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The impact of biopreservatives and storage temperature in the quality and safety of minimally processed mixed vegetables for soup

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Abstract

BACKGROUND: The combined effects of bioactive agents (tea tree essential oil, propolis extract and gallic acid) and storage temperature on the microbiological and sensory quality of fresh-cut mixed vegetables for soup (celery, leek and butternut squash) were studied with the objective of preserving its quality and safety.

RESULTS: Refrigeration temperature was confirmed as the main factor to limit the growth of spoilage and pathogenic microorganisms. Biopreservatives applied on mixed vegetables were effective only when combined with optimal refrigeration temperature (5 °C). Bioactive compounds showed slight effectiveness in controlling the microbiota present in mixed vegetables, although coliforms were greatly reduced by gallic acid and propolis treatments, achieving 0.5–2 log unit reductions during storage. Also, these agents showed antimicrobial activity against endogenous *Escherichia coli* and inoculated *E. coli* O157:H7, exerting a bacteriostatic effect and reducing population counts by 0.9–1.2 log CFU g^{-1} at 10 days of refrigerated storage. The combination of propolis treatment with refrigerated storage conditions effectively preserved the sensory quality and prolonged the sensory shelf life of fresh-cut mixed vegetables by 3 days.

CONCLUSION: The use of natural agents such as propolis extract to preserve the quality and safety of mixed vegetables for soup might be an interesting option to address the concerns of the consumer about the use of synthetic chemical antimicrobials potentially harmful to health.

Keywords: biopreservation; pathogen control; fresh-cut vegetables; shelf life; propolis; sensory attributes

INTRODUCTION

The consumption of minimally processed vegetables, including ready-to-eat or ready-to-use products, has increased worldwide in the last decade owing to their convenience, freshness and improved quality. Raw materials are subjected to preliminary operations such as peeling and cutting that increase tissue damage and cause the release of intracellular contents.[1,2] These operations commonly encourage and increase the activity of pathogenic and saprophytic microorganisms. The main problem that makes fresh-cut vegetables highly perishable products is the ease of microbial growth. Unfortunately, it has been demonstrated that current industrial sanitizing washing treatments do not guarantee the total elimination of pathogens when present.[3] *Escherichia coli* O157:H7 was first recognized as a pathogen in 1982 and is considered the main cause of hemorrhagic colitis and hemolytic uremic syndrome. Recently, two severe outbreaks of *E. coli* O157:H7 infections linked to the consumption of pre-packaged vegetables (leafy greens and romaine lettuce) affected many people in several states of the USA.[4] Also, in 2011, Shiga toxin-producing *E. coli* O104 infected almost 4000 people in Europe and caused the death of 46 of them; authorities suggested that contaminated sprouts were the vehicle of infection.[5]

Safe low temperature maintenance and high relative humidity control are among the most important tools for extending the shelf life of most fresh vegetables. Temperature is the single most important variable, since its improper manipulation causes evident changes in the sensory characteristics of fresh vegetables that condition consumer acceptance. Abusive storage temperatures reduce the levels of some nutrients and favor microorganism proliferation to counts that may exceed tolerable levels.[6] Fresh-cut vegetables probably receive the greatest temperature abuse during retail. Refrigerated storage (<7 °C) can maintain fresh-cut produce quality by slowing the respiration rate, enzymatic processes and microbial activity.[7] However, such low temperature is not always maintained throughout the entire cold chain in some countries, where wholesalers and retailers generally keep produce

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above the recommended temperature to save on cost or owing to a lack of energy and refrigeration equipment.[8] Fresh-cut vegetables, particularly salads, dominate the global production of minimally processed foods.[9] Also, various types of ready-to-cook vegetable soups are present on the fresh-cut market, with more or less simple mixes of vegetables owing to the shelf life limits of some species.[10] Consumption of these products is increasing because of their convenience as ready-to-use products.

The above facts explain the need to develop new technologies to reduce deterioration and safety problems in vegetables. Moreover, since consumers demand less use of chemicals on minimally processed fruits and vegetables, more attention has been paid to the search for natural alternatives for preservation.[11]

A large variety of plant- and spice-based antimicrobials are used in food products, including processed fruits and vegetables, in order to extend their shelf life, to reduce or eliminate pathogenic bacteria and to improve overall quality.[12–18] Tea tree essential oil, steam distilled from *Melaleuca alternifolia* L., was found to be effective in inhibiting the growth of native microflora of Swiss chard leaves stored at 5 °C for 14 days but did not show an antimicrobial effect when the leaves were stored at 0 °C.[13] Moreover, tea tree essential oil was able to reduce the growth of mesophilic aerobes and inoculated *E. coli* O157:H7 in blanched spinach throughout 24 h of storage at 8 and 20–22 °C, showing higher efficacy at the highest storage temperature.[15]

Furthermore, propolis is extensively used in Argentine folk medicine. Some Argentine propolis showed antibacterial activity against antibiotic-resistant human pathogenic bacteria[19] and has been identified as useful for the development of natural food preservers.[20] On the other hand, gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring polyphenol especially present in berries, citrus fruits, cereals, tea, wine and herbs. Gallic acid has a broad biological functionality: it can act as an antioxidant[21] or an antimicrobial agent[22,23] and can also prevent oxidative stress and some kinds of cancer.[24,25]

Preliminary studies demonstrated that agents from natural sources such as tea tree essential oil, propolis extract and gallic acid were effective in controlling *E. coli* and *Listeria monocytogenes* growth by *in vitro* assays.[26] As far as we are aware, there are no reports showing the effects of natural antimicrobials applied on mixed vegetables for soup to preserve safety and quality. Therefore the aim of this study was to investigate the effects of different bioactive compounds (BCs: tea tree essential oil, propolis extract and gallic acid) and storage temperatures (optimal, 5 °C and suboptimal, 15 °C) on the microbiological and sensory quality of mixed vegetables for soup (celery, leek and butternut squash). The effect of BCs on the survival and growth of *E. coli* inoculated in the mixed vegetables (simulating inadequate postharvest management) was also evaluated.

Thus the combined application of two barriers, BCs and storage temperature, was assayed as a natural alternative to microbial control to avoid undesirable sensory changes and to extend the shelf life of minimally processed vegetables.

EXPERIMENTAL

Biopreservative agents

The biopreservative agents used in this work were tea tree (*M. alternifolia*) essential oil (Nelson and Russell, London, UK), propolis extract (Juricich, Mendoza, Argentina) and gallic acid (Sigma Aldrich, Buenos Aires, Argentina).

Tea tree essential oil was extracted by steam distillation from tea tree leaves of Australian origin. The main component determined by gas chromatography/mass spectrometry was terpinen-4-ol (29%). 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 province, Argentina. Extraction was performed with ethanol and water as solvents and the product was standardized to 10% propolis extract. The color was dark brown and the oxidation index, defined as the time of discoloration of 0.1 mol L^{-1} potassium permanganate solution, was 2 s. Total phenolic content was 188 g gallic acid equivalent kg^{-1} propolis.

