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PHYSICAL TREATMENTS AND PROPOLIS EXTRACT TO ENHANCE QUALITY ATTRIBUTES OF FRESH-CUT MIXED VEGETABLES

MARÍA V. ALVAREZ[1,2,3], ALEJANDRA G. PONCE[1,2], ROSARIO GOYENECHE[1,2] and MARÍA R. MOREIRA[1,2]

[1]Grupo de Investigación en Ingeniería en Alimentos, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina
[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Mar del Plata, Argentina

[3]Corresponding author.
TEL: +54 (0223) 481 6600;
FAX: +54 (0223) 481 0046;
EMAIL: mvalvarez@fi.mdp.edu.ar

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ABSTRACT

The impact of individual and combined application of propolis extract (PE) plus ultrasound (ULT) or thermal treatment (TT) on the microbiological, nutritional, physicochemical and sensory quality of fresh-cut mixed vegetables for soup (celery, leek and butternut squash) stored at 5C was studied. The use of PE slightly reduced microbial growth, inhibited the activity of browning related enzymes and improved quality attributes during refrigerated storage. Moreover, PE was effective to reduce ascorbic acid losses in the product maintaining its nutritional quality. TT greatly lowered the initial microbial load (1.7–2.2 log units) and also reduced microbial growth on mixed vegetables extending its microbiological shelf-life to 5 days. Furthermore, TT inactivated browning enzymes improving visual quality; however, ascorbic acid degradation adversely affected nutritional quality. Meanwhile, ULT caused a significant microbial inactivation, reduced browning enzyme activity and also the inhibitory effect on polyphenoloxidase enzyme was enhanced by combining ULT with PE.

PRACTICAL APPLICATIONS

The use of natural agents, as propolis extract, along with physical treatments to preserve the quality of fresh-cut 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 for health.

INTRODUCTION

The consumption of minimally processed vegetables, including ready-to-eat or ready-to-use products, has increased worldwide in last decade due to its convenience, freshness and improved quality. Fresh-cut vegetables, particularly salads, dominate the global production of minimally processed foods (Rojas-Graü *et al.* 2011). Also, various types of ready-to-cook vegetables for 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 (Amodio *et al.* 2006). To prepare these products, raw materials are subjected to preliminary operations, such as peeling and cutting, increasing tissue damage and causing the release of intracellular contents (Rico *et al.* 2007). These operations commonly encourage and increase the activity of pathogenic and saprophytic microorganisms and also produce quality losses.

Decontamination of fresh fruits and vegetables is an important unsolved technological problem. The chlorine and chlorinated compounds have already been used for several decades and these compounds are still the most widely used sanitizers in the food industry (Hua and Reckhow 2014; Al-Zenki *et al.* 2012). However, many researchers mentioned that excessive use of chlorine can be harmful due to the formation of carcinogenic disinfection by-products such as trihalomethanes, chloramines, haloketones, chloropicrins and haloacetic acids caused by the reaction of residual chlorine with organic matter (Ölmez and Akbas 2009; Cao *et al.* 2010). Moreover, since consumers demand less use of chemicals on minimally processed fruits and vegetables, more attention has been paid to the search of natural alternatives for preservation including combined physical and chemical treatments (Bilek and Turantaş, 2013).

Propolis is a bee product collected by honeybees from tree buds; it is used in beehives as a protective barrier against pathogenic microorganisms (Silva *et al.* 2012). Some Argentinean propolis showed antibacterial activity against antibiotic resistant human pathogenic bacteria (Isla *et al.* 2007). Propolis antioxidant, antibacterial and antifungal properties make it useful in food technology to be applied as a natural food preserver (Popova *et al.* 2007; Tosi *et al.* 2001; Viuda-Martos *et al.* 2008). Propolis chemical composition is complex and varies according to its botanical and geographical origin. Most propolis components are of phenolic nature, mainly flavonoids. Substances, which are identified in propolis, generally are typical constituents of food and/or food additives, and are recognized as GRAS (generally recognized as safe) substances (Tosi *et al.* 2001). In a previous work developed by Alvarez *et al.* (2013), it was demonstrated that propolis extract was effective in controlling the growth of *Escherichia coli* O157:H7, *Listeria monocytgenes* and the native microflora of several vegetables by *in vitro* assays. Moreover, previous studies demonstrated that propolis extract improved sensory quality, moderately controlled the native microflora growth and reduced endogenous and inoculated *E. coli* counts in fresh-cut vegetables such as celery, leek and butternut squash (Alvarez *et al.* 2015a,b).

Thermal treatments are well known preservation technologies to reduce the microbial load of food and have proved to be effective in controlling microbial growth in fresh-cut fruits and vegetables when applied at appropriate time and temperature conditions (Amodio *et al.* 2006; Aguayo *et al.* 2008). Heat shocks have been applied by immersion in hot water to preserve quality and reduce enzymatic and microbial spoilage of various vegetables such as lettuce, celery and carrots during storage (Loaiza-Velarde *et al.* 2003; Delaquis *et al.* 1999; Loaiza-Velarde *et al.* 2000; Moreira *et al.* 2006; Moreira *et al.* 2005; Alegria *et al.* 2012). Some biochemical changes occur in plants as a response to stress caused by the heat treatment, such as disruption of protein synthesis including enzymes related to quality (polyphenoloxidase, peroxidase, phenylalanine ammonia-lyase and pectin methyl esterase) and also changes in the kinetic properties of these enzymes (Loaiza-Velarde *et al.* 2000). Moreover, thermal treatments can destroy nutritional components (vitamins) and deteriorate sensory characteristics when applied at high temperatures in fresh vegetables. Besides, ultrasound constitutes a nonthermal technology which contributes to the increase of microbial safety and prolongs shelf-life, especially in food with heat-sensitive nutritional, sensory and functional characteristics (Alegria *et al.* 2009; Cao *et al.* 2010). Higher-power ultrasound applied at low frequencies (20–100 kHz) has the ability to cause cavitation, which is useful in food technology to inactivate microorganisms (Bilek and Turantaş 2013).

