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COMBINED EFFECT OF BIOACTIVE COMPOUNDS AND STORAGE TEMPERATURE ON SENSORY QUALITY AND SAFETY OF MINIMALLY PROCESSED CELERY, LEEK AND BUTTERNUT SQUASH

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ABSTRACT

In this work, the combined effect of bioactive compounds (tea tree essential oil, propolis extract and gallic acid) and storage temperature (15 and 5C) on microbiological and sensory quality of fresh-cut celery, leek and butternut squash was studied. It was confirmed that a good control of refrigeration temperature limits the growth of spoilage and pathogenic microorganisms. Tea tree essential oil and propolis showed significant effectiveness in controlling the microbiota present in vegetables. Also, both endogenous *Escherichia coli* and inoculated *E. coli* O157:H7 counts were significantly reduced in vegetables by the combined application of propolis and tea tree essential oil with optimal refrigeration temperature. Propolis improved visual quality extending the sensory shelf life of celery and butternut squash at 5C. Similar improvement effects were shown by tea tree essential oil when applied on leek and squash, at 5C. These findings strongly suggest that propolis and tea tree essential oil have potential to be good preservatives improving microbiological and sensory quality of celery, leek and squash.

PRACTICAL APPLICATIONS

The industry of fresh-cut produce confronts new challenges such as the development of technologies that allows the maintenance of fresh produce quality and extends its shelf life to reach more distant markets, ensuring the safety of products to protect the health of consumers. Thus, the development of natural food preservatives in the field of fresh produce responds to consumer demands of healthy and natural products. The findings reported in this work strongly suggest that propolis and tea tree essential oil have potential to be good preservatives improving microbiological and sensory quality of celery, leek and squash.

INTRODUCTION

As a response to consumers' demand for healthy, fresh-like and easy to prepare products, along with consumer lifestyle changes, a wide variety of minimally processed fruits and vegetables have been developed. However, mechanical operations during minimal processing impair vegetables tissues and cause the release of intracellular contents, which in turn limits the shelf life of products (Moreira *et al.* 2011).

The main problem that makes fresh-cut vegetables a highly perishable product is the ease of microbial growth. These operations commonly support and increase the activity of pathogenic and saprophytic microorganisms. These facts explain the need to develop new technologies that reduce deterioration and safety problems of vegetables. The interest in the possible use of natural compounds to prevent microbial growth has notably increased in response to consumer awareness when using chemically synthesized additives in foods (Moreira *et al.* 2011). Further investigations are necessary to evaluate the potential of plant extracts and essential oils (EOs) and their active constituents as natural preservatives to improve the shelf life and the safety of minimally processed commodities (Ponce *et al.* 2011; Ramos *et al.* 2013).

A large variety of natural antimicrobials was used in raw and processed fruits and vegetables in order to extend their shelf life, to reduce or eliminate pathogenic bacteria and to improve overall quality (Burt 2004; Ponce *et al.* 2011). The biological properties of tea tree EO and propolis, including antimicrobial, antioxidant, antiviral and antiproliferative activities, have been demonstrated by many researchers (Isla *et al.* 2005; Giordani *et al.* 2006). Tea tree EO is composed by more than 100 different compounds, including terpinen-4-ol, which is one of the main antibacterial components (Carson *et al.* 2006). Likewise, propolis has a complex composition, containing more than 180 constituents and its antimicrobial properties are attributed to phenolic compounds, mainly flavonoids (Castaldo and Capasso 2002).

On the other hand, the most important tools for extending the shelf life of the fresh vegetables are low temperatures and high relative humidity (RH) control. Temperature is the single most important variable, and its improper manipulation causes evident changes in the sensory characteristics of fresh vegetables that conditions consumer's acceptability. However, such low temperature is not always maintained throughout the entire cold chain in some countries, where wholesalers and retailers generally keep produce slightly above the recommended temperature so as to save cost or lack of energy and refrigeration equipments (Moreira *et al.* 2006).

As far as it is concerned, there are no reports showing the effect of natural antimicrobials, such as tea tree, propolis and gallic acid, applied on minimally processed celery, leek and butternut squash to preserve the safety and quality. Therefore, the aim of this study was to investigate the effect of different bioactive compounds (BC; tea tree EO, propolis extract and gallic acid) and storage temperatures (optimal, 5C, and suboptimal, 15C) on the microbiological and sensory quality of vegetables (celery, leek and butternut squash). The effect of BC on the survival and growth of *E. coli* inoculated in the vegetables (simulating an inadequate postharvest management) was also evaluated.

MATERIALS AND METHODS

BC

Biopreservatives used in this work were tea tree (*Melaleuca alternifolia*) EO purchased from Nelson and Russell (London, UK), commercial propolis extract (Juricich, Mendoza, Argentina) and gallic acid (Sigma-Aldrich, Buenos Aires, Argentina). Tea tree EO was extracted by steam distillation from tea tree leaves and the main compound determined by gas chromatography-mass spectrometry was terpinen-4-ol (30%). Other minor constituents detected were γ-terpinene, α-terpinene and p-cymene (data not shown). Propolis extract was prepared from raw material collected in Mendoza (Argentina) province and the product was standardized to 10% propolis extract (dark brown color; total phenolic content 18.8 mg gallic acid equivalents per milliliter).

Sample Preparation

Celery (*Apium graveolens* L.), leek (*Allium porrum* L.) and butternut squash (*Cucurbita moschata* D.) cultivated in the open field were harvested in the early morning and immediately transported to the laboratory, in refrigerated containers. Squashes of uniform size and color were selected and then were peeled, washed thoroughly with tap water and a stainless steel hand slice was used to prepare diced squash (ca. 15 mm). Celery petioles and leeks were also washed and sliced (10 mm thickness). Processed vegetables were dipped in tap water (3 min) and the surface moisture was removed with a manual salad centrifuge.

Culture Maintenance and Inoculum Preparation

E. coli O157:H7, ATCC 43895 provided by CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, La Plata, Argentina) was used. A stock culture was maintained in tryptic soy broth (Britania, Buenos Aires, Argentina) at 4C. Before being used, *E. coli* O157:H7 was cultured in brain heart infusion broth (BHI; Britania, Buenos Aires, Argentina) for 24 h at 37C. A 0.1 mL aliquot of the culture was transferred to 9.9 mL of BHI broth at two consecutive 24 h intervals followed by incubation at 37C before each experiment. A bacterial suspension (approximately 10^8 colony forming unit [cfu]/mL) was prepared by adding 10 mL of the *E. coli* culture to 90 mL of sterile 1 g/L peptone water.

BC Application and Inoculation of Vegetables

BCs were added to celery, leek and butternut squash at selected concentrations according to previous *in vitro* antimicrobial assays. Thus, the concentrations of BC solutions applied were 30 μL/mL for tea tree EO, 64 μL/mL for propolis and 2 mg/mL for gallic acid. To prepare these solutions, BCs were diluted in sterile distilled water and vigorously shaken at 30C for 30 min to obtain reasonably stable dispersions. Minimally processed vegetables were hand-sprayed with the BC solutions (4 mL/300 g fresh-cut vegetable). In control samples, vegetables were sprayed with sterile distilled water. After being treated, vegetable samples were immediately inoculated with *E. coli* O157:H7. To carry this out, the bacterial suspension previously prepared was sprayed (1 mL) on fresh-cut vegetables (celery, leek and butternut squash) to reach a final pathogen concentration of approximately 5.5 log cfu/g. Control samples were noninoculated vegetables and untreated vegetables inoculated with *E. coli*. Finally, the fresh-cut vegetables (with or without pathogen inoculation) were placed in open plastic containers (60 g), covered with a 15 μm polyethylene wrap and sealed. These containers were placed in holding boxes at RH of 95% and at two storage temperatures (15 and 5C) for a maximum of 7–21 days, respectively. Three containers per treatment were removed from storage and used for microbiological analysis and two containers for sensory evaluation, at each sampling time. The experiment was conducted twice.