Sample preparation

Apium graveolens L. (celery), *Allium porrum* L. (leek) and *Cucurbita moschata* D. (butternut squash) cultivated in the open field were harvested in the early morning and immediately (within 1 h) transported to the laboratory in refrigerated containers with polyfreezer (refrigerated gel to maintain the cold chain; Thermics Argentina SA, Mar del Plata, Argentina). Squashes of uniform size and color were selected, hand-peeled, washed thoroughly with tap water and diced (~15 mm^3) using a stainless steel hand slice. The processed dice were dipped in tap water (3 min) and the surface moisture was removed with a manual salad centrifuge. A similar methodology was carried out with celery and leek. Mixed vegetable samples (300 g) were placed in open plastic containers, treated with different biopreservatives and finally covered with 15 µm polyethylene wrap (O_2 permeability 600 cm^3 m^{-2} day^{-1}, CO_2 permeability 4000 cm^3 m^{-2} day^{-1} and water vapor permeability 4 g m^{-2} day^{-1}). The containers were placed in holding boxes at a relative humidity of 95% and at two storage temperatures (5 and 15 °C).

Culture maintenance and inoculum preparation

Escherichia 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 4 °C. Before use, *E. coli* O157:H7 was cultured in brain heart infusion (BHI) broth (Britania) for 24 h at 37 °C. 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 37 °C before each experiment. A bacterial suspension was prepared by adding 10 mL of the *E. coli* culture to 90 mL of sterile 1 g L^{-1} peptone water.

Biopreservative application and inoculation of samples

BCs were added to mixed vegetables for soup in concentrations previously determined from results of preliminary *in vitro* and *in vivo* assays. Tea tree essential oil and propolis extract were applied at 15 µL mL^{-1} and gallic acid at 2 mg mL^{-1}. The BCs were diluted in sterile distilled water and vigorously shaken at 30 °C for 30 min to obtain reasonably stable dispersions. The minimally processed vegetables were hand-sprayed with the BC solutions (4 mL per container); these solutions remained in contact with the surface of the vegetables during the 7–14 days of storage. In control samples, vegetables were sprayed with sterile distilled water.

Samples treated with bioactive compounds were immediately inoculated with *E. coli* O157:H7. To carry this out, the bacterial suspension previously prepared was sprayed (1 mL) on fresh-cut mixed vegetables to reach a final pathogen concentration of ~5 log colony-forming units (CFU) g^{-1}. Control samples were

non-inoculated mixed vegetables and untreated mixed vegetables inoculated with *E. coli*.

After being treated, the fresh-cut vegetables (with or without pathogen inoculation) were stored in refrigerated chambers at 15 and 5 °C for 7 and 14 days respectively. Three replications per temperature (three containers) were performed and 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 15 °C; three replicates were used. For microbiological analysis, ∼10 g of treated mixed vegetables (celery, leek and squash) corresponding to inoculated and non-inoculated samples were macerated in 90 mL of phosphate buffer solution (0.1 mol L^{-1}) and homogenized in a Stomacher 400 Circulator Homogenizer (LAB CIMA, Buenos Aires, Argentina) (pH 7.2). Serial dilutions (1:10) of each homogenized sample were made in the same diluents and surface spread in duplicate. The enumeration and differentiation of microorganisms were performed using the following culture media and conditions: mesophilic aerobic bacteria on plate count agar (PCA) incubated at 30–32 °C for 48–72 h; psychotropic bacteria on the same medium incubated at 5–7 °C for 5–7 days; total coliforms in MacConkey agar incubated at 30–32 °C for 24 h. Molds and yeasts were counted in yeast/glucose/chloramphenicol (YGC) medium incubated at 25 °C for 5 days. Viable *E. coli* counts were monitored as follows: 0.1 mL samples of each treatment were spread on the surface of eosin methylene blue (EMB) agar plates and the colonies were counted after incubation at 37 °C for 24–48 h.[11,26] 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 media used were purchased from Britania. Microbial counts were expressed as log CFU g^{-1}.

Qualitative sensory evaluation

At each storage time, mixed vegetables (untreated and treated with natural agents) were subjected to a panel of testers to evaluate the sensory quality of treated and untreated samples. A panel comprising nine members of the UNMdP Food Engineering Group, aged 30–50 years and with sensory evaluation experience in vegetable quality, was trained and carried out the evaluation of celery, leek and squash quality. In preliminary studies the panel defined five critical sensory attributes to be evaluated on the mixed vegetables, namely overall visual quality (OVQ), odor of the product, celery browning, celery firmness and butternut squash firmness. The firmness of squash cubes was measured by squeezing the product between the forefinger and the thumb (deformation test), while celery firmness was evaluated by holding the piece of celery with both hands, one at each end, and bending it (flexure test).[27]

Evaluations were performed in duplicate immediately after vegetable removal from storage conditions. The coded (three-digit) samples were presented one at a time in random order to the panel members, who sat at a round table and made independent evaluations. The intensity of the attributes evaluated was quantified on a continuous, unstructured intensity scale from 0 to 5. OVQ was scored from 0 (highly deteriorated aspect) to 5 (fresh aspect). Celery browning was rated from 0 (very severe) to 5 (no presence), odor from 0 (intense off-odors) to 5 (fresh) and firmness from 0 (very soft) to 5 (stiff-crispy). The limit of acceptance was 2.5 (value corresponding to 50% of the scale), indicating that a score below this limit for any of the attributes evaluated was deemed to indicate the end of shelf life.[26,28]

Statistical analysis

The experimental design used in this study was completely randomized with each 300 g sample being the experimental unit. Results reported in this paper are mean values obtained from population data previously transformed to log scale. Data were analyzed using SAS Version 9.0 (SAS Institute, Cary, NC, USA). The general linear model procedure (PROC GLM) was used for the analysis of variance (ANOVA) applied to each factor (antimicrobial treatment, storage temperature and storage time). Antimicrobial treatment was defined in four levels (control, tea tree essential oil, propolis extract and gallic acid). Storage temperature was defined in two levels (5 and 15 °C) and storage time in five levels (0, 2, 5, 7 and 10 days). Differences between means were evaluated by Tukey's multiple comparison test. Wherever differences are reported as significant, a 95% confidence level was used.[29]

RESULTS

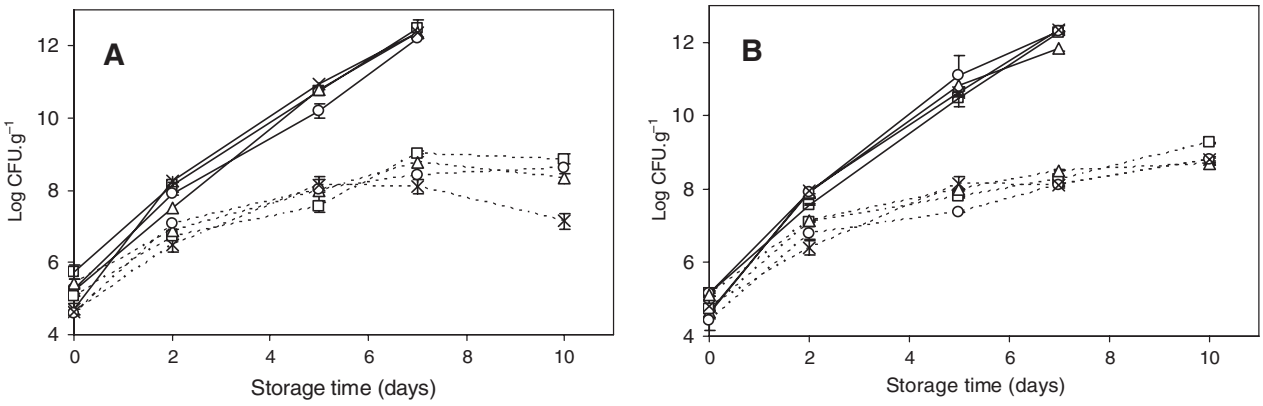
Evolution of native microflora in treated mixed vegetables non-inoculated and inoculated with *E. coli* O157:H7 during storage at optimal and abusive temperatures

The antimicrobial effects of tea tree essential oil, propolis extract and gallic acid on the growth of native microflora in mixed vegetables (celery, leek and butternut squash) non-inoculated and inoculated with *E. coli* O157:H7 are shown in Figs 1 and 2 at two storage temperatures, 15 and 5 °C.