The concept of multitarget preservation of foods, by employing a combination of treatments to increase the product stability, hence extending shelf life, is highly applicable to fresh-cut fruits and vegetables processing. This hurdle approach is based on the assumption that different techniques applied in a food might not have just an additive preservation effect, but they could act synergistically (Leistner 2000). Nevertheless, combination of techniques could also generate antagonistic effects. This is especially relevant when considering the combination of physical and chemical treatments. The combination of ultrasound/heat shock with other natural preservation alternative could be an attractive approach to enhance microbial inactivation and prevent decay; hurdle effects due to the use of ultrasound plus sanitizer solutions have been demonstrated in different raw materials such as plum fruit, strawberries, apples, lettuce and red bell pepper (Cao *et al.* 2010; Chen and Zhu 2011; Alexandre *et al.* 2012; Alexandre *et al.* 2013).

However, so far the combined effects of physical treatments with natural preservatives to inhibit microbial growth and to extend the shelf-life of fresh-cut mixed vegetables have not been evaluated. On the basis of these considerations, the aim of this study was to investigate the effects of ultrasound and thermal treatments combined with a natural antimicrobial agent such as propolis extract on the microbiological, nutritional, physicochemical and sensory quality of fresh-cut mixed vegetables for soup (celery, leek and butternut squash). Hence, in this work we evaluated the antimicrobial effect of individual and combined preservation treatments on the evolution of the native microflora (mesophilic, psychrotrophic bacteria, coliforms and yeasts/molds), activity of enzymes related to browning of vegetables (polyphenoloxidase and peroxidase), ascorbic acid content and sensory attributes during refrigerated storage of mixed vegetables.

MATERIALS AND METHODS

Biopreservative Agent

Propolis extract (PE) from commercial origin (Juricich, Mendoza, Argentina) was used as biopreservative agent in this work. It was prepared from raw material collected in Mendoza province (Argentina) using ethanol and water for extraction and was standardized to a concentration of 10 g propolis/100 mL extract. PE showed a total phenolic content of 18.8 mg gallic acid equivalents/mL, determined following the method proposed by Singleton *et al.* (1999); total flavonoids content was 37 mg quercetin equivalents/mL, determined according to Kim *et al.* (2012).

Sample Preparation and Application of Treatments

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 transported to the laboratory in refrigerated containers with polyfreezer (refrigerated gel to maintain cold chain, Thermics Argentina SA). Squashes of uniform size and color were selected and then were hand-peeled, washed thoroughly with tap water and a stainless steel hand slice was used to prepare diced squash (15 mm). The processed dices were dipped in tap water (3 min) to remove intracellular contents released after cutting operation; after that, the surface moisture was removed with a manual salad centrifuge. A similar methodology was carried out with celery petioles and leek using slices of 10 mm thickness. Mixed vegetables (1:1:1) were subjected to the following individual and combined treatments: ultrasound (ULT), thermal treatment (TT), propolis extract (PE), ultrasound plus propolis extract (ULT-PE), thermal treatment plus propolis extract (TT-PE). Untreated control samples were prepared washing, cutting, dipping in tap water and drying the vegetables. A conventional washing procedure using chlorinated water (CL) was also included as a positive control given that it is traditionally used by the fresh produce industry.

To apply chlorine, the cut produce was placed in a basket and immersed in a chlorinated solution (sodium hypochlorite, 120 ppm) for 2 min (Martín-Diana *et al.* 2012); subsequently were rinsed in tap water for 2 min. For ultrasonic treatment, vegetables were immersed in an ultrasonic bath (Testlab, Argentina) during 40 min at a constant frequency of 40 kHz (conditions were selected based on preliminary experiments). Thermal treatment was carried out by immersion of vegetables in a well-stirred water bath for 90 s at 70C. These conditions were selected based on preliminary experiments. For this, vegetables were subjected to different thermal treatments (temperature 60, 70 and 80C/time 60 and 90 s) and a high microbial reduction along with minimal damage to texture and appearance were considered to select the optimum TT. Afterward, vegetables were removed from the hot water and dipped in cold water (5C, 1 min). In all cases, immersion treatments (CL, ULT and TT) were developed using 100 g vegetable product per liter of water and the surface moisture was removed with a manual salad centrifuge.

PE was applied to mixed vegetables at 64 μL/mL; this concentration was selected based on previous *in vitro* and *in vivo* assays. To prepare this solution, PE was diluted in sterile distilled water and vigorously shaken at 30C for 30 min. Minimally processed vegetables were hand-sprayed with the PE solution (4 mL per 300 g) and the solution remained in contact with the surface of the vegetables during the storage. In ULT-PE and TT-PE treatments, propolis was added after physical treatments application.

After being treated, the mixed vegetables were placed in plastic containers (approximately 100 g), covered with a 15 μm polyethylene wrap sealed with heat. These containers were placed in a refrigerated chamber and stored at 5C for 10 days. Three containers per treatment were removed from storage and used for physicochemical and microbiological analysis and two containers for sensory evaluation, at each sampling time.