Microbiological Analysis

Microbial counts were determined within 1–2 h of treatment application and after 2, 5, 7 and 10 days of storage at 5 and 15C where three replicates were used. Microbiological analysis (mesophilic psychrotrophic *Enterobacteriaceae* and total coliforms, molds and yeasts) was developed according to Moreira *et al.* (2011). *E. coli* counts were determined using eosin methylene blue (EMB) agar and the colonies were counted after incubation at 37C for 24–48 h. EMB is a selective medium that allows the characterization of typical *E. coli* colonies; those that were dark centered, flat and with a metallic sheen were taken into account. Randomly, selected *E. coli* colonies were confirmed using an *E. coli* chromogenic test kit (Chromobrit, Britania). All culture mediums were purchased from Britania.

Sensory Analysis and Shelf Life

Overall Visual Quality Assessment. At each storage time, two individual containers of celery, leek and butternut squash were subjected to sensory evaluation by a panel of testers; nine members of the UNMdP Food Engineering Group, aged 30–50 years with sensory evaluation experience in vegetable quality was trained and carried out the evaluation of celery, leek and butternut squash quality. Preliminary tests were carried out to identify those defects most likely to appear due to prolonged storage of fresh-cut butternut squash, celery and leek. Then, the panel defined overall visual quality (OVQ) as a critical sensory attribute to be evaluated on vegetables, and also agreed on the methods for assessing this attribute. OVQ included attributes visually perceived as freshness, surface brightness, uniformity of color and texture. For the experiment, the coded (three digits) samples were presented one at the time randomly to the members who sat at a round table and made independent evaluations. Evaluations were performed under artificial daylight-type illumination, at room temperature (22–24C). The intensity of OVQ attribute was quantified on a 5-cm unstructured intensity scale. OVQ was scored from 0 (highly deteriorated aspect) to 5 (fresh aspect). The limit of acceptance was 2.5 (value corresponding to 50% of the scale), indicating that a score below this limit was deemed to indicate end of shelf life (Piagentini *et al.* 2005).

Mathematical Modeling of Quality Changes. The following general equation may be used to describe the rate of quality changes in foods:

$$\pm\frac{dQ}{dt} = k_q[Q]^n$$

where Q is a quality attribute, t is the time, n is the reaction order and k_q is the quality change rate constant for the attribute Q. The sign (+) refers to attributes with increasing values during time and the sign (−) to attributes with decreasing values (as OVQ or general appearance) (Piagentini *et al.* 2005). Traditionally, quality change processes of foods stored under controlled environmental conditions are described with zero-order ($n = 0$) and/or first-order ($n = 1$) rate functions (Labuza 2000).

The regression analysis was used to determine the kinetic order of the visual quality change rate. An analysis of the coefficient of determination (R^2) and residuals (differences between observed and fitted data) was performed to select the adequate models. Finally, the obtained fitting models were used to estimate sensory shelf life period values (t_s), when the OVQ attribute exceeded its limit of acceptability (score below 2.5).

Statistical Analysis

The results showed in this study are expressed as mean values obtained from population data previously transformed to $\log_{10}$ scale, with their standard deviations. Experiments were developed at two storage temperatures (5 and 15C) and were established with two factors (antimicrobial treatment and storage time) using a completely randomized design. Antimicrobial treatment was defined in four levels (control, tea tree EO, propolis extract and gallic acid) and storage time in five levels (0, 2, 5, 7 and 10 days). Analysis of variance was applied to each factor. Differences between means were evaluated by Tukey's multiple comparison test. Wherever differences are reported as significant, a 95% confidence level was used (Khuel 2001). OVQ data were fitted to the corresponding models (zero and first order) and the regression analyses were carried out. The 95% confidence intervals of estimated parameters were calculated. Data were analyzed using R 2.12.2 statistical software (R Foundation for Statistical Computing, Vienna, Austria) and InfoStat 2013 (Universidad Nacional de Córdoba, Argentina).

RESULTS AND DISCUSSION

Evolution of Native Microflora in Treated and Untreated Vegetables during Storage at Optimal and Abusive Temperatures

The antimicrobial effects of tea tree EO, propolis extract and gallic acid on the native microflora growth of different vegetables are shown in Figs. 1 (celery), 2 (leek) and 3 (butternut squash) at two storage temperatures: 15 and 5C.

Figure 1A–C shows the evolution of total mesophilic aerobes, psychrotrophics and yeast and molds of minimally processed celery treated with BC during storage at 5 and 15C. It was observed that the growth rate of mesophilic bacteria in minimally processed celery stored at 15C was significantly ($P < 0.05$) higher than in those samples stored at 5C. For celery samples stored during 7 days at 15 and 5C, significant differences ($P < 0.05$) were observed in microbial counts (1.6–2 log cfu/g lower in samples stored at 5C). When tea tree EO and gallic acid were applied to control the celery native microflora growth, they did not produce any inhibitory effects on mesophilic, psychrotrophic and yeast and mold counts during all the storage period (7 days) at 15C. However, propolis slightly reduced mesophilic, psychrotrophic and yeast and mold counts (0.5–0.7 log cfu/g reductions) between days 5–7 of storage, compared with control sample. On the other hand, when treated samples were stored at 5C, the BC did not show any significant inhibitory effect until day 7 of storage; only propolis extract showed a slight inhibitory effect (0.7 log cfu/g reduction) on yeast and mold counts up to 10 days of storage (Fig. 1C).

Figure 2A–C shows microbial evolutions in minimally processed leek treated with BC and stored at 15 and 5C. Similar differences compared with those detailed for celery were seen in all microbial counts corresponding to leek stored at 15 and 5C (1.2–1.5 log cfu/g lower in samples stored at 5C, at day 7) (Fig. 2A–C). When minimally processed leek was treated with tea tree EO, propolis and gallic acid and stored both at 5 and 15C, the BC did not produce any inhibitory effect on mesophilic, psychrotrophic and yeast and mold counts during all the storage period. The only exception was that tea tree EO treatment produced a significant reduction ($P < 0.05$) on psychrotrophic and yeast and mold counts, compared with control sample, at the end of storage at 5C (Fig. 2B,C).

The antimicrobial effects of tea tree EO, propolis extract and gallic acid on the native microflora growth of minimally processed butternut squash stored at 15 and 5C are shown in Fig. 3A–C. Psychrotrophic and yeast and mold populations (Fig. 3B,C) on squash samples stored at 15C showed similar growth patterns than mesophilic population (Fig. 3A). At day 5 of storage, final mesophilic, psychrotrophic and yeast and mold counts were significantly higher (approximately 2.5 log cfu/g) in samples stored at 15C compared with those stored at 5C (Fig. 3A–C). When tea tree EO, propolis and gallic acid were applied to control native microflora growth in butternut squash stored at 15C, they did not produce any inhibitory effect on mesophilic and psychrotrophic counts during all storage period (7 days). However, gallic acid and tea tree EO significantly reduced ($P < 0.05$) yeast and mold counts (1.0–1.3 log cfu/g reductions compared with control) at day 7 of storage at 15C. Furthermore, when minimally processed squash was treated with tea tree, this treatment significantly reduced (0.7–1.0 log cfu/g) mesophilic and psychrotrophic counts after the application and throughout the storage period at 5C.

Ragaert *et al.* (2007) reported total microbial counts on fresh-cut vegetables after minimal processing in the range from 3.0 to 6.0 log cfu/g. Accordingly, in the present study, initial counts of mesophilic aerobic microorganisms and yeast and molds in minimally processed celery, leek and butternut squash were in the same range. Furthermore, Vandekinderen *et al.* (2009) reported initial mesophilic population of minimally processed leek in the range of 5.6 and 7.3 log cfu/g. This high initial microbial load could be attributed to pre-harvest contamination due to direct contact with soil through which bacteria have the possibility to form biofilms, to attach or to infiltrate into vegetable tissues. In the same way, Roura *et al.* (2004) found that diced butternut squash contained initial mesophilic bacterial counts of 4.8 log cfu/g. The microbial load of squash is expected to be lower than that of vegetables with stems and leaves as the squash's shell protects the fruit against microbial infiltration. With regard to celery, Vandamm *et al.* (2013) reported total aerobic bacterial counts ranging from 6 to 10 log cfu/g in fresh-cut celery ready to eat for sale in a supermarket.

