated, control and CH lettuce heads, respectively) (Fig. 2a). On the contrary, those lettuce heads that did not receive any preharvest treatment or inoculation (control plants) showed a significant increase in EC during refrigerated storage, from less than 2.0 log cfu/g at harvest to 4.4 log cfu/g after 7 days (Fig. 2b). Although higher initial counts of EC were found for all inoculated plants (with a mean value of 4.1 log cfu/g), during postharvest storage, an effect of the CH preharvest application on the EC survival rate was evident. While EC in inoculated lettuce heads increase from 2.0 log cfu/g at harvest to 7.2 log cfu/g at day 7, CH-treated plants maintained similar counts during the same period, reaching day 7 with a mean value of 4.3 log cfu/g. These results indicated that preharvest treatment with CH exerted a significant bacteriostatic effect on *E. coli* growth in refrigerated lettuces (7–9C) (Fig. 2b).

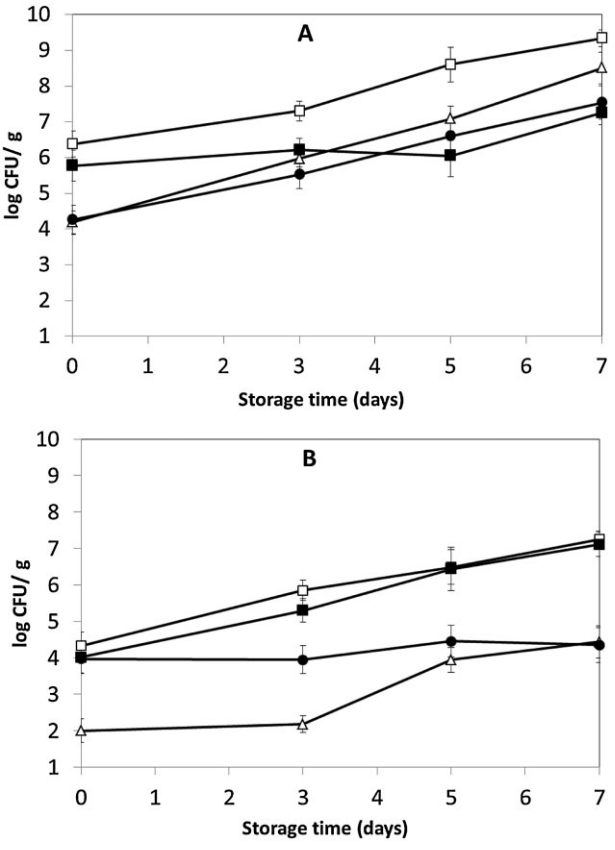

FIG. 2. POSTHARVEST MICROBIAL COUNTS EVOLUTION OF COLIFORMS (A) AND *E. COLI* (B) DURING REFRIGERATED STORAGE OF LETTUCE HEADS INOCULATED WITH *E. COLI* O157:H7 (□), PREHARVEST TREATED WITH CHITOSAN AND INOCULATED (●), PREHARVEST TREATED WITH TEA TREE AND INOCULATED (■) AND CONTROL (◆)

Internalization of *E. coli* is one of the main reasons why postharvest complete elimination of pathogen is not possible. Microbial cells can penetrate the tissue through stomata, fissures and cracks in the leaves, where it remained protected against decontamination agents. CH is a high molecular weight polymer (Badawy and Rabea 2009; Dutta *et al.* 2009) and can prevent the internalization of the microbial cells acting as a physical barrier in the access points of the leaves. CH is also known to elicit several defense responses in the host plant in response to microbial infections, including the accumulation of phytoalexins, pathogen-related proteins and proteinase inhibitors, lignin synthesis and callose formation (El Hadrami *et al.* 2010; Badawy and Rabea 2011).

Preharvest treatment with the TT solution produced lower inhibitory effects on TC and EC counts during postharvest refrigerated storage (Fig. 2). TT lettuce plants had lower TC counts than inoculated plants, with 1.1 log cfu/g reduction by day 3 and 2.3 log cfu/g reduction for the final days of storage (day 5 and 7). However, preharvest application of TT did not exert any inhibitory effects on EC counts during storage, indicating limited residual antimicrobial activity against *E. coli* O157:H7 (Fig. 2).

In the present work, TT used as a preharvest antimicrobial agent did not control native microflora or exogenous *E. coli* growth. Low effectiveness was observed for this biopreservative during postharvest storage. These results are in conflict with previous studies of TT when it was used

in vitro or as a postharvest biopreservative. Ponce *et al*. (2003) reported a significant antimicrobial effect of TT when applied postharvest on organically produced Swiss chard. Also, Ponce *et al*. (2011) reported that TT, applied in different forms (spray, immersion), exerted a significant inhibitory effect on different microbial populations present in lettuce leaves, during refrigerated storage.

Nevertheless, TT antimicrobial activity appears significantly reduced when applied preharvest and during refrigerated storage. Most essential oils and their active compounds are highly volatile. Therefore, the application of essential oils as antimicrobials in food is often discouraged because of the potential loss of antimicrobial action because of their volatility and lipophilicity (Bagamboula *et al*. 2004). This statement could explain the lack of long-term antimicrobial activity of TT in the present study. Higher doses of TT essential oil could be studied in the future taking into consideration the sensory effect on the plant because of the characteristic flavor and odor of the essential oil, which may result in the rejection from the consumers' point of view.

Results obtained in this work indicate that a CH solution was a good alternative for controlling not only the microorganisms present in lettuce heads during postharvest storage, but also reducing the survival of *E. coli* inoculated in the vegetable at harvest. The reductions on EC obtained throughout refrigerated storage of lettuce heads treated with successive doses of CH during the preharvest may be attributed to the residual antimicrobial actions of this biopreservative. In agreement with our results, Dutta *et al*. (2009), Moreira *et al*. (2011a,b) and Alvarez *et al*. (2013) reported that CH applied postharvest inhibited various spoilage and pathogenic bacteria. This antimicrobial effect of CH could be attributed to its ability to bind water and inactivate enzymes and also to its ability to chelate nutrients therefore competing with the bacteria present (Devlieghere *et al*. 2004; Ansorena *et al*. 2011).

Preharvest application of CH could be presented as a potential technology that is capable of reducing the microbial population present in leafy vegetables. The spray application of the CH solution could be considered practical and easy to implement because it does not require expensive or complex equipment. Therefore, it presents a viable natural alternative from commercial antimicrobials to significantly reduce the microbial populations present in lettuce at harvest and to control the native microflora during postharvest storage.

In conclusion, preharvest application of CH solution exerted a significant inhibitory effect on the native microflora of butterhead lettuce heads. Moreover, CH solutions showed a bacteriostatic effect on *E. coli* inoculated in the vegetable. On the other hand, TT essential oil application showed no significant reduction of lettuce native microflora throughout postharvest refrigerated storage and it did not manage to reduce exogenous *E. coli* growth. Therefore, CH appears to be a promising method for enhancing the safety of lettuce heads, exposed to an inadequate postharvest handling. Preharvest application of CH in the late stages of lettuce head development (14, 10, 7, 3 and 0 days before harvest) could easily be introduced in the good agricultural practice routine, without consequences on the environment as CH is a generally recognized as safe substance.

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