Microbiological Analysis

Microbial counts were determined within 1 h of treatment application (day 0) and after 2, 5, 7 and 10 days of storage at 5C and three replicates were used. For microbiological analysis, about 10 g of treated mixed vegetables (celery, leek and squash at 1:1:1) were macerated in 90 mL phosphate buffer solution (0.1 mol/L) and were homogenized with a Stomacher 400 Circulator Homogenizer (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 by using the following culture media and culture conditions: mesophilic aerobic bacteria on Plate Count Agar (PCA) incubated at 30–32C for 48–72 h; psychotropic bacteria on the same medium incubated at 5–7C for 5–7 days; Enterobacteriaceae and total coliforms in Mac Conkey agar incubated at 30–32C for 24 h and molds and yeasts in Yeast-Glucose-Chloramphenicol (YGC) medium incubated at 25C for 5 days. All culture mediums used were purchased from Britania (Buenos Aires, Argentina). Microbial counts were expressed as log CFU/g.

Ascorbic Acid Content

Ascorbic acid (AA) contents were determined by the titrimetric assay described by Moreira *et al.* (2009). Twenty grams of mixed vegetables were homogenized with 40 mL of oxalic acid solution at 20 g/kg. This mixture was vacuum filtered through glass fiber. Five milliliter aliquots of the filtrate were titrated with 2,6 dichloroindophenol. Ascorbic acid contents were reported as mg/100 g of sample on a wet basis. Determinations of ascorbic acid contents were performed using three separate replicates (three containers) per treatment at each storage time.

Polyphenoloxidase and Peroxidase Activity

For polyphenoloxidase (PPO) assay, 10 g of mixed vegetables (1:1:1) were homogenized at a 1:2 (g:mL) ratio with 0.5 mol/L phosphate buffer (pH 7.0) in the presence of 50 g/L polyvinylpyrrolidone (ICN Biomedicals, Inc., Irvine, CA) with a

commercial mixer and centrifuged at 12,700 × g for 30 min. The supernatant, which contained PPO activity, was used as the experiment enzyme source (PPO crude vegetable extract). Crude extract was maintained at 0C until use. The substrate mixture contained 20 mmol/L of catechol in 5 mmol/L sodium phosphate buffer (pH 7). The reaction cuvette contained 2.9 mL of substrate mixture and 0.1 mL PPO crude vegetable extract. The rate of catechol oxidation was followed spectrophotometrically at 25C with a UV-1601 PC UV-visible spectrometer (Shimadzu Corporation, Kioto, Japan) at 400 nm for 60 and 120 s. One unit of activity (UA) was defined as a 0.001 change in absorbance per min under the assay conditions.

For peroxidase (POD) assay, 10 g of mixed vegetables (1:1:1) were homogenized with 30 mL of distilled water in a commercial blender. The slurry was filtered through two layers of cheesecloth and centrifuged during 15 min at 10,000 × g. All steps were carried out at 4C. The supernatant, which contained POD activity, was used as the enzyme source for the experiment (Ponce *et al.* 2004). The activity was determined spectrophotometrically at 25C with a UV-1601 PC UV-visible spectrometer (Shimadzu Corporation, Kioto, Japan) at 470 nm using guaiacol as substrate and H_2O_2 as hydrogen donor (Hemeda and Klein 1990). The substrate mixture contained 10 mL of guaiacol solution at 10 μl/mL, 10 mL of hydrogen peroxide solution at 3 mg/mL and 100 mL of 0.05 mol/L sodium phosphate buffer (pH 6.5). The reaction cuvette contained 2.9 mL substrate mixture and 0.1 mL of POD crude extract in a total volume of 3 mL. In order to use the right levels of enzymatic activity, the assay volume was adjusted to an adequate dilution to ensure linearity of the assay. One unit of activity (UA) was defined as a change in absorbance of 0.001/min (Ponce *et al.* 2004).

For PPO and POD assays, the reference cuvette contained only the substrate mixture. Extractions and enzyme activity determinations were conducted using three separate replicates (three containers) per treatment at each storage time. Enzyme activity results were expressed as relative activity according to $UA(PPO)/UA(PPO)_0$ and $UA(POD)/UA(POD)_0$, where $UA(PPO)_0$ and $UA(POD)_0$ represent the initial enzyme activity for PPO and POD, respectively, showed by the control sample at day 0.

Sensory Evaluation

At each storage time, mixed vegetables (untreated and treated with individual and combined treatments) were subjected to a panel of testers to evaluate sensory quality of samples. Sensory analysis was carried out as described by Alvarez *et al.* (2015a). A panel comprised of nine members of the UNMdP Food Engineering Group, aged 25-50 years, and with sensory evaluation experience in vegetable quality, was trained and carried out the evaluation of celery, leek and 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. Hence, the panel defined five critical sensory attributes to be evaluated on the mixed vegetables, such as overall visual quality (OVQ), celery browning, odor of the product, celery firmness and butternut squash firmness and also agreed on the methods for assessing these attributes.

Mixed vegetables were removed from storage conditions and tempered at room temperature before sensory evaluations. The coded (three digit) samples were presented one at the time in random order 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 the attributes evaluated was quantified on a 5-cm unstructured intensity scale. OVQ is a unique value given by panelists on the basis of attributes visually perceived as freshness, surface brightness and uniformity of color; it was scored from 0 (highly deteriorated aspect) to 5 (appealing/fresh aspect). Celery browning was rated from 0 (very severe) to 5 (no presence); odor from 0 (intense off-odors) to 5 (fresh); firmness from 0 (very soft) to 5 (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 end of shelf-life (Jacxsens *et al.* 2005; Piagentini *et al.* 2005).

Statistical Analysis

The results showed in this study are expressed as mean values with their standard deviations. Experiments were established with two factors (treatment and storage time) using a completely randomized design ($n = 3$). Analysis of variance (ANOVA) 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. Data were analyzed using InfoStat 2013 statistical software.