Several authors reported that a good control of refrigeration temperature limits the growth of spoilage and pathogenic microorganisms in minimally processed fruits and vegetables (Zhan *et al.* 2012). However, adequate storage temperature is not always maintained throughout the entire cold chain (Olaimat and Holley 2012).

In a previous study, Alvarez *et al.* (2013) reported that tea tree EO and propolis extract exerted a bacteriostatic effect on mesophilic and psychrotrophic populations, when applied on minimally processed broccoli (stored at 5–7C).

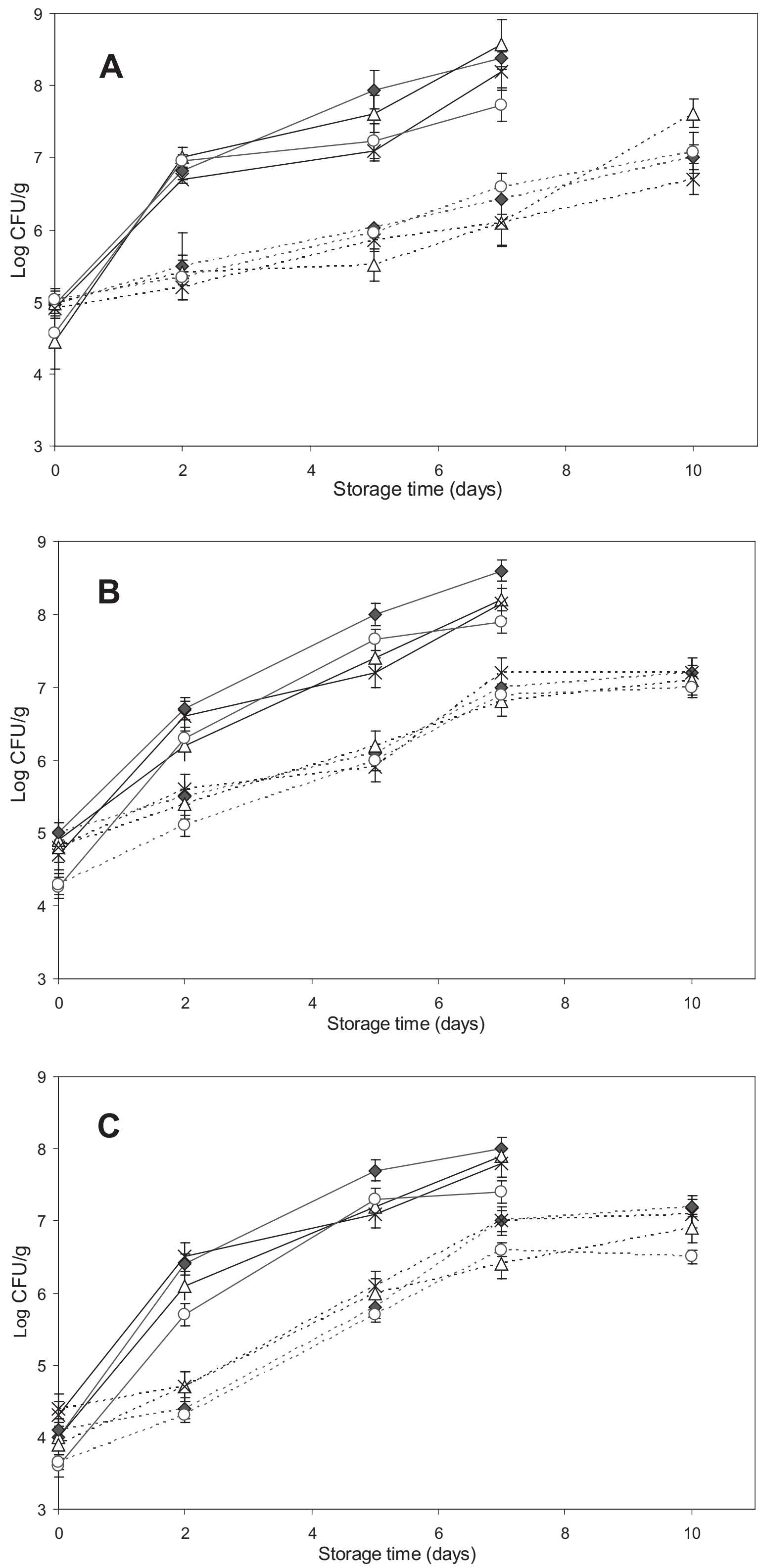

FIG. 1. EVOLUTION OF (A) MESOPHILIC AEROBES, (B) PSYCHROTROPHICS AND (C) YEAST AND MOLDS IN MINIMALLY PROCESSED CELERY TREATED WITH BIOACTIVE COMPOUNDS DURING STORAGE AT 15C (WHOLE LINES) AND 5C (DOTTED LINES); (◆) CONTROL; (△) TEA TREE ESSENTIAL OIL; (○) PROPOLIS; (X) GALLIC ACID

Data represent mean values ($n = 6$) and vertical bars represent standard deviation.

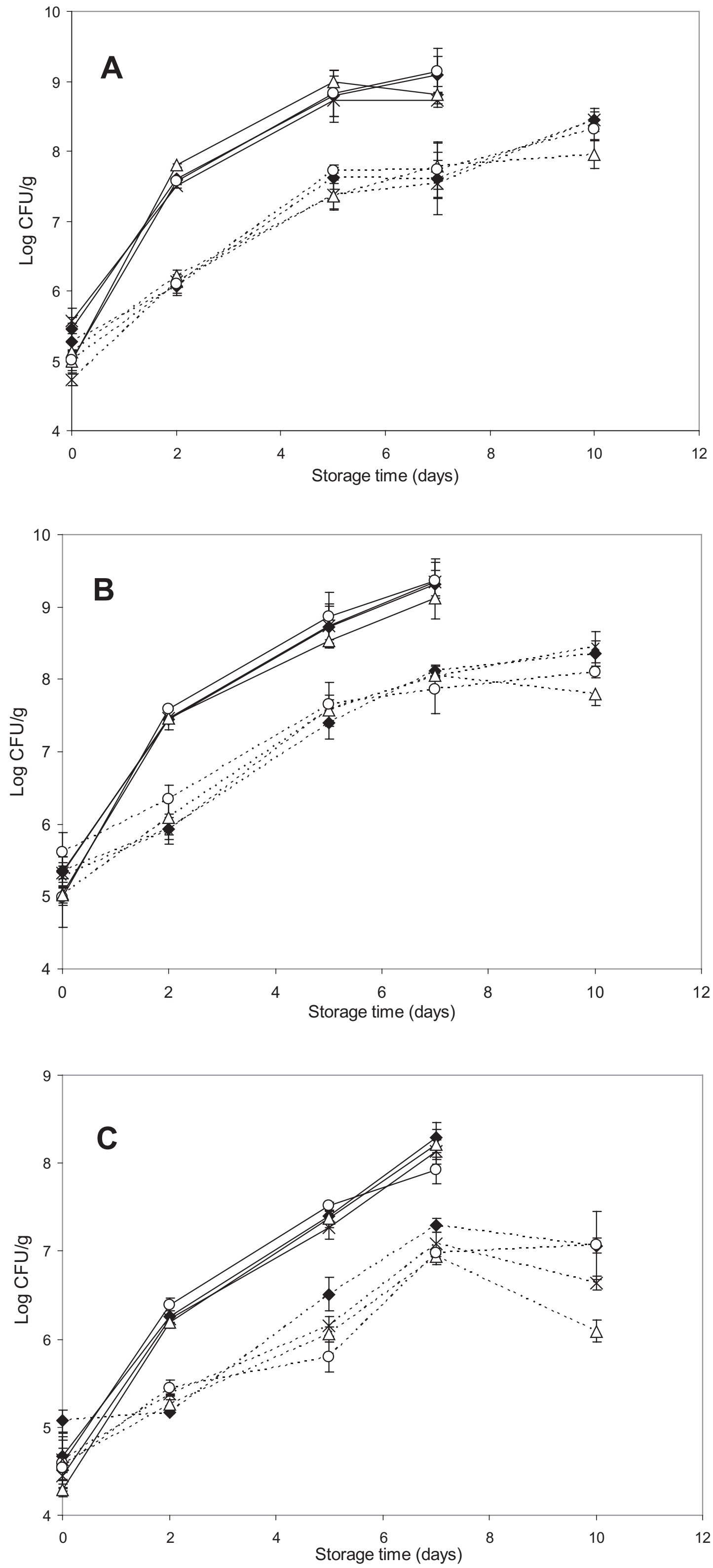

FIG. 2. EVOLUTION OF (A) MESOPHILIC AEROBES, (B) PSYCHROTROPHICS AND (C) YEAST AND MOLDS IN MINIMALLY PROCESSED LEEK TREATED WITH BIOACTIVE COMPOUNDS DURING STORAGE AT 15C (WHOLE LINES) AND 5C (DOTTED LINES); (◆) CONTROL; (△) TEA TREE ESSENTIAL OIL; (○) PROPOLIS; (X) GALLIC ACID

Data represent mean values ($n = 6$) and vertical bars represent standard deviation.