Figures 1A–1D show the evolution of total mesophilic aerobes, psychrotrophics, coliforms and yeasts/molds respectively during the storage of mixed vegetables (non-inoculated with *E. coli*) treated with BCs.

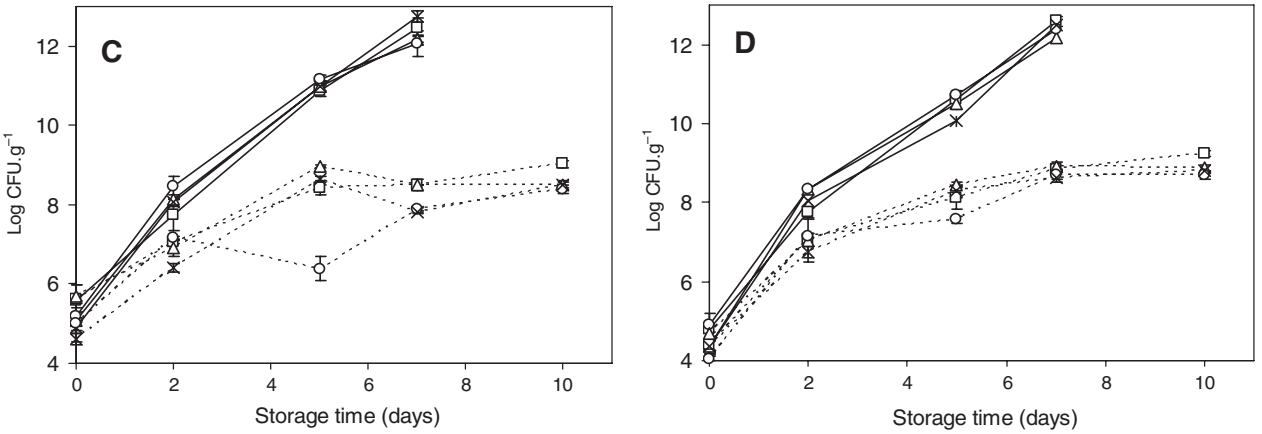
The growth rate of mesophilic bacteria in mixed vegetables stored at 15 °C was significantly ($P < 0.05$) higher than that in samples stored at 5 °C. For vegetables stored at 15 and 5 °C, final microbial counts (day 7) were in the range 12.2–12.5 and 8.1–9.0 log CFU g^{-1} respectively (Fig. 1A). When mixed vegetables were stored at 15 °C, increases occurred in mesophilics as the storage time increased, regardless of the treatment applied. However, when vegetables were stored at optimal refrigeration temperature (5 °C), the maximum growth was achieved after 7 days and no significant increase was observed until the end of storage (Fig. 1). When biopreservatives were applied to control native microflora in vegetables stored at 15 °C, only gallic acid significantly reduced initial mesophilic counts, but this effect was not observed at later storage stages. When treated samples were stored at 5 °C, the BCs did not show any significant inhibitory effect until day 7 of storage. However, from day 7 until the end of storage a significant inhibitory effect of gallic acid on mesophilic bacteria was observed (1.6 log CFU g^{-1} reduction) compared with the control sample (Fig. 1A).

Psychrotrophic, coliform and yeast/mold populations (Figs 1B–1D) on mixed vegetables non-inoculated with *E. coli* and stored at 15 °C showed similar growth patterns to the mesophilic population (Fig. 1A). At the end of the storage period, final psychrotrophic, coliform and yeast/mold counts were significantly higher (∼4 log CFU g^{-1}) in samples stored at 15 °C compared with those stored at 5 °C (Figs 1B–1D). At 15 °C, gallic acid and tea tree slightly reduced initial psychrotrophic and yeast/mold counts ($P < 0.05$) and only the inhibitory effect of tea tree was observed



15°C	0	2	5	7	
Control	5.8 a w	8.2 a x	10.7 a y	12.5 a z	
Tea tree	5.2 ab w	7.5 b x	10.8 a y	12.4 a z	
Propolis	5.2 ab w	7.9 ab x	10.2 a y	12.2 a z	
Gallic acid	4.7 b w	8.3 a x	11.0 a y	12.4 a z	
5°C	0	2	5	7	10
Control	5.1 ab w	6.7 ab x	7.6 a y	9.0 a z	8.8 a z
Tea tree	5.4 a x	6.9 a y	8.0 a z	8.7 ab z	8.3 a z
Propolis	4.6 b w	7.1 a x	8.0 a y	8.4 ab yz	8.6 a z
Gallic acid	4.6 b w	6.5 b y	8.1 a z	8.1 b z	7.2 b y

15°C	0	2	5	7	
Control	5.2 a w	7.6 a x	10.8 a y	12.3 a z	
Tea tree	4.7 b w	7.9 a x	10.8 a y	11.8 b z	
Propolis	5.2 a w	7.9 a x	11.3 a y	12.3 a z	
Gallic acid	4.6 b w	8.0 a x	10.7 a y	12.4 a z	
5°C	0	2	5	7	10
Control	4.8 ab v	7.1 a w	7.8 a x	8.3 a y	9.3 a z
Tea tree	5.1 a w	7.2 a x	8.0 a y	8.5 a z	8.7 b z
Propolis	4.4 b w	6.8 ab x	7.4 b x	8.1 a y	8.8 b z
Gallic acid	4.8 ab w	6.4 b x	8.1 a y	8.2 a y	8.8 b z



15°C	0	2	5	7	
Control	5.6 a w	7.8 a x	10.9 a y	12.5 a z	
Tea tree	5.1 a w	8.1 a x	11.0 a y	12.2 a z	
Propolis	5.2 a w	8.5 a x	11.2 a y	12.1 a z	
Gallic acid	4.9 a w	8.1 a x	11.0 a y	12.7 a z	
5°C	0	2	5	7	10
Control	5.6 a w	7.1 a x	8.4 a y	8.5 a y	9.0 a z
Tea tree	5.7 a x	6.9 a y	9.0 a z	8.5 a z	8.5 b z
Propolis	5.0 ab w	7.2 a y	6.4 b x	7.9 b z	8.4 b z
Gallic acid	4.6 b x	6.4 b y	8.6 a z	7.8 b z	8.5 b z