RESULTS AND DISCUSSION

Microbial Growth

The inhibitory effects of individual and combined hurdles on the growth of native microflora in mixed vegetables (celery, leek and butternut squash) were evaluated. Figure 1a,b show the evolution of aerobic mesophilic and psychrotrophic bacteria during the storage of mixed vegetables. The growth of Enterobacteriaceae and yeasts and molds populations was also studied, although the corresponding results

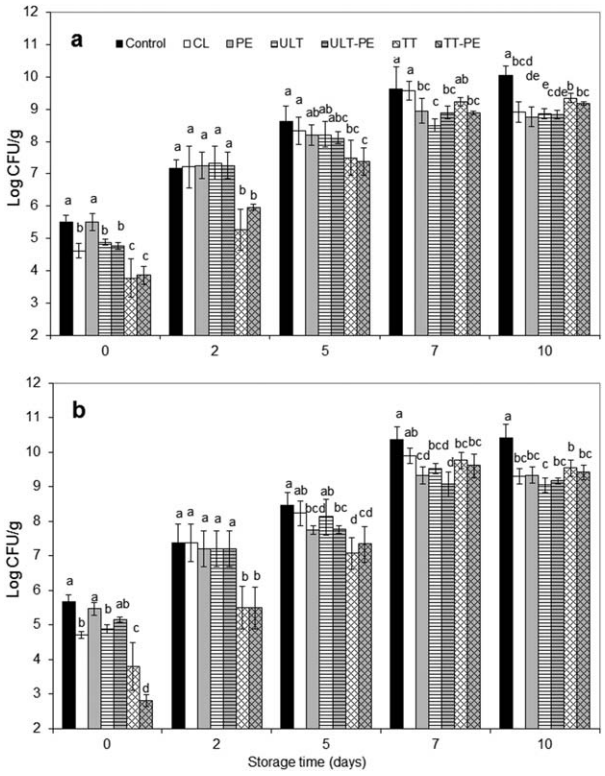

FIG. 1. MICROBIAL GROWTH FROM MIXED VEGETABLES SUBJECTED TO INDIVIDUAL AND COMBINED PRESERVATION TREATMENTS AND STORED AT 5C DURING 10 DAYS. (a) MESOPHILIC BACTERIA AND (b) PSYCHROTROPHIC BACTERIA
Vertical bars represent standard deviation of the means ($n = 3$). Letters differentiate statistically significant values within the same sampling time.

are not shown since the effects of the treatments were similar compared to mesophilic and psychrotrophic bacteria.

Initial mesophilic count in untreated mixed vegetables was 5.5 $\log_{10}$ (CFU/g) and significantly increased (1.7 log) between days 0 and 2. Untreated sample exceeded the microbiological limit imposed by French law for total mesophilic bacteria (7.7 log) (Lucera *et al.* 1997) on the fifth day (Fig. 1a). The evolution of psychrotrophics (Fig. 1b), Enterobacteriaceae and yeasts and molds (data not shown) in untreated samples was similar to that described for mesophilics during refrigerated storage. CL treatment reduced by 0.9–1.0 log the initial microbial load of the analyzed microbial populations (Fig. 1a,b). However, it was not effective in controlling microbial growth during storage. Besides, PE applied as a single hurdle did not affect the initial microbial load of mixed vegetables; however, it significantly reduced counts of mesophilic (Fig. 1a) and Enterobacteriaceae by 0.8–1.3 log from the seventh day of storage with compared with control. For psychrotrophics, the inhibitory effect of PE (0.8–1.0 log reductions) was observed from day 5 until the end of the storage period (Fig. 1b).

ULT treatment showed a slight but significant microbial inactivation (0.7–0.8 log) on the studied populations. Also, this treatment reduced mesophilic and psychrotrophic counts by 1.2 log compared with control, in particular, at the end of the storage (Fig. 1a,b). The use of ultrasound combined with propolis treatment (ULT-PE) did not enhance the individual effects of each hurdle. In general, the combination of both treatments did not show significant differences compared with the inhibitory effects of each individual treatment ($P > 0.05$) (Fig. 1a,b).

As was expected, the application of thermal treatment (TT) on mixed vegetables caused a significant decrease on the initial microbial load of the product with reductions of 1.7–1.9 log in mesophilic and psychrotrophic bacteria (Fig. 1a,b) and 2.0–2.2 log in Enterobacteriaceae and yeasts and molds (data not shown). Moreover, TT was able to extend the shelf-life of the product from a microbiological point of view keeping mesophilics below the microbiological limit for 5 days in contrast to what was observed for control samples (Fig. 1a). However, a high nutrient availability and a reduced competition from other microorganisms allowed the growth of surviving bacteria to high levels similar to those observed in control sample at the end of storage. Besides, combining TT with a subsequent application of propolis (TT-PE) was not effective to inhibit the growth of microorganisms that survived to the applied temperature, since no significant differences ($P > 0.05$) were observed between the individual application of TT and the combination TT-PE along the storage for the studied populations (Fig. 1a,b). As an exception, TT-PE caused a greater initial reduction on psychrotrophic bacteria (2.8 log) compared with that observed for TT (1.8 log).

Propolis is constituted by a wide variety of substances such as polyphenols, quinones, coumarins, steroids, amino acids and inorganic compounds. It is known that simple

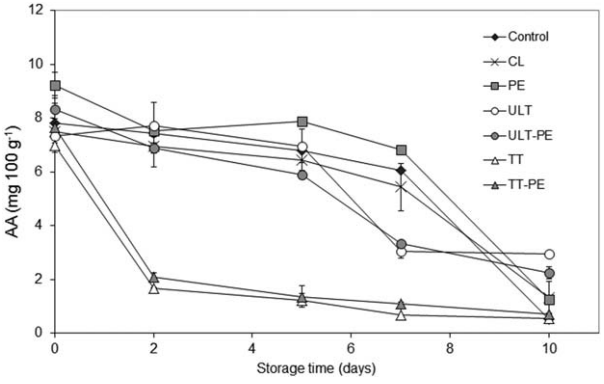

FIG. 2. ASCORBIC ACID CONTENT OF MIXED VEGETABLES SUBJECTED TO INDIVIDUAL AND COMBINED PRESERVATION TREATMENTS AND STORED AT 5C DURING 10 DAYS
Vertical bars represent standard deviation of the means ($n = 3$).