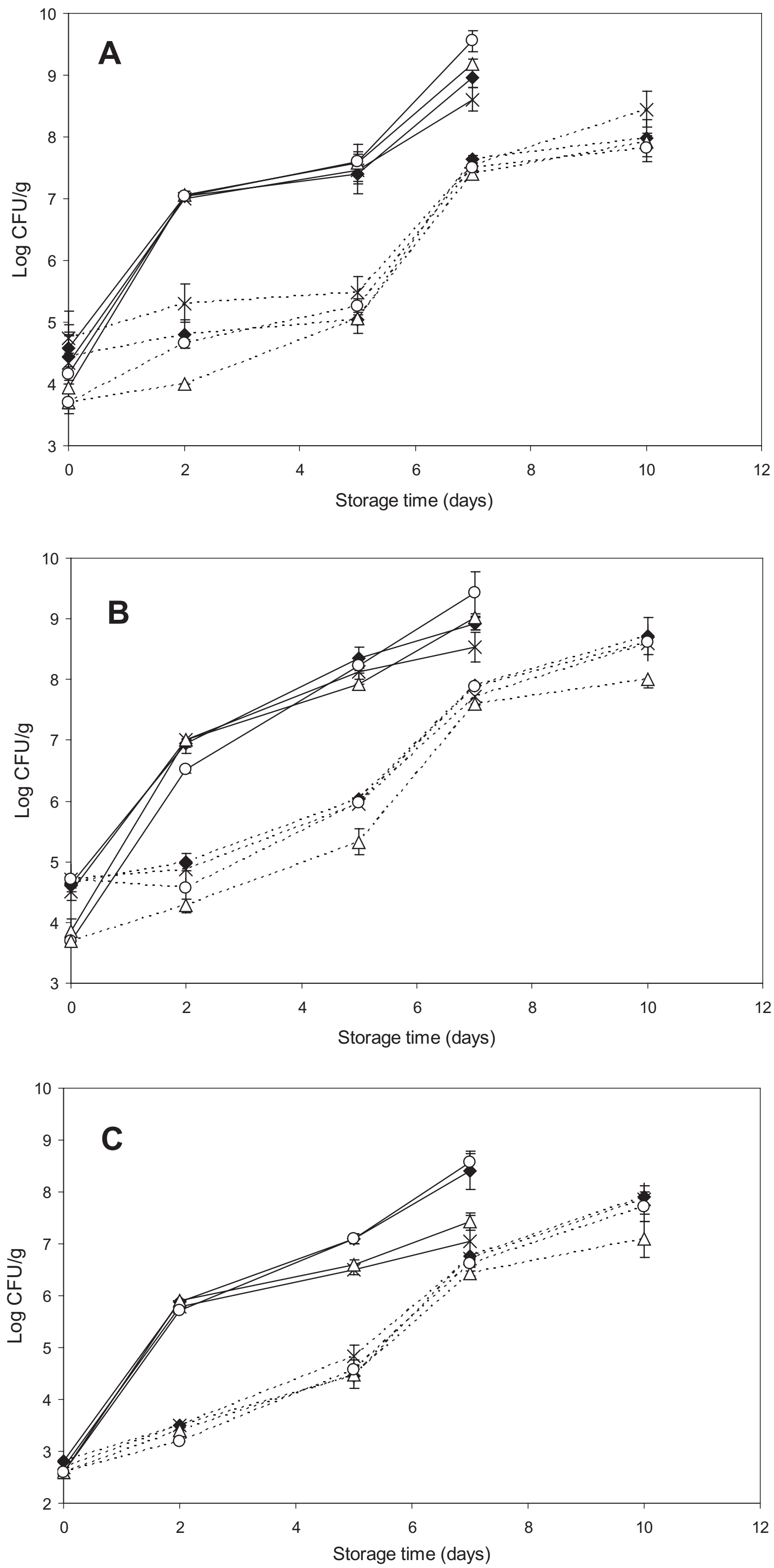

FIG. 3. EVOLUTION OF (A) MESOPHILIC AEROBES, (B) PSYCHROTROPHICS AND (C) YEAST AND MOLDS IN MINIMALLY PROCESSED BUTTERNUT SQUASH TREATED WITH BIOACTIVE COMPOUNDS DURING STORAGE AT 15C (WHOLE LINES) AND 5C (DOTTED LINES); (◆) CONTROL; (△) TEA TREE ESSENTIAL OIL; (○) PROPOLIS; (X) GALLIC ACID
Data represent mean values ($n = 6$) and vertical bars represent standard deviation.

On the contrary, in a previous work we found that these biopreservatives were not effective in controlling mesophilic bacteria growth on mixed vegetables for soup stored at 5C (Alvarez *et al.* 2015). In the present work, we observed that tea tree EO and propolis extract exerted a significant inhibitory action on the native microflora growth in minimally processed celery, leek and butternut squash. Although in all experiments BC were applied by spraying, and the concentrations used were similar, it is clear that the type of substrate greatly influenced the effectiveness of treatments applied. Differences between vegetable substrates regarding native microflora, chemical composition, interactions with added BC and the amount of cut surface are some of the reasons that might explain the differences in the obtained results (Gutierrez *et al.* 2008; Juneja *et al.* 2012; Davidson *et al.* 2013).

Propolis is constituted by a wide variety of substances such as polyphenols, quinones, coumarins, steroids, amino acids and inorganic compounds. Most propolis components are of phenolic nature, mainly flavonoids (Tosi *et al.* 2007). It is known that simple phenols, phenolic acids and polyphenols are active antimicrobial agents; moreover, many authors have attributed propolis antimicrobial capacity to the high level of flavonoids (Castaldo and Capasso 2002; Popova *et al.* 2007). Particularly, flavan-3-ols (e.g., catechins) and flavonols (e.g., myricetin and quercetin) are the most effective flavonoids due to their broad-spectrum antimicrobial action (Daglia 2012). At present, there are few publications describing the use of propolis as a natural preservative in vegetables. Recently, Feás *et al.* (2014) demonstrated that a propolis treatment (by immersion using 2% aqueous solution) applied on minimally processed lettuce was able to reduce 1.4, 1.6 and 0.8 log cfu/g of the initial load of mesophilic bacteria, psychrophilic and fecal coliforms, respectively. Furthermore, Ordóñez *et al.* (2011) demonstrated the ability of a hydroalcoholic extract of propolis to control the development of pathogenic bacteria such as *Pseudomonas syringae* in tomatoes and prevent microbial spoilage. In another study, Pastor *et al.* (2011) evaluated the effectiveness of hydroxypropyl methylcellulose coatings (HPMC) enriched with a propolis extract to preserve microbiological and sensory quality of grapes. The results indicated that the HPMC coating with 1.5% of propolis slightly reduced the growth of mesophilic bacteria and yeasts and molds during refrigerated storage as compared with uncoated control.

