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Antimicrobial efficiency of chitosan coating enriched with bioactive compounds to improve the safety of fresh cut broccoli

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ABSTRACT

Antimicrobial properties of chitosan (CH) coatings and CH enriched with bioactive compounds (BC) and essential oils (EO) were determined by *in vitro* and *in vivo* assays on minimally processed broccoli. The efficiency of CH plus BC/EO in improving the safety of broccoli was tested against the native microflora. Also, its effects on the survival of *Escherichia coli* and *Listeria monocytogenes* inoculated in broccoli were evaluated.

In vitro assays performed in tea tree, rosemary, pollen and propolis demonstrated significant inhibitory effects on *E. coli* and *L. monocytogenes* counts while pomegranate and resveratrol presented reduced activity. *In vivo* application of these BC on broccoli exerted a bacteriostatic effect on mesophilic and psychrotrophic populations except for rosemary. The application of CH alone or enriched with BC/EO resulted in a significant reduction in mesophilic and psychrotrophic counts. Between days 5 and 7, significant reductions (2.5 log) were observed in samples treated with CH + BC. The enrichment with BC improved the antimicrobial action of CH. The application of these coatings did not introduce deleterious effects on the sensory attributes of broccoli.

CH coatings enriched with BC/EO were a good alternative for controlling not only the microorganisms present in broccoli, but also the survival of *E. coli* and *L. monocytogenes*.

1. Introduction

In recent years, the demand for broccoli for fresh or ready to eat salad consumption has increased greatly (Vallejo, García-Viguera, & Tomás-Barberán, 2003). The main problem that makes fresh cut broccoli a highly perishable product is the ease of microbial growth (Rivera-Lopez, Vasquez-Ortiz, Ayala-Zavala, Sotelo-Mundo, & Gonzalez-Aguilar, 2005). Cutting or slicing operations greatly increase tissue damage and cause the released of intracellular contents (González-Aguilar et al., 2009). The release of cellular substrates supports and increases the activity of pathogenic and saprophytic microorganisms. This is why the development of new technologies to reduce broccoli deterioration and safety problems is much needed.

There is a new tendency in food technology preservation that consists of developing materials with film-forming capacity and antimicrobial properties which help improve food safety and shelf life. Edible coatings, formed with Generally Recognized As Safe materials, offer several advantages over synthetic materials, such as being biodegradable and environmentally friendly (Tharanathan, 2003). Moreover, some edible coatings have the potential to improve food appearance and delay or inhibit the growth of pathogenic and spoilage microorganisms (Dutta, Tripathi, Mehrotra, & Dutta, 2009; Quintavalla & Vicini, 2002).

The incorporation of antimicrobial agents in coatings is emerging as a promising technology, as it establishes contact with food and inhibits the growth of microorganisms present on the surface (Santiago-Silva et al., 2009).

In this context, chitosan coatings result adequate for their application in food preservation (Dutta et al., 2009). The chitosan coating creates a semipermeable barrier that controls gas exchange and reduces water loss, thereby maintaining tissue firmness and reducing microbial decay of harvested vegetables for extended periods (Devlieghere, Vermeulen, & Debevere, 2004; Dong, Cheng, Tan, Zheng, & Jiang, 2004; Thommohaway, Kanlayanarat, Uthairatanakij, & Jitareerat, 2007).

Various natural compounds could be used to improve the antimicrobial activity of chitosan coatings. The essential oils and

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bioactive compounds are an attractive option of natural preservatives. The information available on their biological activity in edible coatings is still scarce.

There are a few scientific works describing the effects of chitosan edible coatings enriched with biopreservatives on minimally processed broccoli to control microbial spoilage and to ensure the vegetable's safety. The present study had the objective to develop and evaluate the antimicrobial effect of chitosan edible coatings enriched with bioactive compounds (BC) and essential oils (EO). Native microflora evolution (mesophilic and psychrotrophic) of fresh cut broccoli was followed during refrigerated storage. Also, the effects of chitosan coatings combined with BC or EO on the survival and growth of *Escherichia coli* and *Listeria monocytogenes* inoculated in broccoli were evaluated. Moreover, as the sensory quality is the property with greater impact on purchase decision it is essential to evaluate how the coating treatment impacts the sensory quality of the product.

2. Materials and methods

2.1. Plant material

Broccoli heads (*Brassica oleracea* L. var. *Italica*) were directly obtained from a local producer in Mar del Plata, Argentina. Heads were immediately transported to the laboratory within 1 h of harvesting, in refrigerated containers with polyfreezer (refrigerated gel for maintaining cold chain, Thermics Argentina SA). Before the application of the chitosan coating, heads were separated into florets and stems and rinsed with chlorinated water (100 μL/L) for 3 min, then washed by immersion in tap water for 1 min and drained.

2.2. Essential oils and bioactive compounds

The essential oils used in this work were purchased from Nelson and Russell (London, England), which supplies food grade oils. The essential oils used for *in vitro* test were: tea tree (*Melaleuca alternifolia*), rosemary (*Rosmarinus officinalis*), clove (*Syzygium aromaticum*), lemon (*Citrus limonum*), oreganum (*Origanum vulgare*), calendula (*Calendula officinalis*) and aloe vera (*Aloe ferox*). The bioactive compounds used in this study were: bee pollen (Crinway S.A., Argentina), ethanolic extract of propolis (Jurisich, Argentina), pomegranate dried extract (*Punica granatum* L.) and resveratrol (3, 4′, 5-Trihydroxy-*trans*-stilbene). Pomegranate and resveratrol (Sigma) were initially dissolved in 1 mL of DMSO (Biopack, Argentina).