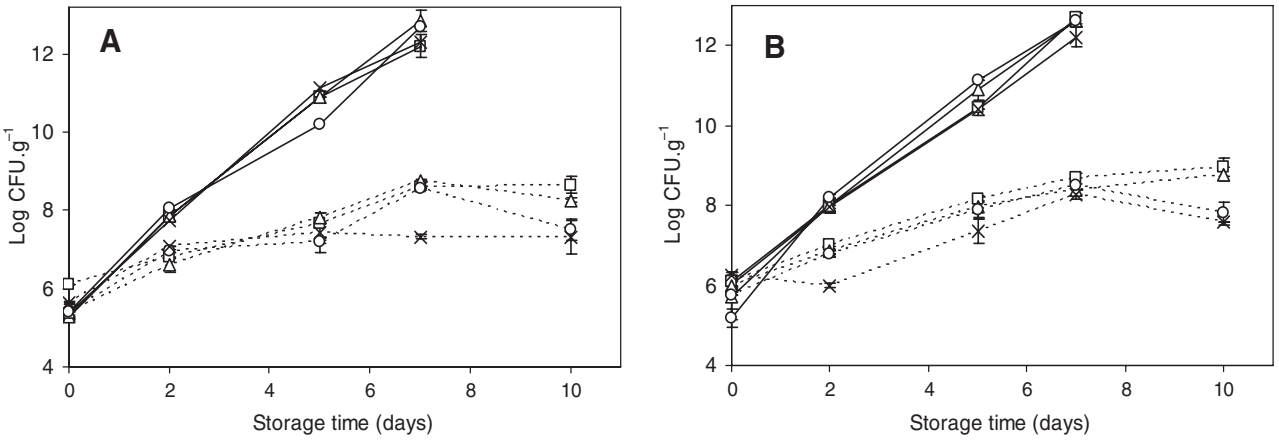
15°C	0	2	5	7	
Control	4.8 a w	7.8 a x	10.6 ab y	12.6 a z	
Tea tree	4.4 b w	8.4 a x	10.5 ab y	12.2 b z	
Propolis	5.0 a w	8.3 a x	10.7 a y	12.4 ab z	
Gallic acid	4.4 b w	8.0 a x	10.1 b y	12.5 a z	
5°C	0	2	5	7	10
Control	4.4 a w	7.1 a x	8.1 a y	8.8 a yz	9.2 a z
Tea tree	4.7 a x	7.0 a y	8.4 a z	8.9 a z	8.9 b z
Propolis	4.0 a w	7.2 a x	7.6 a y	8.7 a z	8.7 b z
Gallic acid	4.4 a x	6.7 a y	8.4 a z	8.6 a z	8.8 b z

Figure 1. Evolution of (A) mesophilic aerobes, (B) psychrotrophics, (C) coliforms and (D) yeasts and molds of mixed vegetables (uninoculated with *Escherichia coli*) treated with different bioactive agents during storage at 15 °C (full lines) and 5 °C (dotted lines): □, control; △, tea tree essential oil; ○, propolis; ×, gallic acid. Data represent the mean of six determinations and vertical bars represent standard deviation of the mean. For each storage temperature, different letters (a, b) within columns indicate significant differences ($P < 0.05$) of corresponding populations among the treatments, and different letters (w, x, y, z) within rows indicate significant differences ($P < 0.05$) among the storage times.

again at day 7 of storage (Figs 1B and 1D). Coliform growth in samples stored at 15 °C was not significantly affected by any of the applied agents during the storage period (Fig. 1C).

At 5 °C, all treatments exerted slight but significant ($P < 0.05$) inhibitory effects on psychrotrophic and yeast/mold growth, although these results were observed mainly at day 10 (0.5–0.6 log unit reductions). Besides, coliform counts in samples stored at 5 °C were significantly reduced by propolis and gallic acid treatments. Thus mixed vegetables treated with propolis showed a significant reduction (2.0 log units) at 5 days of storage and counts kept under control until the end of the storage period (Fig. 1C). Also, coliforms were significantly ($P < 0.05$) inhibited by gallic acid in samples stored at 5 °C during almost the entire storage period (0.5–1 log unit reductions compared with control) (Fig. 1C). With regard to tea tree, a slight inhibitory effect on coliforms was observed only at the end of storage (Fig. 1C).

Figures 2A and 2B show the evolution of mesophilic and coliform counts respectively during the storage of treated mixed vegetables inoculated with *E. coli*. Similar differences to those detailed for mixed vegetable samples uninoculated with pathogen were observed in all microbial counts corresponding to 15 and



15°C	0	2	5	7	
Control	5.3 a w	7.9 a x	10.9 a y	12.2 a z	
Tea tree	5.3 a w	7.9 a x	10.9 a y	12.9 a z	
Propolis	5.4 a w	8.1 a x	10.2 b y	12.7 a z	
Gallic acid	5.4 a w	7.8 a x	11.2 a y	12.3 a z	
5°C	0	2	5	7	10
Control	6.1 a w	6.8 ab x	7.7 a y	8.6 a z	8.7 a z
Tea tree	5.4 b w	6.6 b x	7.8 a y	8.8 a z	8.3 ab yz
Propolis	5.4 b x	7.0 ab y	7.2 a y	8.6 a z	7.5 b y
Gallic acid	5.6 ab y	7.1 a z	7.4 a z	7.3 b z	7.3 b z

15°C	0	2	5	7	
Control	6.1 a w	8.0 a x	10.4 ab y	12.7 a z	
Tea tree	5.7 a w	8.0 a x	10.9 ab y	12.6 a z	
Propolis	5.2 a w	8.2 a x	11.2 a y	12.6 a z	
Gallic acid	6.0 a w	8.0 a x	10.3 b y	12.2 a z	
5°C	0	2	5	7	10
Control	6.1 a w	7.0 a x	8.2 a y	8.7 a yz	9.0 a z
Tea tree	6.0 a w	6.9 a x	8.0 a y	8.4 a yz	8.8 a z
Propolis	5.8 a x	6.9 a y	7.9 a y	8.5 a z	7.8 b z
Gallic acid	6.3 a y	6.0 b y	7.4 a z	8.3 a z	7.6 b z

Figure 2. Evolution of (A) mesophilic aerobes and (B) coliforms of mixed vegetables inoculated with *Escherichia coli* and treated with different bioactive agents during storage at 15 °C (full lines) and 5 °C (dotted lines): □, control; △, tea tree essential oil; ○, propolis; ×, gallic acid. Data represent the mean of six determinations and vertical bars represent standard deviation of the mean. For each storage temperature, different letters (a, b) within columns indicate significant differences ($P < 0.05$) of corresponding populations among the treatments, and different letters (w, x, y, z) within rows indicate significant differences ($P < 0.05$) among the storage times.

5 °C (~3–4 log CFU g^{-1} higher in samples stored at abusive temperature).

When vegetable samples were stored at optimal refrigeration temperature, a significant inhibitory effect of gallic acid was observed (1.4 log CFU g^{-1} reduction) on mesophilic and coliforms counts at 10 days of storage (Fig. 2). Propolis also showed a significant inhibitory effect on both populations (1.2 log CFU g^{-1} reduction) at 10 days.

Moreover, no significant differences were observed between the evolution of microbial counts in vegetable samples with and without pathogen inoculation when samples were stored at 15 °C, with similar final values (~12 log CFU g^{-1}) being reached (Figs 1 and 2).