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). In a previous study, Alvarez *et al*. (2013) reported that PE exerted a bacteriostatic effect on mesophilic and psychrotrophic populations, when applied on minimally processed broccoli (stored at 5–7C). A recent study developed by Alvarez *et al*. (2015b) showed that PE exerted a significant inhibitory action on the native microflora growth in minimally processed celery, leek and butternut squash treated as individual substrates and stored at 5C. In all experiments PE was applied by spraying, and the concentrations used were similar; however it is clear that the type of substrate, regarding its native microflora, chemical composition and amount of cut surface greatly influenced the effectiveness of treatments applied (Gutierrez *et al*. 2008; Juneja *et al*. 2002; Davidson *et al*. 2013). Mixing of different fresh-cut vegetables as squash, celery and leek results in a complex product where the interaction of three living plant substrates occur into the container; furthermore, the mixture is composed of a much more diverse indigenous microflora which can greatly affect the effectiveness of antimicrobial treatments.

According to several reported studies, high power ultrasound applications showed moderate microbial reductions when used in the wash-water decontamination process of fresh fruits and vegetables. Thereby, applying 45 kHz for 10 min reduced mesophilic and yeasts/molds counts by 0.6 and 0.5 log, respectively, in strawberries (Cao *et al*. 2010). In shredded carrots, Alegria *et al*. (2009) found 1.3 log reduction in mesophilic counts when a 45 kHz treatment during 1 min was applied.

Besides, Alegria *et al*. (2012) working with grated carrot reported that a heat treatment with water at 100C for 45 s (applied before grating) reduced 2.5 log the initial load of aerobic mesophilic bacteria; meanwhile, washing the grated carrot with chlorinated water (200 ppm, 1 min) showed a 1.9 log reduction. Contrarily to our results, these authors affirmed that the heat treatment greatly reduced microbial growth finding a total increase of only 0.6 log in microbial counts for 7 days of storage at 5C probably due to the higher temperature applied. However, in grated carrot washed with chlorinated water, at 7 days of storage the microbial counts reached the initial level observed in untreated control (Alegria *et al*. 2012).

Ascorbic Acid Retention

Ascorbic acid (AA) is one of the most important nutritional quality factors in many horticultural crops and has many activities in the human body. AA is heat and oxygen labile and therefore gives a sensitive indication of the effects of processing on the relative nutritional quality of vegetables (Moreira *et al*. 2008). AA content in untreated and treated mixed vegetables (celery, leek and butternut squash) stored at 5C is shown in Fig. 2. The control sample initially contained 7.8 ± 0.7 mg AA/100 g (day 0). For fresh cut butternut squash, Moreira *et al*. (2008) reported a AA content of 12.5 mg/100 g. In minimally processed celery, Viña and Chaves (2008) found 3.6 mg AA/100 g. Besides, Bernaert et al. (2012) evaluated AA content in thirty different leek cultivars and reported results between 0.89 and 3.55 mg AA/g in dry basis.

With regard to the effect of treatments, none of them significantly reduced AA content immediately after being applied. Contrarily, PE application showed a significant increase in initial AA content when compared to untreated control ($P < 0.05$), showing values of 9.2 mg/100 g at day 0 (Fig. 2). It is not possible that the increase observed in mixed vegetables AA content is due to the addition of vitamin when applying PE, since the AA content of this extract is actually very low. Consequently, since the contribution of AA that could exert PE is much lower than the increase observed in the PE treated sample, it could be hypothesized that PE would produce a protective effect on AA, avoiding vitamin degradation after vegetable processing.

AA degradation in untreated samples during the first 7 days of storage was 23% compared to the initial content; however, at day 10, AA retention was almost nil (Fig. 2). In this regard, Lee and Kader (2003) affirmed that fruit and vegetables exhibit a gradual reduction ascorbic acid content as the temperature and/or storage time increases.

In samples subjected to thermal treatments (TT), no significant changes in AA content were obtained compared to untreated sample immediately after treatments application. However, AA degradation was induced later, up to the second day, with AA losses of 75% (Fig. 2). Regarding the effect of ULT, AA content in treated samples was lower compared to control only at day 7 although a higher AA retention was observed at the end of storage (Fig. 2).

PE applied alone was the only treatment that achieved a greater retention of AA in the product compared with control showing significant differences ($P < 0.05$) during refrigerated storage (Fig. 2). As was mentioned above, PE would protect AA preventing its degradation due to the antioxidant power of flavonoids and polyphenols present in the extract. Nevertheless, in general, PE spraying after physical treatments as TT and ULT did not enhance AA retention in mixed vegetables. In the case of CL treatment, no significant differences were observed ($P > 0.05$) in AA content compared to control along the storage (Fig. 2).