With regard to *in vivo* application of tea tree EO, Ponce *et al.* (2004a) reported that this antimicrobial was found to be effective in inhibiting the growth of indigenous microflora of Swiss chard leaves throughout 14 days of storage at 5C. These results are in accordance with those obtained in our study, mainly when tea tree EO was applied on fresh-cut butternut squash.

Evolution of Enterobacteriaceae and *E. coli* Populations in Treated Vegetables Noninoculated and Inoculated with *E. coli* O157:H7 during Storage at Optimal and Abusive Temperatures

Figure 4A,B shows the evolution of *Enterobacteriaceae* and endogenous *E. coli* counts in celery samples treated with BC, without pathogen inoculation and stored at 15 and 5C. No significant differences ($P > 0.05$) were observed in enterobacterial and endogenous *E. coli* counts between celery samples treated with tea tree EO and gallic acid compared with control sample, at both storage temperatures. However, a significant initial reduction (1.0–1.5 log cfu/g) of enterobacterial and endogenous *E. coli* counts was observed when propolis extract was used as biopreservative on minimally processed celery. These reductions were maintained up to the end of storage at 15C (day 7). On the other hand, in all treated and untreated samples stored at 5C, an initial bactericidal effect of refrigeration storage temperature on endogenous *E. coli* was found (reductions of 1.5–2.3 log cfu/g). Moreover, between days 7 and 10 of storage, only propolis extract exerted a slight inhibitory effect (0.5–0.7 log reductions compared with control) on endogenous *E. coli* growth. At the end of storage at 5C, enterobacterial and *E. coli* counts remained in similar values compared with day 0, while at 15C of storage these counts considerably increased (Fig. 4 A,B). Similar differences compared with those detailed for mesophilic counts in celery samples (Fig. 1A) were observed in enterobacterial and endogenous *E. coli* populations corresponding to 15 and 5C (approximately 2–2.5 log cfu/g higher in samples stored at abusive temperature).

Figure 4C,D shows the evolution of *Enterobacteriaceae* and total *E. coli* (endogenous and exogenous) populations in celery samples treated with BC and inoculated with pathogen, stored at 15 and 5C. Initial enterobacterial and total *E. coli* counts in BC treated and untreated samples were in the range of 5.8–6.1 log cfu/g remaining at these levels up to the end of storage at 5C. No differences were observed in enterobacterial and total *E. coli* counts obtained for treated and untreated celery samples along the entire storage period at 15C. However, a significant inhibitory effect ($P < 0.05$) was found on pathogen counts (0.7–1.0 log reductions) when propolis and gallic acid were used as antimicrobials on celery samples, at days 7 and 10 of storage at 5C (Fig. 4D).

Figure 5A,B shows the evolution of *Enterobacteriaceae* and endogenous *E. coli* populations in minimally processed leek treated with BC, without pathogen inoculation and stored at 15 and 5C. No significant differences ($P > 0.05$) were observed in enterobacterial and endogenous *E. coli*

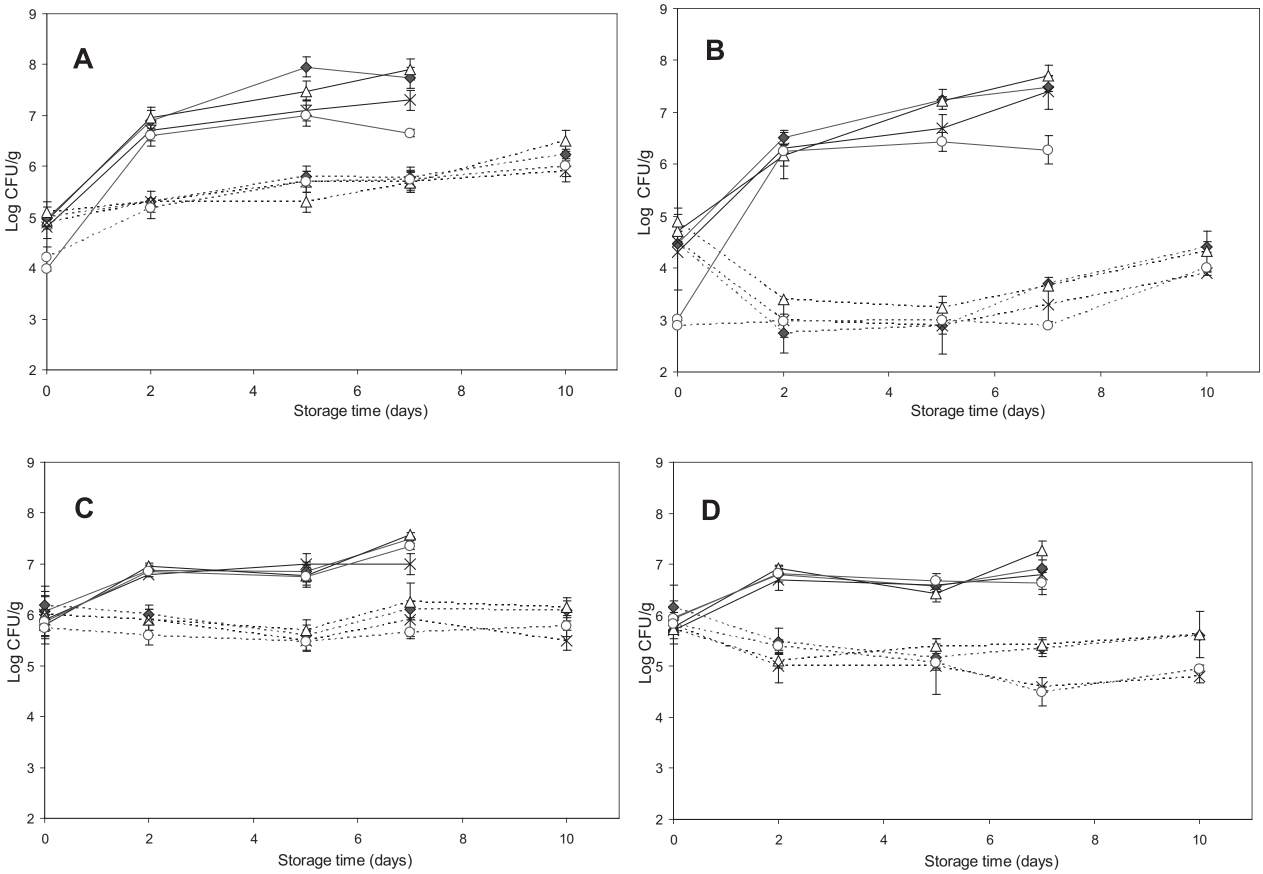

FIG. 4. EVOLUTION OF ENTEROBACTERIAL AND *E. COLI* POPULATIONS IN TREATED CELERY WITHOUT PATHOGEN INOCULATION (A AND B, RESPECTIVELY) AND IN TREATED CELERY WITH PATHOGEN INOCULATION (C AND D, RESPECTIVELY) STORED AT 15C (WHOLE LINES) AND 5C (DOTTED LINES); (◆) CONTROL; (△) TEA TREE ESSENTIAL OIL; (○) PROPOLIS; (X) GALLIC ACID
Data represent mean values ($n = 6$) and vertical bars represent standard deviation.

counts between treated and untreated leek samples, at both storage temperatures.

Figure 5C,D shows the evolution of *Enterobacteriaceae* and total *E. coli* populations in leek samples treated with BC and inoculated with pathogen, stored at 15 and 5C. Initial enterobacterial and total *E. coli* counts in BC treated and untreated samples stored at 5 and 15C were in the range of 5.8–6.0 log cfu/g. When leek samples were inoculated with *E. coli* O157:H7, treated with BC and stored at 15C, it was observed that tea tree EO was able to significantly reduce ($P < 0.05$) enterobacterial and total *E. coli* counts, since day 5 up to the end of storage (Fig. 5C,D). Gallic acid and propolis did not exert any inhibitory effects on enterobacteria along the storage at both temperatures (Fig. 5C). It was observed that between days 0 and 5 of storage at 5C, total *E. coli* counts remained without major changes, compared with initial values, in all samples (Fig. 5D). Up to 7 days of storage, propolis extract and gallic acid exerted a significant ($P < 0.05$) inhibitory effect (1.0 log reductions) on total *E. coli* counts. After 7 days of storage, *E. coli* population in samples stored at 5C significantly increased ($P < 0.05$) reaching values of approximately 7.3 log units, without significant differences between treatments (Fig. 5D).