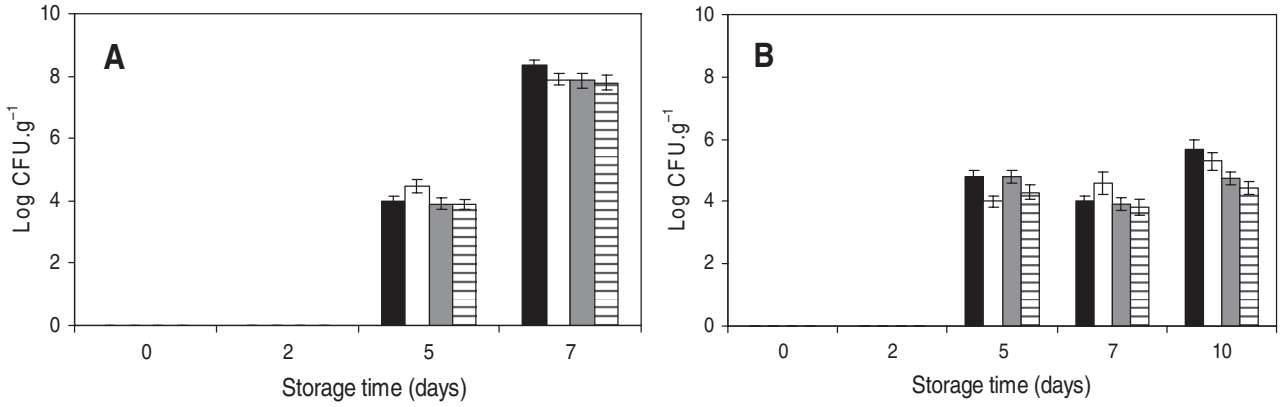
Evolution of *E. coli* counts in treated mixed vegetables non-inoculated and inoculated with *E. coli* O157:H7 during storage at optimal and abusive temperatures

Figures 3A and 3B show the evolution of endogenous *E. coli* counts in samples treated with BCs without pathogen inoculation and stored at 15 and 5 °C respectively. It was observed that, between days 0 and 2 of storage, endogenous *E. coli* counts remained at undetectable levels at both storage temperatures (Figs 3A and 3B). After 7 days of storage the *E. coli* population in samples stored at 15 °C increased significantly ($P < 0.05$), reaching 7.8–8.3 log units, without significant differences between treatments (Fig. 3A).

At 5 °C, endogenous *E. coli* counts also increased, though with a lower growth rate, and reached 5.7 log units in untreated samples after 10 days of storage. However, propolis and gallic acid treatments in combination with optimal refrigeration storage temperature were able to stop the growth of *E. coli* from day 5 until the end of storage. Thus, at day 10, samples treated with propolis and gallic acid showed significantly ($P < 0.05$) lower *E. coli* counts compared with the control sample (0.9 and 1.2 log unit reductions respectively) (Fig. 3B).

Figures 4A and 4B show the evolution of total *E. coli* counts in samples treated with BCs, inoculated with *E. coli* O157:H7 and stored at 15 and 5 °C respectively. The initial *E. coli* concentration in inoculated mixed vegetables was between 4.4 and 4.9 log CFU g^{-1} (Figs 4A and 4B). The *E. coli* population remained constant for the first 2 days of storage at 15 °C and significant increases ($P < 0.05$) were observed later at each time point regardless of the BC treatment applied (Fig. 4A). The inoculated pathogen could be showing an adaptation period (0–2 days), taking into account that it was artificially added to the substrate. At the end of storage at 15 °C, total *E. coli* counts in untreated samples (control) reached 9.6 log CFU g^{-1}. There were no significant differences ($P > 0.05$) between total *E. coli* counts in samples treated with tea tree and gallic acid and those untreated throughout storage at 15 °C. Nevertheless, propolis treatment exerted an inhibitory effect that became significant ($P < 0.05$) at day 7 with a reduction of 1.6 log units in *E. coli* counts compared with the control sample (Fig. 4A).

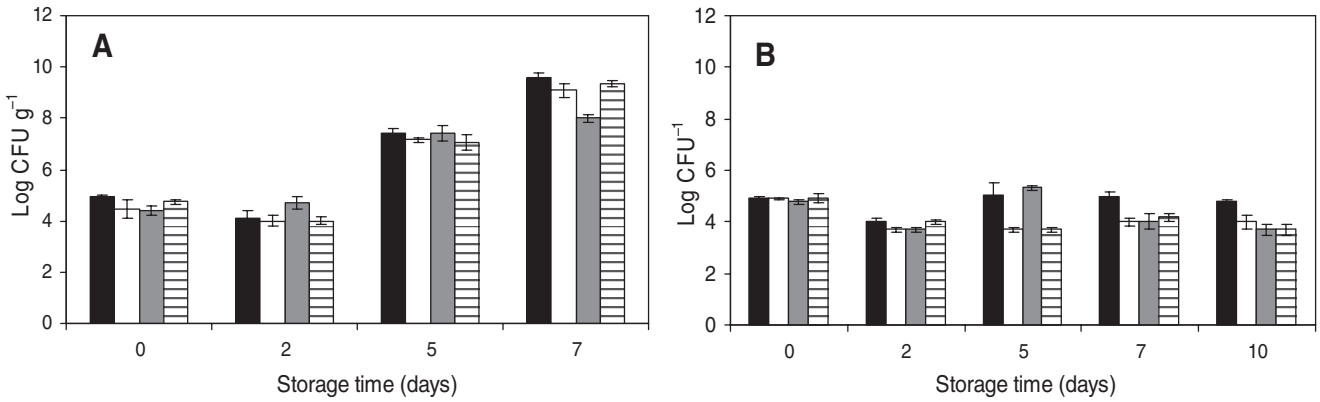
At 5 °C the total *E. coli* population (endogenous and inoculated O157:H7) did not grow but survived throughout storage in treated and untreated samples (Fig. 4B). The pathogen concentration remained at 4.8–5 log CFU g^{-1} in untreated samples during refrigerated storage. On the contrary, when fresh-cut mixed vegetables were treated with tea tree essential oil, propolis and gallic acid and stored at 5 °C, the population decreased significantly ($P < 0.05$) by 0.8, 1.1 and 1.1 log units in 10 days (Fig. 4B).



15°C	0	2	5	7
Control	ND x	ND x	4.0 a y	8.3 a z
Tea tree	ND x	ND x	4.5 a y	7.9 a z
Propolis	ND x	ND x	3.9 a y	7.9 a z
Gallic acid	ND x	ND x	3.9 a y	7.8 a z

5°C	0	2	5	7	10
Control	ND w	ND w	4.8 a y	4.0 a x	5.7 a z
Tea tree	ND x	ND x	4.0 b y	4.6 a y	5.3 ab z
Propolis	ND x	ND x	4.8 a z	3.9 a y	4.8 bc z
Gallic acid	ND x	ND x	4.3 ab yz	3.8 a y	4.5 c z

Figure 3. Endogenous *Escherichia coli* counts in mixed vegetables (uninoculated with *E. coli*) treated with bioactive compounds and stored at (A) 15 and (B) 5 °C: ■, control; □, tea tree essential oil; ▩, propolis; ⊟, gallic acid. Data represent the mean of six determinations and vertical bars represent standard deviation of the mean. For each storage temperature, different letters (a, b) within columns indicate significant differences ($P < 0.05$) of *E. coli* populations among the treatments, and different letters (w, x, y, z) within rows indicate significant differences ($P < 0.05$) among the storage times. ND means non-detectable level.