In accordance with our results, Viña and Chaves (2007) reported that AA content in minimally processed celery was reduced by 10% after a heat treatment at 50C for 90 s; after 14 days of storage at 0C, total AA losses of 17% were observed. Moreover, Gómez and Artés (2005) also

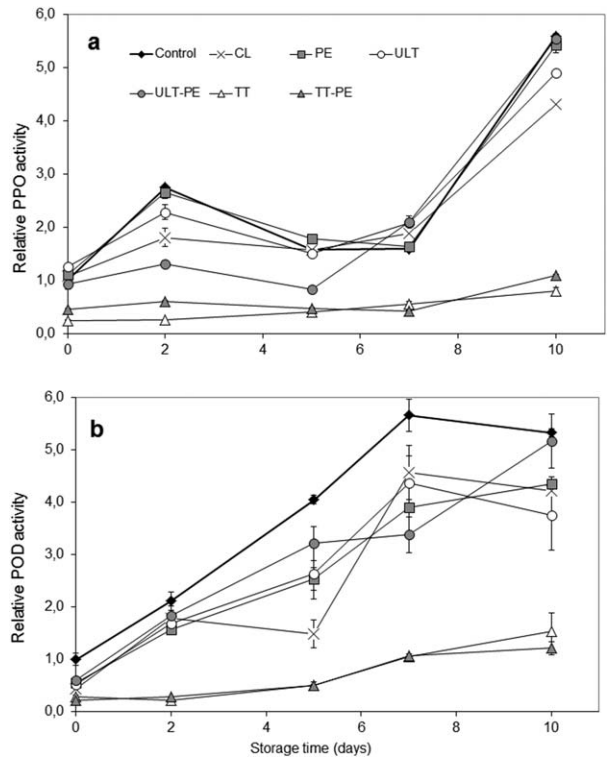

FIG. 3. RELATIVE PPO (a) AND POD (b) ACTIVITIES IN MIXED VEGETABLES SUBJECTED TO INDIVIDUAL AND COMBINED PRESERVATION TREATMENTS AND STORED AT 5C DURING 10 DAYS
Vertical bars represent standard deviation of the means ($n = 3$).

demonstrated that AA content in minimally processed celery significantly decreased after 15 days of storage at 4C. Furthermore, similarly to the observed for PE in our study, Martin-Diana *et al.* (2008) showed that the application of a green tea extract rich in flavonoids (catechins) on minimally processed lettuce exerted a protective effect by reducing AA losses during storage at 4C.

Browning-Related Enzymes Activities

The main enzymes responsible for the browning of fruits and vegetables are PPO and POD. The first, mainly located in plastids, catalyzes the oxidation of phenolics with subsequent formation of dark compounds. POD performs single electron oxidation on a wide variety of compounds in the presence of hydrogen peroxide (Cefola *et al.* 2012). Although PODs are widely distributed in plants, their role in the enzymatic browning of vegetables is still being studied, because the internal level of hydrogen peroxide in plants limits POD activity (Cefola *et al.* 2012). However, during the oxidation of phenolic compounds in PPO catalyzed reactions, a hydrogen peroxide generation can be occurred. Hence, the main agent responsible for enzymatic browning in fruits and vegetables is PPO, although a possible synergistic effect between PPO and POD cannot be excluded (Tomás-Barberán and Espin 2001).

In this study, PPO and POD activities were monitored as indicators of browning in mixed vegetables subjected to the individual and combined treatments along the refrigerated storage (Fig. 3). Total increases for PPO and POD activities in the control (untreated) sample after 10 days of storage were similar, reaching almost six times the initial activity values observed for the fresh control sample. However, it can be observed that the evolution of PPO and POD activities through time was different (Fig. 3a,b). In this regard, Cefola *et al.* (2012) reported that PPO and POD activities measured in six artichoke cultivars during cold storage were on average correlated by 74%; however the behavior of the cultivars was not uniform and both enzymes followed different kinetics.

Regarding to the effect of treatments on PPO activity, TT and TT-PE were the only procedures that significantly inactivated PPO in mixed vegetables, reducing the initial PPO activity by more than 50% when compared to control (Fig. 3a). However, the application of PE after TT did not cause any additional inhibitory effect. Inactivation of PPO enzyme in vegetables by the use of heat shocks has been described in several reports (Saltveit 2000; Martín-Diana *et al.* 2012). Martín-Diana *et al.* (2005) studied the effect of heat shocks at 50C on PPO and POD activity of fresh-cut iceberg lettuce. These authors demonstrated that a heat shock for 1 min only caused a 6% loss of activity for both enzymes, as direct inactivation. However, the reduction of enzymatic activity in lettuce during refrigerated storage could be attributed to a possible indirect effect caused by lack of phenolic

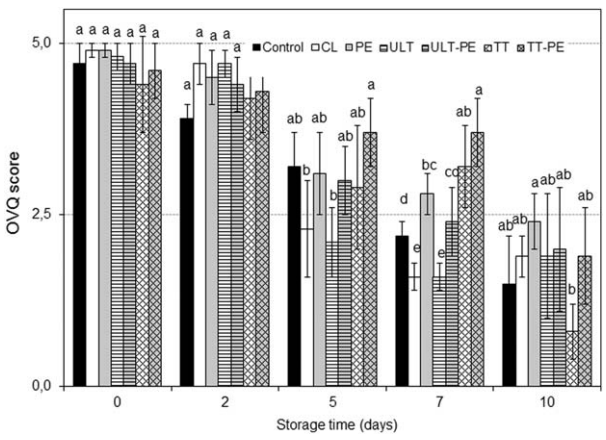

FIG. 4. OVERALL VISUAL QUALITY (0 = HIGHLY DETERIORATED ASPECT TO 5 = APPEALING/FRESH ASPECT) OF MIXED VEGETABLES SUBJECTED TO INDIVIDUAL AND COMBINED PRESERVATION TREATMENTS AND STORED AT 5C DURING 10 DAYS
Vertical bars represent standard deviation of the means ($n = 9$). Letters differentiate statistically significant values within the same sampling time.