Figure 6A,B shows the evolution of *Enternecedoramente* and endogenous *E. coli* populations in butternut squash samples treated with BC, without pathogen inoculation and stored at 15 and 5C. Initial enterobacterial and endogenous *E. coli* counts were in the range of 2.5–3.5 log cfu/g. No significant inhibitory effects on enterobacteria and endogenous *E. coli* were observed when squash samples were treated with BC and stored at 15C. On the contrary, tea tree EO and propolis treatments exerted significant ($P < 0.05$) inhibitory effects on enterobacterial and *E. coli* counts (1.0–2.5 log unit reductions compared to control) during the entire refrigerated storage period.

Figure 6C,D shows the evolution of enterobacteria and total *E. coli* during the storage of minimally processed butternut squash treated with BC and inoculated with *E. coli*

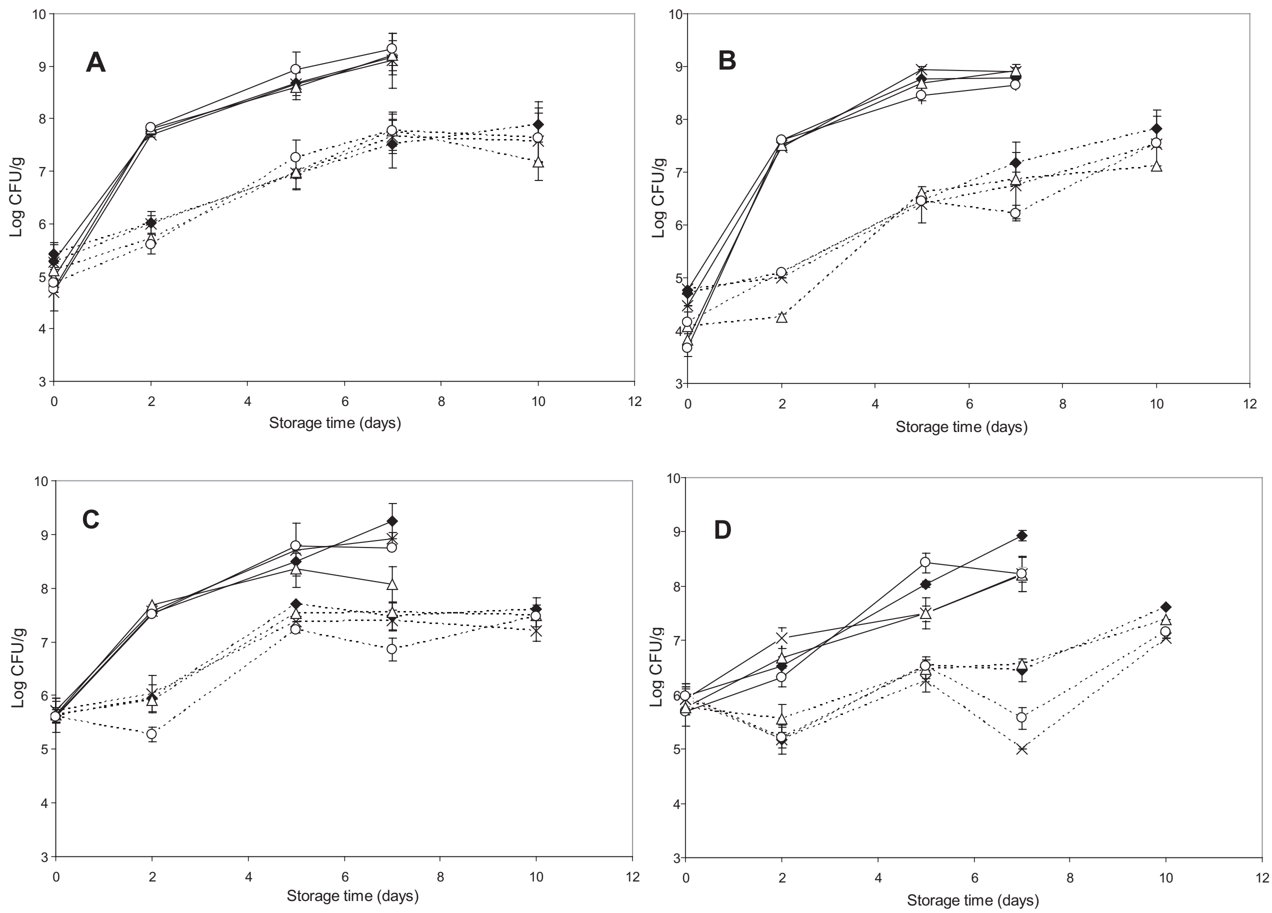

FIG. 5. EVOLUTION OF ENTEROBACTERIAL AND *E. COLI* POPULATIONS IN TREATED LEEK WITHOUT PATHOGEN INOCULATION (A AND B, RESPECTIVELY) AND IN TREATED LEEK WITH PATHOGEN INOCULATION (C AND D, RESPECTIVELY) STORED AT 15C (WHOLE LINES) AND 5C (DOTTED LINES); (◆) CONTROL; (△) TEA TREE ESSENTIAL OIL; (○) PROPOLIS; (X) GALLIC ACID
Data represent mean values (n = 6) and vertical bars represent standard deviation.

O157:H7. No significant inhibitory effects on enterobacterial and total *E. coli* counts were observed when squash samples were treated with propolis extract and gallic acid and stored at 15C. On the contrary, tea tree EO treatments exerted significant ($P < 0.05$) inhibitory effects on enterobacteria and *E. coli* (1.0–1.5 log unit reductions compared with control), up to 7 days of storage at 15C. Up to day 7, total *E. coli* counts in samples stored at 5C remained without significant changes (in the range of 5.5 log cfru/g); from days 7 to 10 these counts significantly increased. The inoculated pathogen might be showing an adaptation period (0–7 days) taking into account that it was artificially added to the substrate. When squash samples were stored at optimal refrigeration temperature, a significant inhibitory effect of propolis extract was observed (0.9–1.4 log cfu/g reductions) on enterobacterial counts, between days 5 and 10 of storage (Fig. 6C). Furthermore, propolis extract exerted a significant reduction on total *E. coli* counts (1.0 log cfu/g) at 5 and 7 days of the refrigerated storage (5C) (Fig. 6D).

In the present work, we evaluated the growth and survival of endogenous *E. coli* and inoculated *E. coli* O157:H7 on vegetables (celery, leek and butternut squash untreated and treated with BC). Both endogenous and inoculated *E. coli* grew on celery, leek and squash samples stored at 15C. Similarly, Palumbo *et al.* (1995) reported that several pathogenic *E. coli* strains could easily grow at 10C and thus suggested the potential for growth in temperature-abused refrigerated foods. In our study, it was demonstrated that naturally occurring *E. coli* population was able to grow under optimal refrigeration temperature. On the contrary, *E. coli* counts in inoculated vegetable samples stored at 5C slightly increased. This behavior may be explained taking into account that this temperature is lower than the minimal level (8C)