15°C	0	2	5	7
Control	4.9 a x	4.1 a x	7.4 a y	9.6 a z
Tea tree	4.5 a x	4.0 a x	7.2 a y	9.1 a z
Propolis	4.4 a x	4.7 a x	7.4 a y	8.0 b z
Gallic acid	4.8 a x	4.0 a x	7.1 a y	9.3 a z

5°C	0	2	5	7	10
Control	4.9 a z	4.0 a y	5.0 a z	5.0 a z	4.8 a z
Tea tree	4.9 a z	3.7 a y	3.7 b y	4.0 b y	4.0 b y
Propolis	4.8 a z	3.7 a y	5.3 a z	4.0 b y	3.7 b y
Gallic acid	4.9 a z	4.0 a y	3.7 b y	4.2 b y	3.7 b y

Figure 4. *Escherichia coli* counts in mixed vegetables inoculated with *E. coli*, treated with bioactive compounds and stored at (A) 15 and (B) 5 °C: ■, control; □, tea tree essential oil; ▩, propolis; ⊟, gallic acid. Data represent the mean of six determinations and vertical bars represent standard deviation of the mean. For each storage temperature, different letters (a, b) within columns indicate significant differences ($P < 0.05$) of *E. coli* populations among the treatments, and different letters (w, x, y, z) within rows indicate significant differences ($P < 0.05$) among the storage times.

Qualitative sensory evaluation

Table 1 shows the results obtained from sensory analysis of mixed vegetables untreated and treated with bioactive agents (tea tree, propolis and gallic acid) and stored at 15 and 5 °C. Immediately after treatment application, the panelists did not find any significant difference between control and treated samples, showing OVQ scores close to the optimal score (5) (Table 1).

At 5 days of storage, samples treated with propolis and tea tree and stored at 15 °C showed similar OVQ scores compared with control samples and were acceptable. However, at that time, samples treated with gallic acid showed sensory quality scores lower than the acceptability level (2.5) as a result of moderate celery browning, presence of off-odors and loss of butternut squash firmness (Table 1).

Up to 7 days of storage, all samples kept in refrigerated conditions (5 °C) were organoleptically acceptable (Table 1). At 10 days the only sample that showed an OVQ score higher than the acceptability level was the one treated with propolis. This improvement in the soup mix visual quality is associated with a delay in the appearance of browning in celery for samples treated with propolis (Table 1). Moreover, based on comments made by panelists, this treatment exerted a significant retention of the green color in leek (data not shown). Thus propolis treatment was effective in extending the sensory shelf life of refrigerated mixed vegetables by 3 days.

Table 1. Sensory evaluation of mixed vegetables for soup (celery, leek and butternut squash) treated with biopreservative agents during 7 days of storage at 15 °C and 14 days at 5 °C

		Sensory attribute									
		Overall visual quality		Celery browning		Odor		Celery firmness		Butternut squash firmness	
Time (days)	Treatment	15 °C	5 °C	15 °C	5 °C	15 °C	5 °C	15 °C	5 °C	15 °C	5 °C
0	Control	4.4 ± 0.6a	4.4 ± 0.6a	4.8 ± 0.2a	4.7 ± 0.2a	4.9 ± 0.2a	4.8 ± 0.2a	4.8 ± 0.2a	4.8 ± 0.2a	4.8 ± 0.2a	4.8 ± 0.2a
	Propolis	4.7 ± 0.3a	4.8 ± 0.2a	4.9 ± 0.2a	4.9 ± 0.2a	4.9 ± 0.2a	4.9 ± 0.2a	4.8 ± 0.2a	4.8 ± 0.2a	5.0 ± 0.2a	4.9 ± 0.2a
	Tea tree	4.7 ± 0.4a	4.7 ± 0.3a	4.9 ± 0.1a	4.9 ± 0.2a	4.1 ± 0.7a	4.2 ± 0.5a	4.8 ± 0.4a	4.8 ± 0.2a	5.0 ± 0.1a	4.8 ± 0.3a
	Gallic acid	4.2 ± 0.6a	4.5 ± 0.5a	4.3 ± 0.3a	4.8 ± 0.2a	4.9 ± 0.2a	4.9 ± 0.3a	4.9 ± 0.1a	4.6 ± 0.4a	4.7 ± 0.3a	4.5 ± 0.4a
2	Control	3.8 ± 0.6a	4.4 ± 0.4a	3.9 ± 0.8a	4.6 ± 0.4a	3.8 ± 0.7a	4.8 ± 0.3a	3.9 ± 0.5a	4.7 ± 0.4a	3.7 ± 0.4a	4.5 ± 0.4a
	Propolis	3.6 ± 0.9a	4.6 ± 0.2a	3.9 ± 0.5a	4.5 ± 0.4a	3.9 ± 0.7a	4.6 ± 0.6a	4.1 ± 0.6a	4.6 ± 0.4a	4.2 ± 0.3a	4.6 ± 0.4a
	Tea tree	4.1 ± 0.6a	4.2 ± 0.7a	4.3 ± 0.6a	4.3 ± 0.6a	3.9 ± 0.8a	3.9 ± 0.4a	4.5 ± 0.7a	4.2 ± 0.8a	4.2 ± 0.5a	4.6 ± 0.5a
	Gallic acid	3.2 ± 0.6a	3.9 ± 0.7a	3.6 ± 0.8a	4.2 ± 0.4a	4.5 ± 0.6a	4.7 ± 0.4a	3.6 ± 0.5a	4.5 ± 0.8a	3.7 ± 0.6a	4.4 ± 0.5a
5	Control	3.6 ± 0.6a	4.6 ± 0.3a	3.3 ± 0.7a	4.4 ± 0.4a	3.7 ± 0.6a	4.6 ± 0.3a	3.5 ± 0.4a	4.6 ± 0.2a	3.9 ± 0.4a	4.6 ± 0.3a
	Propolis	3.2 ± 0.7a	4.4 ± 0.5a	3.2 ± 0.6a	4.2 ± 0.6a	3.8 ± 0.5a	4.5 ± 0.4a	4.1 ± 0.5a	4.4 ± 0.2a	4.2 ± 0.2a	4.3 ± 0.7a
	Tea tree	3.2 ± 0.8a	4.3 ± 0.3a	2.9 ± 0.5a	3.7 ± 0.7a	3.8 ± 0.5a	4.1 ± 0.4a	4.1 ± 0.3a	4.3 ± 0.4a	4.2 ± 0.6a	4.3 ± 0.7a
	Gallic acid	1.6 ± 0.1b	3.3 ± 0.2b	2.7 ± 0.7b	3.7 ± 0.6a	2.4 ± 0.5b	4.4 ± 0.3a	3.6 ± 0.8a	4.5 ± 0.3a	1.8 ± 0.5b	4.1 ± 0.7a
7	Control	1.1 ± 0.6a	3.9 ± 0.7a	1.9 ± 0.8a	3.9 ± 0.8a	2.9 ± 0.6a	4.1 ± 0.8a	2.5 ± 0.4a	3.7 ± 0.6a	2.2 ± 0.7a	3.7 ± 0.7a
	Propolis	0.9 ± 0.5a	3.4 ± 0.8a	2.2 ± 0.8a	3.3 ± 0.7a	1.1 ± 0.7b	3.7 ± 0.9a	1.9 ± 0.6a	3.7 ± 0.4a	1.9 ± 0.5a	3.5 ± 0.4a
	Tea tree	0.8 ± 0.5a	3.7 ± 0.7a	1.8 ± 0.7a	3.3 ± 0.6a	1.4 ± 0.6b	3.2 ± 0.2a	2.9 ± 0.6a	3.5 ± 0.4a	1.8 ± 0.6a	3.8 ± 0.5a
	Gallic acid	0.7 ± 0.3a	3.3 ± 0.7a	1.4 ± 0.7a	3.2 ± 0.5a	2.3 ± 0.6ab	4.4 ± 0.3a	2.6 ± 0.5a	3.7 ± 0.5a	2.1 ± 0.7a	3.8 ± 0.5a
10	Control		1.4 ± 0.4b		1.9 ± 0.5b		3.3 ± 0.3ab		3.1 ± 0.5a		2.7 ± 0.5ab
	Propolis		4.0 ± 0.5a		3.5 ± 0.5a		3.9 ± 0.4a		3.9 ± 0.5a		3.5 ± 0.5a
	Tea tree		2.0 ± 0.8b		1.7 ± 0.4b		2.7 ± 0.6b		3.4 ± 0.4a		2.2 ± 0.5b
	Gallic acid		1.9 ± 0.8b		1.1 ± 0.7b		3.3 ± 0.5ab		3.7 ± 0.5a		2.5 ± 0.4ab
14	Control		0.5 ± 0.2b		0.6 ± 0.3a		1.7 ± 0.1a		1.3 ± 0.3a		0.7 ± 0.3a
	Propolis		1.4 ± 0.2a		1.5 ± 0.7a		1.9 ± 0.7a		1.5 ± 0.4a		0.5 ± 0.2a
	Tea tree		0.4 ± 0.1b		1.0 ± 0.6a		1.9 ± 0.6a		2.2 ± 0.4a		0.7 ± 0.3a
	Gallic acid		0.8 ± 0.1b		1.2 ± 0.4a		2.2 ± 0.2a		2.1 ± 0.3a		0.5 ± 0.3a