TABLE 1. SENSORY EVALUATION (CELERY BROWNING, ODOR, CELERY FIRMNESS, BUTTERNUT SQUASH FIRMNESS) OF MIXED VEGETABLES SUBJECTED TO INDIVIDUAL AND COMBINED PRESERVATION TREATMENTS AND STORED AT 5C DURING 10 DAYS ($n = 9$)

Time (days)	Treatment	Celery browning	Odor	Celery firmness	Butternut squash firmness
	Control	4.9 ± 0.1^{a}	4.8 ± 0.2^{a}	$4.5 \pm 0.6^{a,b,c}$	4.9 ± 0.2^{a}
	CL	4.9 ± 0.2^{a}	4.8 ± 0.2^{a}	$4.7 \pm 0.4^{a,b}$	4.8 ± 0.1^{a}
	PE	4.9 ± 0.1^{a}	4.8 ± 0.3^{a}	4.8 ± 0.1^{a}	$4.8 \pm 0.1^{a,b}$
0	ULT	4.9 ± 0.1^{a}	4.8 ± 0.3^{a}	4.8 ± 0.2^{a}	$4.8 \pm 0.1^{a,b}$
	ULT-PE	4.9 ± 0.1^{a}	4.3 ± 0.5^{a}	$4.5 \pm 0.4^{a,b,c}$	4.9 ± 0.2^{a}
	TT	4.9 ± 0.2^{a}	4.4 ± 0.8^{a}	3.0 ± 1.2^{c}	4.2 ± 0.4^{c}
	TT-PE	5.0 ± 0.1^{a}	4.4 ± 0.5^{a}	3.3 ± 0.9^{bc}	$4.3 \pm 0.3^{b,c}$
	Control	4.4 ± 0.5^{a}	4.2 ± 0.6^{a}	4.6 ± 0.4^{a}	4.9 ± 0.2^{a}
	CL	4.8 ± 0.2^{a}	4.4 ± 0.7^{a}	4.7 ± 0.5^{a}	4.6 ± 0.4^{a}
	PE	4.6 ± 0.6^{a}	4.3 ± 0.8^{a}	4.8 ± 0.2^{a}	4.7 ± 0.4^{a}
2	ULT	4.7 ± 0.4^{a}	4.4 ± 0.6^{a}	$4.3 \pm 0.7^{a,b}$	4.8 ± 0.2^{a}
	ULT-PE	4.6 ± 0.3^{a}	4.6 ± 0.3^{a}	4.8 ± 0.2^{a}	4.8 ± 0.2^{a}
	TT	4.5 ± 0.7^{a}	3.8 ± 1.2^{a}	$3.7 \pm 0.7^{b,c}$	4.5 ± 0.5^{a}
	TT-PE	4.6 ± 0.6^{a}	4.0 ± 0.8^{a}	3.4 ± 0.9^{c}	4.4 ± 0.4^{a}
	Control	$3.5 \pm 0.7^{a,b}$	3.7 ± 0.9^{a}	4.4 ± 0.3^{a}	3.8 ± 0.7^{a}
	CL	2.2 ± 1.1^{b}	2.6 ± 0.6^{a}	4.0 ± 1.0^{a}	$3.1 \pm 1.3^{a,b}$
	PE	3.7 ± 0.6^{a}	3.8 ± 1.2^{a}	4.5 ± 0.4^{a}	3.8 ± 0.6^{a}
5	ULT	$3.1 \pm 1.1^{a,b}$	3.3 ± 0.9^{a}	3.7 ± 0.5^{a}	2.1 ± 1.0^{b}
	ULT-PE	3.6 ± 0.9^{a}	4.0 ± 0.4^{a}	4.2 ± 0.5^{a}	$3.1 \pm 0.3^{a,b}$
	TT	4.2 ± 0.8^{a}	3.1 ± 1.2^{a}	1.7 ± 0.5^{b}	1.9 ± 0.3^{b}
	TT-PE	4.3 ± 0.9^{a}	4.1 ± 0.4^{a}	2.5 ± 0.9^{b}	$3.2 \pm 0.2^{a,b}$
	Control	3.3 ± 0.6^{b}	$2.4 \pm 0.9^{a,b}$	4.1 ± 0.5^{a}	$1.9 \pm 0.6^{b,c}$
	CL	2.5 ± 0.8^{b}	1.4 ± 0.6^{b}	3.4 ± 0.6^{a}	0.8 ± 0.6^{c}
	PE	$3.1 \pm 0.3^{b,c}$	$2.8 \pm 1.0^{a,b}$	4.1 ± 0.5^{a}	3.2 ± 0.3^{a}
7	ULT	2.4 ± 0.4^{c}	$2.2 \pm 0.4^{a,b}$	3.5 ± 0.6^{a}	$1.8 \pm 1.1^{b,c}$
	ULT-PE	$3.1 \pm 0.7^{b,c}$	3.5 ± 1.0^{a}	3.8 ± 0.5^{a}	$2.3 \pm 1.2^{a,b}$
	TT	4.5 ± 0.4^{a}	3.7 ± 0.7^{a}	1.7 ± 0.8^{b}	$1.9 \pm 0.9^{b,c}$
	TT-PE	4.5 ± 0.5^{a}	3.9 ± 0.7^{a}	2.0 ± 0.5^{b}	4.0 ± 0.5^{a}
	Control	$2.9 \pm 1.0^{a,b,c}$	2.1 ± 1.0^{a}	3.5 ± 0.9^{a}	1.3 ± 0.5^{a}
	CL	1.7 ± 1.3^{c}	1.5 ± 0.5^{a}	3.1 ± 0.7^{a}	1.6 ± 0.6^{a}
	PE	$3.3 \pm 1.2^{a,b}$	2.2 ± 0.6^{a}	3.8 ± 0.9^{a}	1.4 ± 0.6^{a}
10	ULT	$2.8 \pm 1.2^{a,b,c}$	2.0 ± 0.6^{a}	3.9 ± 0.4^{a}	1.3 ± 0.5^{a}
	ULT-PE	$2.2 \pm 0.9^{b,c}$	1.7 ± 0.7^{a}	3.0 ± 0.8^{a}	1.2 ± 0.4^{a}
	TT	$3.6 \pm 1.1^{a,b}$	1.3 ± 0.6^{a}	0.6 ± 0.3^{b}	0.9 ± 0.4^{a}
	TT-PE	4.2 ± 0.5^{a}	2.4 ± 1.0^{a}	1.4 ± 0.5^{b}	1.8 ± 0.5^{a}