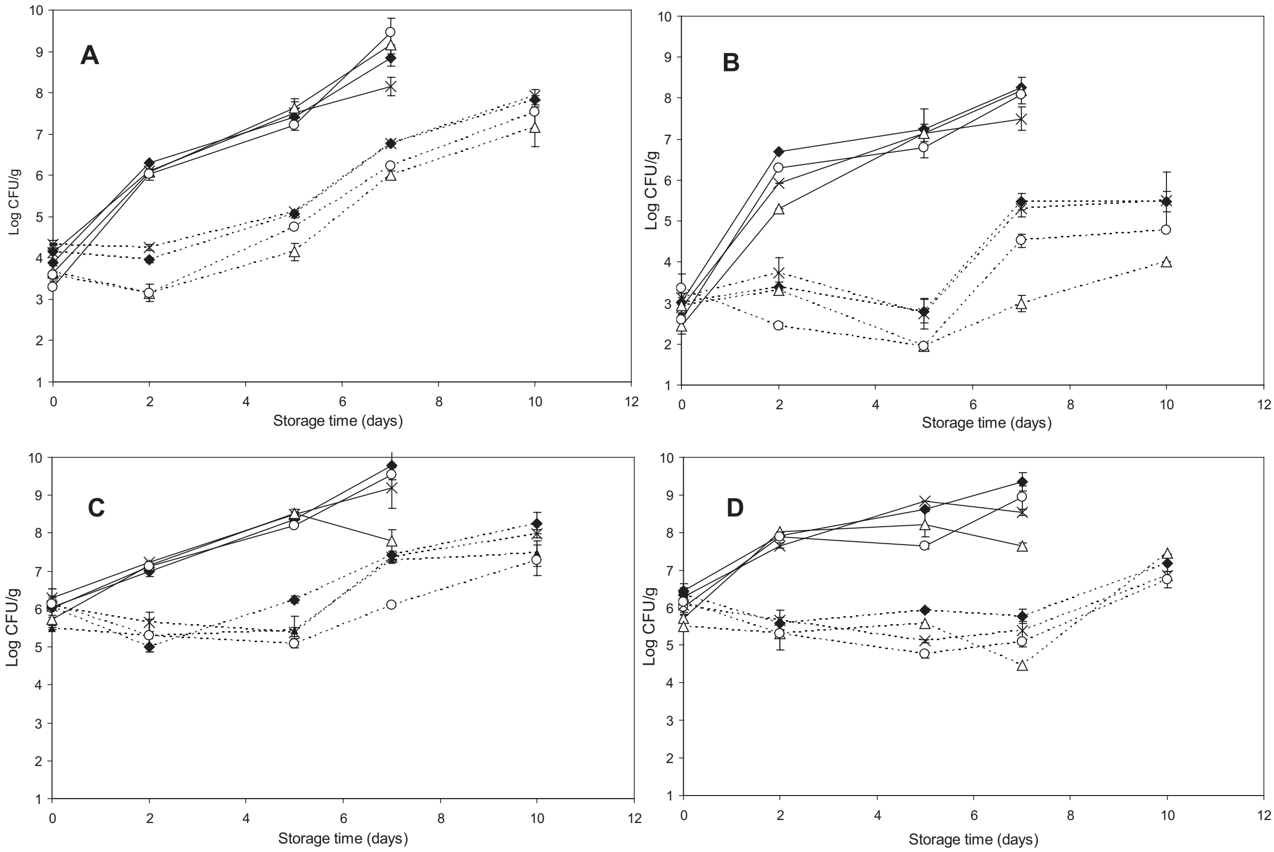

FIG. 6. EVOLUTION OF ENTEROBACTERIAL AND *E. COLI* POPULATIONS IN TREATED BUTTERNUT SQUASH WITHOUT PATHOGEN INOCULATION (A AND B, RESPECTIVELY) AND IN TREATED BUTTERNUT SQUASH WITH PATHOGEN INOCULATION (C AND D, RESPECTIVELY) STORED AT 15C (WHOLE LINES) AND 5C (DOTTED LINES); (◆) CONTROL; (△) TEA TREE ESSENTIAL OIL; (○) PROPOLIS; (X) GALLIC ACID
Data represent mean values (n = 6) and vertical bars represent standard deviation.

required for *E. coli* O157:H7 to grow. Probably, natural occurring *E. coli* can adapt to the substrate and grow even at low temperatures, in contrast with artificially added *E. coli*. Accordingly, Abadias *et al.* (2012) found that *E. coli* O157:H7 did not grow but survived throughout refrigerated storage (5C) when inoculated on fresh-cut fruits and vegetables. Also, in this study we reported that treated and untreated celery, leek and butternut squash samples with and without *E. coli* inoculations presented pathogen counts in the same range, at both storage temperatures, up to the end of storage. This fact could indicate that the inoculation with *E. coli* O157:H7 did not produce any increase of the pathogen counts, showing the few opportunities to colonize and prosper or develop in this kind of substrates. Similar results were reported by Alvarez *et al.* (2013), working with minimally processed broccoli.

With regard to biopreservative treatments applied on celery, leek and butternut squash, it was demonstrated that tea tree EO and propolis extract effectively reduced *Enterobacteriaceae* and *E. coli* populations, while gallic acid did not show any activity. Besides, it was observed that the intensity of BC inhibitory effects depended on the substrate and storage temperature applied. In a previous study, Alvarez *et al.* (2013) showed that propolis extracts and tea tree EO were significantly effective in inhibiting *E. coli* growth by *in vitro* assays. Also, in another study, Alvarez *et al.* (2015) reported that the inhibitory effects showed by propolis and tea tree EO treatments on inoculated pathogen control in vegetables for soup were less significant when compared with those obtained by *in vitro* assays. According to our results, Larraínzar *et al.* (2012) analyzed the antimicrobial activity of natural phenolic compounds against foodborne pathogens and spoilage bacteria by *in vitro* assays, reporting that gallic acid was effective in the control of *Staphylococcus aureus*, although it was less effective in the control of *E. coli*.

Furthermore, Tosi *et al.* (2007) studied the antibacterial activity of several Argentine propolis and demonstrated that all tested ethanolic extracts of propolis successfully inhibited *E. coli*'s development *in vitro*. Samples with a high antimicrobial activity also showed a high content of coumaric acid + syringic acid, quercetin, galangin, caffeic acid + crisine and total soluble compounds.

Besides, Carson and Riley (1995) studied the antimicrobial activity of several components of tea tree EO by *in vitro* assays and demonstrated that terpinen-4-ol, α-terpineol, linalool and α-terpinene were the most effective in inhibiting the growth of *E. coli* (minimum inhibitory concentrations ranging 0.6–2 μL/mL) while γ-terpinene and p-cymene were not active. In an *in vivo* study, tea tree EO was able to reduce the growth of inoculated *E. coli* O157:H7 in blanched spinach throughout 24 h of storage at 8 and 20–22C, showing higher efficacy at the highest storage temperature (Moreira *et al.* 2007). On the other hand, Goñi *et al.* (2014) tested the application of natural antimicrobial during the preharvest of lettuce, as an early intervention strategy for controlling the growth of native microflora and *E. coli* O157:H7 inoculated on lettuce at postharvest. When lettuce was treated by applying a solution of tea tree EO (27 μL/mL), the growth of the pathogen during storage at 7–9C was not affected (Goñi *et al.* 2014). In our study, it was observed that the application of tea tree EO on fresh-cut vegetables showed a higher effectiveness in controlling the growth of endogenous *E. coli* compared with inoculated *E. coli* O157:H7, mainly in refrigerated butternut squash and leek.

Modeling OVQ Decay and Determination of Sensory Shelf Life

The sensory shelf life of vegetables can be defined as the length of time at which they can maintain an appearance that appeals to the consumer. An issue associated with ready-to-use vegetables is the short shelf life, which is usually no longer than 7 days, when stored in adequate conditions (Zhou *et al.* 2004). In general, little information is known about the relationship between the outgrowth of spoilage microorganisms, their production of metabolites and how consumers perceive decay in minimally processed vegetables (Olaimat and Holley 2012).

In this work, the impact of the antimicrobial treatments combined with storage conditions on the visual quality and acceptability of fresh-cut vegetables was studied. Moreover, the addition of BC should not negatively affect the sensory properties of vegetable products. OVQ changes for treated fresh-cut celery, leek and butternut squash stored at optimal refrigeration temperature and abusive conditions were modeled so as to estimate its sensory shelf life. The first step in selecting an appropriate model to represent the OVQ changes for fresh-cut vegetables was to perform regression analysis to determine the kinetic order. The performance of the fitted models was analyzed. Based on the best coefficient of determination (R^2), zero order was the apparent order of the OVQ change reactions in most of the cases (model comparison is not shown). Finally, the plots of residuals versus predicted values for each model (zero and first order) indicated that the distribution around zero was more random for the first-order model. These results were taken into account to select the zero-order reaction model in order to describe changes in visual quality of celery, leek and butternut squash throughout storage at both temperature conditions.