Data represent mean score ± standard deviation. Treatments at each storage temperature and time of storage were compared. Means followed by different letters are significantly different ($P < 0.05$).

DISCUSSION

In this work we present an approach to extend the shelf life of mixed vegetables for soup aimed to control quality decay through the application of BCs and appropriate refrigerated storage throughout the entire cold chain.

Total microbial counts on fresh-cut vegetables after processing usually range from 3.0 to 6.0 log CFU g^{-1} and depend greatly on the type of vegetable. The dominating bacterial population during low-temperature storage consists mainly of species belonging to the Pseudomonadaceae (especially *Pseudomonas fluorescens*) and Enterobacteriaceae and some species of lactic acid bacteria.[30] In contrast with bacteria, many different yeast species of comparable quantitative importance have been identified in these products. Additionally, molds are less important in fresh-cut vegetables owing to the intrinsic properties such as a slightly acid to neutral pH favoring bacteria and yeasts.[30] Accordingly, in our study, initial populations of mesophilic aerobic microorganisms and yeasts/molds in mixed vegetables (equal ratios of celery, leek and butternut squash) were 5.1–5.8 and 4.4–4.8 log CFU g^{-1} respectively. The initial mesophilic population of minimally processed leek was reported by Vandekinderen *et al.*[31] to vary between 5.6 and 7.3 log CFU g^{-1}. This high initial microbial load is related to preharvest contamination due to direct contact with soil, whereby the bacteria have the possibility to form biofilms that attach to or infiltrate vegetable tissues.[31] Roura *et al.*[32] reported that diced butternut squash contained initial mesophilic bacterial counts of 4.8 log CFU g^{-1} and initial yeast/mold counts of 2.9 log CFU g^{-1}. The microbial load of squash is expected to be lower than that of vegetables with stems and leaves, since the squash's shell protects the fruit against microbial infiltration. With regard to celery, Vandamm *et al.*[33] reported total aerobic bacterial counts ranging from 6 to 10 log CFU g^{-1} in fresh-cut ready-to-eat celery for sale in a supermarket. Minimally processed vegetables can also be contaminated before, during or after harvest with human pathogens. Possible contamination sources are seeds, soil, irrigation water, animals and human manipulation at harvesting, processing and packaging.[30]

An adequate storage temperature can maintain fresh-cut produce quality by slowing the respiration rate, enzymatic processes and microbial activity.[7] The generally recommended storage temperature is 0–4 °C for most fresh vegetables, as this temperature level keeps vegetables turgid and slows microbial contamination.[28,34] However, such low temperature is not always maintained throughout the entire cold chain. In this study we recorded significantly higher final mesophilic, psychrotrophic, yeast/mold and coliform counts in samples stored at 15 °C compared with those maintained at 5 °C. Likewise, Ukuku and Sapers[35] reported that populations of coliforms, aerobic mesophilic bacteria, yeasts/molds and *Pseudomonas* spp. in fresh-cut melons left at room temperature were significantly higher than those

in samples stored at 5 °C. Furthermore, Zhan *et al.*[8] and Olaimat and Holley,[34] working with minimally processed fresh fruits and vegetables, reported that good control of the refrigeration temperature limits the growth of spoilage and pathogenic microorganisms.

As reported above, the biopreservative effect of natural agents was observed when treatments were combined with optimal refrigeration storage temperature, in most cases. Abusive temperature conditions (15 °C) together with the high availability of nutrients in vegetables for soup allowed microbes to reach very high counts in a few hours and accelerate biochemical reactions. This fact might explain why the applied concentrations of bioactive agents were not effective in samples stored at 15 °C.

In this work the antimicrobial effects of tea tree essential oil, propolis extract and gallic acid sprayed on a vegetable mix were studied. The biological properties of tea tree and propolis, including antioxidant, antiviral, antiproliferative and antimicrobial activities, have been demonstrated by many researchers.[19,36,37] Tea tree essential oil consists of more than 100 different compounds, including terpinen-4-ol, which is one of the main antibacterial components.[38] Propolis samples usually contain more than 180 constituents and differ greatly owing to variation in their geographical and botanical origin.[39] The chemical compounds present in propolis are mainly polyphenols (flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones) and also sesquiterpene quinones, coumarins, steroids, amino acids and inorganic compounds. Many authors have attributed the antimicrobial properties of propolis to its phenolic constituents, mainly flavonoids.[39–41]

In a previous study, Alvarez *et al.*[26] reported significant antimicrobial effects of tea tree and propolis by *in vitro* assay against several indicator bacteria (*E. coli* and *L. monocytogenes*). They also reported that *in vivo* application of tea tree essential oil and propolis extract exerted a bacteriostatic effect on mesophilic and psychrotrophic populations when applied on minimally processed broccoli (stored at 5–7 °C). However, in the present study we found that these biopreservatives were not effective in controlling mesophilic bacteria growth on mixed vegetables for soup stored at 5 °C and showed a slight inhibitory action on psychrotrophics evidenced towards the end of refrigerated storage (10 days). Although in both experiments the BCs were applied by spraying and the concentrations used were similar, it is clear that the type of substrate greatly influenced the effectiveness of the treatments applied. Differences between vegetable substrates regarding native microflora, chemical composition, interactions with added BCs and the amount of cut surface are some of the reasons that could explain the differences in the results obtained.