For each sensory attribute, data represent mean score ± standard deviation. Treatments at each time of storage were compared. Means followed by different letters are significantly different ($P < 0.05$).

compounds (substrate) or to a direct effect on a receptor implicated in the synthesis of PPO and POD. Besides, PE and ULT as individual treatments did not produce significant changes in PPO activity during refrigerated storage when compared to control (Fig. 3a). However, the combined use of both treatments (ULT-PE) significantly ($P < 0.05$) reduced PPO activity in mixed vegetables at days 2 and 5 of storage; the application of ultrasound on vegetable tissues may facilitate the penetration of propolis to exert its antioxidant activity.

Moreover, all the applied treatments caused a significant ($P < 0.05$) decrease of initial POD activity in mixed vegetables; reductions of 40–56% were observed for CL, ULT, PE and ULT-PE (Fig. 3b). Similarly to the observed for PPO, TT inactivated POD by 72% while the addition of PE to the TT treated samples did not enhance the inhibitory effect. TT and TT-PE kept relative POD activity (with respect to POD activity of control at day 0) below 1.0 throughout 7 days of storage (Fig. 3b).

According to our results, Alegria *et al.* (2012) demonstrated that a heat shock at 100C for 45 s applied on carrots was effective in inhibiting POD activity by 30%; in the same study, a chlorine treatment (200 ppm, 1 min) resulted less effective than heat shock although both procedures reduced POD activity with respect to untreated control during storage. Furthermore, one of the major changes affecting quality of minimally processed celery is the vascular browning in cut surfaces. Loaiza-Velarde *et al.* (2000) reported that a

thermal treatment at 70C for 1 min reduced browning in celery slices. This treatment significantly reduced phenylalanine ammonia-lyase activity (involved in phenolic compounds synthesis as a response to stress conditions) and browning potential in celery stored at 10C.

Qualitative Sensory Evaluation

OVQ scores obtained from sensory analysis of mixed vegetables untreated and treated with combined or individual procedures and stored at 5C are shown in Fig. 4. Scores obtained for other quality attributes such as celery browning, odor of the product, celery firmness and butternut squash firmness are described in Table 1.

Immediately after treatments application, the panelists did not find significant differences ($P > 0.05$) between control and treated samples, showing OVQ scores close to the optimal score (5) (Fig. 4). PE, ULT and CL treatments did not affect quality attributes after being applied (day 0, Table 1). However, significant losses in vegetables firmness (mainly in squash cubes) were observed in thermally treated samples (Table 1).

Up to 5 days of storage all the samples, with the exception of CL and ULT, resulted organoleptically acceptable with OVQ scores higher than the acceptability level (2.5) (Fig. 4). Between days 5 and 7, squash firmness was the attribute that suffered major changes limiting the shelf-life of the product (Table 1). At 7 days of storage, the only samples that showed acceptable OVQ scores were those treated with PE, TT and combined TT-PE while other samples including control were unacceptable (Fig. 4). When PE was applied alone or in combination with TT, the improvement in visual quality of mixed vegetables was observed along with a higher retention of squash firmness with respect to control (day 7) (Table 1). Moreover, at that time, a significant ($P < 0.05$) inhibition of celery browning in samples subjected to the thermal treatment (TT and TT-PE) was observed. Finally, in general, no significant differences ($P > 0.05$) were observed in quality attributes of samples treated with ULT and CL when compared to control (Table 1).

Recently, Hernández *et al.* (1990) assessed the impact of a heat shock (immersion in water at 60C for 120 s) on the sensory quality of several minimally processed vegetables. These authors reported that this treatment significantly reduced respiration rate and preserved the sensory quality of celery, carrot and zucchini after 24 h of storage at 8C; however, the odor, texture and color of broccoli and cauliflower was negatively affected.

CONCLUSIONS

The use of PE on mixed vegetables for soup was able to reduce microbial growth, inhibit enzymatic activity and improve quality attributes during refrigerated storage. Moreover, it was effective to reduce ascorbic acid losses in the product. Besides, TT greatly lowered the initial microbial load and also reduced microbial growth on mixed vegetables extending its microbiological shelf-life. Furthermore, TT significantly inhibited enzymatic activity related to browning of vegetables and also improved visual quality of the product. However, nutritional quality was adversely affected by TT due to the degradation of ascorbic acid. As an advantage, combined TT-PE treatment had a positive effect on the retention of squash firmness. Meanwhile, ULT caused a slight microbial inactivation and reduced POD activity but it was not effective to maintain sensory quality of vegetables. Also, the inhibitory effect on PPO enzyme was enhanced by combining ULT with PE. Finally, it is remarkable that washing vegetables with chlorine solutions is widely used in the fresh-cut industry; nevertheless, in this study, chlorine showed a limited effectiveness as antimicrobial agent and was not able to extend the shelf-life of mixed vegetables.

The use of natural agents as PE along with physical treatments to preserve quality of mixed vegetables for soup might be an interesting option. The hurdle methods assessed in this study improved some of the quality indicators in mixed vegetables; antagonistic effects between treatments were not observed. However, in general the preservative effect of combined treatments was not enhanced with respect to the individual application of each treatment. Therefore, an alternative in the search of more effective preservation methods would be the use of PE in combination with other obstacles such as ozone washing, UV-light or edible coatings, besides using hygienic processing conditions and adequate storage temperatures.

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