Estimated values of sensory shelf life (t_s) (corresponding to OVQ score 2.5) were obtained from zero-order fitting models. Thus, Table 1 shows the rate constants (k_q) for OVQ attribute, the corresponding R^2 and the estimated t_s values for fresh-cut celery, leek and butternut squash untreated and treated with biopreservatives and stored at 15 and 5C. OVQ scores of all fresh-cut vegetables decreased at higher rates (k_q) when stored at 15C, compared with the storage at 5C; as a consequence, estimated sensory shelf life of untreated vegetables was significantly higher when stored at 5C.

In celery samples, propolis treatment improved significantly the visual quality both at optimal refrigeration temperature and abusive conditions. Based on the confidence intervals of mean values (95%) for t_s, it can be observed that propolis treatment extended sensory shelf life of celery untreated control from 9.53 to 14 days at 5C and from 4.18 to 6.41 at 15C (Table 1). According to some comments made by panelists, this treatment delays browning appearance, the main visual defect observed in cut celery. Celery samples treated with tea tree EO showed no differences compared with untreated celery stored at 5 and 15C. On the other hand, when gallic acid was applied, the sensory shelf life of treated samples was greatly reduced due to a quicker appearance of browning for both 15 and 5C storage temperature.

In the case of untreated fresh-cut leek, the estimated sensory shelf life period was 5.7 days when stored at 15C and 15 days at 5C. Based on some comments made by panelists, the sensory attributes that suffered deterioration over time were green color, brightness, texture and fresh appearance. At 15C, only propolis treatment improved the visual quality and was effective in extending the sensory shelf life of leek, showing an estimated value of 8.4 days. On the contrary, in untreated leek samples stored at 5C the sensory shelf life was 15 days, and the only treatment able to significantly extend the shelf life period of refrigerated leek was tea tree EO, which showed an estimated value of 17.5 days (Table 1).

Butternut squash cubes without treatment remained acceptable from a sensory point of view for 11.4 and 5.2 days when stored at 5 and 15C, respectively. The results indicated that both propolis and tea tree EO applications improved visual quality of butternut squash in samples stored at 5C. At 15C, none of the treatments were able to

TABLE 1. MODELING OVERALL VISUAL QUALITY CHANGES AND ESTIMATED SENSORY SHELF LIFE OF FRESH-CUT CELERY, LEEK AND BUTTERNUT SQUASH UNTREATED AND TREATED WITH BC AND STORED AT ABUSIVE AND OPTIMAL REFRIGERATION TEMPERATURE

			Zero-order model				
						Sensory shelf life (t_s, days)	
Vegetable	Storage temperature (C)	Treatment	Rate constant * k_q (day)		Coefficient of determination (R^2)	Estimated value	Confidence interval at 95%
Celery	5	Control	–0.26	± 0.06	0.87	*9.53*	(8.50–10.55)
		Tea tree EO	–0.27	± 0.06	0.87	*9.81*	(9–10.87)
		Propolis	–0.17	± 0.03	0.89	*14.00*	(12.51–15.39)
		Gallic acid	–0.28	± 0.08	0.80	*2.93*	(2–3.95)
	15	Control	–0.57	± 0.05	0.98	*4.18*	(3.94–4.41)
		Tea tree EO	–0.69	± 0.11	0.95	*3.74*	(3.35–4.13)
		Propolis	–0.34	± 0.07	0.93	*6.41*	(5.78–7.05)
		Gallic acid	–0.64	± 0.13	0.86	*1.45*	(0.80–2.11)
Leek	5	Control	–0.17	± 0.03	0.93	15.02	(14.15–15.89)
		Tea tree EO	–0.16	± 0.03	0.94	*17.52*	(16.36–18.68)
		Propolis	–0.17	± 0.03	0.92	*16.46*	(14.81–18.19)
		Gallic acid	–0.18	± 0.02	0.96	13.43	(12.64–14.23)
	15	Control	–0.44	± 0.09	0.89	*5.72*	(5.03–6.40)
		Tea tree EO	–0.40	± 0.08	0.95	*6.36*	(5.72–7.01)
		Propolis	–0.28	± 0.06	0.94	*8.35*	(7.47–9.23)
		Gallic acid	–0.46	± 0.07	0.95	*4.65*	(4.21–5.09)
Butternut squash	5	Control	–0.27	± 0.06	0.91	*11.44*	(10.28–12.60)
		Tea tree EO	–0.21	± 0.04	0.94	*14.17*	(12.81–15.53)
		Propolis	–0.19	± 0.04	0.93	*13.78*	(12.76–14.80)
		Gallic acid	–0.39	± 0.12	0.80	*4.29*	(2.56–6.01)
	15	Control	–0.47	± 0.10	0.90	*5.23*	(4.51–5.94)
		Tea tree EO	–0.50	± 0.08	0.97	*5.02*	(4.47–5.56)
		Propolis	–0.48	± 0.09	0.96	*5.01*	(4.38–5.65)
		Gallic acid	–0.61	± 0.23	0.75	*2.77*	(1.76–3.78)

* k_q ± confidence interval at 95%.
BC, bioactive compound; EO, essential oil.

improve visual quality with respect to untreated control. When gallic acid was applied on squash cubes, the sensory shelf life of treated samples was significantly lower compared with control at both 15 and 5C storage temperature (Table 1).

The physical damage caused by processing operations increases respiration and ethylene production in vegetables within minutes and associated increases occur in the rate of other biochemical reactions responsible for changes in color, flavor, texture and nutritional quality (Allende *et al.* 2006). Specifically, enzymatic browning in vegetable tissues can cause undesirable quality changes during handling, processing and storage. This reaction results mostly from polyphenol oxidase (PPO) and peroxidase (POD) activity (Toivonen and Brummell 2008). Biopreservatives used in this study, mainly propolis and tea tree EO, could also act as antioxidant agents avoiding oxidative processes catalyzed by enzymes as PPO and POD, and therefore, preventing browning and maintaining the fresh appearance of vegetables. In this regard, Chang *et al.* (2012) demonstrated the effectiveness of a propolis extract as antibrowning agent, significantly reducing PPO activity in apples after 24 h of storage at room temperature. The antioxidative properties of propolis were reported by Nagai *et al.* (2001) who associated this activity with the presence of flavonoids, such as quercetin, flavones, isoflavones, flavonones, catechin and isocatechin. Moreover, Ponce *et al.* (2004b) demonstrated the effectiveness of several EOs including tea tree EO in reducing peroxidase activity of leafy vegetables by *in vitro* assays.

CONCLUSIONS

The obtained results demonstrated that the effectiveness of the biopreservative treatments applied varied according to the vegetable substrate and storage conditions. It was confirmed that a good control of refrigeration temperature limits the growth of spoilage and pathogenic microorganisms. In fresh-cut celery, the most effective BC was propolis, slightly reducing the native microflora counts toward the end of storage, both at optimal and abusive temperature. Besides, propolis treatment significantly reduced initial endogenous *E. coli* counts, and was slightly effective in reducing both endogenous *E. coli* and inoculated *E. coli* O157:H7 growth. Also, this treatment considerably improved visual quality of celery and allowed to extend its sensory shelf life at both storage conditions. On the other hand, when diced butternut squash was treated with BC, the most effective antimicrobial was tea tree EO. Both tea tree EO and propolis treatments controlled endogenous and inoculated *E. coli* growth throughout refrigerated storage. Moreover, butternut squash sensory shelf life was extended by the application of tea tree EO and propolis combined with optimal storage temperature. In fresh-cut leek, the effects of natural antimicrobials on the native microflora growth were less significant, meanwhile propolis extract and tea tree EO significantly retained leek visual quality. Therefore, the use of natural agents such as propolis and tea tree EO to preserve the quality and safety of minimally processed vegetables might be an interesting option. An alternative to produce more significant inhibitory effects would be the use BCs in combination with other barriers such as ultrasound, mild heat shock or edible coatings, besides using hygienic processing conditions and adequate storage temperatures.

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