In accordance with our results, Vega-Vega *et al.*[42] reported the effectiveness of mango seed extract (gallic acid 59%) as an antimicrobial agent applied on fresh-cut mango. This treatment showed 1.5 and 0.72 log unit reductions in yeasts/molds and mesophilic aerobes respectively after 15 days of refrigerated storage (5 °C).

In the present work we evaluated the growth and survival of endogenous *E. coli* and inoculated *E. coli* O157:H7 on a vegetable mix for soup (untreated and treated with BCs). Both endogenous and inoculated *E. coli* grew on samples stored at 15 °C. Similarly, Palumbo *et al.*[43] reported that several pathogenic *E. coli* strains can easily grow at 10 °C and thus suggested the potential for growth in temperature-abused refrigerated foods. Minimal temperature levels that allow the growth of generic *E. coli* and *E. coli* O157:H7 are generally believed to be 5 and 8 °C respectively.[44] In our study it was demonstrated that the naturally occurring *E. coli* population was able to grow under optimal refrigeration temperature. Thus it increased greatly up to day 5 of storage and then the growth rate declined. On the contrary, *E. coli* counts in inoculated samples at 5 °C did not increase but survived. This behavior may be explained by taking into account that this temperature is lower than the minimal level (8 °C) required for *E. coli* O157:H7 to grow. Probably, naturally occurring *E. coli* can adapt to the substrate and grow even at low temperatures, in contrast with artificially added *E. coli*. Similarly, Abadias *et al.*[45] found that *E. coli* O157:H7 did not grow but survived throughout refrigerated storage (5 °C) when inoculated on fresh-cut fruits (melon and pineapple) and vegetables (carrot and escarole). Also, Moreira *et al.*[11] found that the endogenous *E. coli* population on minimally processed broccoli increased slightly when samples were stored at 5 °C, while, on the contrary, inoculated *E. coli* O157:H7 did not grow and even decreased by 1 log unit during 20 days of storage.

With regard to the biopreservative treatments applied in our study, propolis extract and gallic acid effectively reduced the *E. coli* population on mixed vegetables for soup stored at 5 °C. Both agents showed antimicrobial activity against endogenous *E. coli* and also against inoculated *E. coli* O157:H7. Argentine propolis samples were studied as potential food preservers by Tosi *et al.*[20] These authors demonstrated that all tested ethanolic extracts of propolis successfully inhibited *E. coli*'s development *in vitro* and related higher antimicrobial activity to higher content of coumaric acid + syringic acid, quercetin, galangin, caffeic acid + crisine and total soluble compounds. Moreover, in a previous study, Alvarez *et al.*[26] demonstrated that propolis extract applied at 10–20 μL mL^{-1} is effective in significantly inhibiting the growth of this pathogen by *in vitro* assays. The inhibitory effects shown by propolis treatment in the present study were less significant compared with those obtained by *in vitro* assays. In this sense, several works reported that high concentrations of antimicrobial compounds were necessary to obtain significant inhibitory effects, because their effectiveness decreases when they are applied on a food substrate.[11,14,15,46]

Moreover, gallic acid was found to be effective in controlling the development of human pathogens. In this sense, Gutierrez-Larraínzar *et al.*[23] analyzed the *in vitro* antimicrobial activities of natural phenolic compounds against foodborne pathogens and spoilage bacteria and reported that gallic acid was effective in controlling *Staphylococcus aureus* (minimum inhibitory concentration range 0.09–1.6 mg mL^{-1}) at lower concentrations than those used in foods (maximum use level 2 mg mL^{-1}) according to the European flavoring industry. On the other hand, gallic acid was less effective in inhibiting the growth of *E. coli* (minimum inhibitory concentration range 3.2–6.4 mg mL^{-1}).

The sensory shelf life of vegetables can be defined as the length of time during which they maintain an appearance that appeals to the consumer.[47] An issue associated with ready-to-use vegetables is their short shelf life, which is usually no longer than 7 days under adequate storage conditions.[48] In general, little is known about the relationship between the outgrowth of spoilage microorganisms and their production of metabolites and how consumers perceive decay in minimally processed vegetables.[34]

Besides, the addition of BCs should not negatively affect the sensory properties of vegetable products. In this study, gallic acid treatment reduced the quality of the vegetable samples mainly under abusive temperature conditions, accelerating celery browning appearance, promoting off-odors and deteriorating the texture of pumpkin cubes. However, propolis treatment positively affected the quality of vegetables. Thus mixed vegetables for

soup treated with propolis and stored at 5 °C showed a sensory shelf life of 10 days, while untreated samples and those treated with tea tree and gallic acid showed a shorter sensory shelf life (7 days). As shown, the preservation of visual quality in mixed vegetables treated with propolis is related to a delay in the appearance of browning in celery. The antioxidative properties of propolis were reported by Nagai *et al.*,[49] who associated this activity with the presence of flavonoids such as quercetin, flavones, isoflavones, flavonones, catechin and isocatechin. Moreover, Chang *et al.*[50] demonstrated that propolis extract was effective as an anti-browning agent and greatly inhibited polyphenol oxidase (PPO) activity in treated sliced apples after 24 h of room storage. Also, a grape seed extract rich in flavonoids effectively delayed the appearance of browning and exerted inhibitory effects on PPO when applied on fresh-cut lettuce.[51]

CONCLUSIONS

The obtained results demonstrated that biopreservatives were effective only when combined with optimal refrigeration temperature. It was confirmed that good control of refrigeration temperature limits the growth of spoilage and pathogenic microorganisms. BCs (at the tested concentrations) showed slight effectiveness in controlling the microbiota present in mixed vegetables for soup (celery, leek and butternut squash), with the exception of coliforms that were greatly reduced by gallic acid and propolis treatments. Both agents combined with refrigerated conditions also showed antimicrobial activity against endogenous *E. coli* and inoculated *E. coli* O157:H7.

Moreover, the combination of propolis treatment with refrigerated storage conditions (5 °C) effectively preserved the quality and prolonged the sensory shelf life of fresh-cut mixed vegetables by 3 days. On the contrary, gallic acid damaged the sensory attributes of vegetables, making it unsuitable for technological application.

The use of natural agents to preserve the quality and safety of mixed vegetables for soup might be an interesting option. An alternative to produce more significant inhibitory effects, even under temperature abuse, would be the use of higher concentrations of these BCs, but this could be questionable owing to the organoleptic impact. Therefore, based on the concept of hurdle technologies, the use of BCs in combination with other barriers such as ultrasound, mild heat shock or edible coatings, besides using hygienic processing conditions and adequate storage temperatures, may contribute to assure the safety of minimally processed mixed vegetables, controlling pathogen growth and minimizing undesirable changes in organoleptic properties.

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