2.3. Preparation of coating-forming solutions

Medium molecular weight Chitosan (deacetylation degree (DD) = 98%) was supplied by ACOFAR (Argentina), and food grade glycerol from Mallinckrodt (Paris, KY, USA). Chitosan solutions (2 g/100 mL) (Xu, Kim, Hanna, & Nag, 2005) were prepared by dispersing chitosan powder in acetic acid solution (1 mL/100 mL) with magnetic stirring at 23 °C. To achieve complete chitosan dispersion, the solution was stirred overnight at room temperature and centrifuged to remove impurities. Then, it was sterilized at 121 °C for 15 min (Park, Daeschel, & Zhao, 2004). Glycerol was added as plasticizer to obtain flexible coatings that could be folded and manipulated without breakage. Glycerol content was added to achieve a glycerol/chitosan (Gly/CH) weight ratio of 0.28.

2.4. Culture maintenance and inoculum preparation

E. coli O157:H7, ATCC 43895 (American Type Culture Collection), provided by CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, La Plata, Argentina) and *L. monocytogenes* provided by CERELA (Centro de Referencia de Lactobacilos, Tucumán, Argentina) were used. A stock culture was maintained on tryptic soy broth (Britania, Buenos Aires, Argentina) at 4 °C. Before it was used, the O157:H7 and *L. monocytogenes* were cultured in Brain–Heart Infusion (BHI, Britania, Buenos Aires, Argentina) for 24 h at 37 °C. 0.1 mL of culture was transferred to 9.9 mL of BHI at two consecutive 24 h intervals immediately before each experiment.

2.5. In vitro assay

2.5.1. Preparation of broccoli native microflora

Native microflora from broccoli was prepared from 10 g of raw material macerated in 90 mL of phosphate buffer solution (0.1 mol/L), using a Stomacher 400 Circulator Homogenizer (pH 7.2) and incubated overnight at 37 °C, in agreement with the procedure reported by Moreira, Ponce, del Valle, and Roura (2007).

2.5.2. Determination of sensitivity

The sensitivity of the broccoli native microflora to different EO and BC was determined by the agar diffusion method. Sterile paper discs (Whatman N° 40; 6.0 mm in diameter, Britania) were soaked with pure tea tree and rosemary EO, pure pollen and propolis extract and diluted (60 mg/mL) pomegranate and resveratrol. DMSO was included as a negative control for pomegranate and resveratrol. Then, the paper discs were placed on the surface of the inoculated BHI agar plates. The dishes were incubated at 37 °C for 24–48 h and the zones of inhibition were measured. The sensitivity to the different biopreservatives was classified by the diameter of the inhibition halos as: not sensitive, for diameter less than 8 mm; sensitive, for diameter 9–14 mm; very sensitive, for diameter 15–19 mm and extremely sensitive, for diameter larger than 20 mm (Moreira, Ponce, Del Valle, & Roura, 2005; Ponce, Fritz, Del Valle, & Roura, 2003). Each assay was performed in duplicate on 3 separate experimental runs.

2.5.3. Tube-assay method

Test tubes with 5 mL of BHI broth were inoculated with 1 mL of inoculum obtained from the native microflora of broccoli (approximately 10^4–10^5 CFU/mL). Then, 4 mL of CH coating-forming solutions and acetic acid solvent (2 mL/100 mL) were added. At 0 h and after 24 h incubation at 37 °C the optical density of the broths at 610 nm was measured with the UV–Visible spectrophotometer (Shimadzu Corporation UV 1601 PC UV–Visible, Kyoto, Japan) (Moreira, Roura, & Ponce, 2011). Each assay was performed in duplicate on 3 separate experimental runs.

2.5.4. Microdilution agar plate method

Aliquots of 10 mL of Luria–Bertani broth (LB, triptone 1 g/100 mL, yeast extract 0.5 g/100 mL and NaCl 1 g/100 mL) were agitated vigorously with the BC or EO to achieve different final biopreservative concentrations (0.5–8.0 μL/mL for tea tree and rosemary; 60–180 μg/mL for pomegranate and resveratrol; and 1.0–40.0 μL/mL for pollen and propolis). DMSO (3 μL/mL) was included as a negative control for pomegranate and resveratrol, taking into account the maximum concentration used to dissolve these BC. Then, 100 μL of an overnight culture of *E. coli* and *L. monocytogenes* were added. Inoculated solutions were mixed followed by incubation at 37 °C during 32 h. The viable *E. coli* and *L. monocytogenes* counts were monitored as follows: 0.1 mL sample of each treatment were spread on the surface plating on LB agar. The plates were incubated at 37 °C for 24–48 h and the numbers of colonies were determined. Microbial counts were expressed as log CFU/mL. Each assay was performed in duplicate on 3 separate experimental runs.

2.6. In vivo assay

2.6.1. Essential oils and bioactive compounds application

Essential oils (EOs) were applied to the minimally processed broccoli (as was described in 2.1 Plant material section) in different concentrations (5, 10 and 15 μL/mL for tea tree and 6, 12 and 18 μL/mL for rosemary). Bioactive compounds (BC) were used in different concentrations (30, 60, 80, 100, 120 and 180 μg/mL for pomegranate and resveratrol; 3, 6, 10 and 12 μL/mL for pollen and propolis). EOs and BC were diluted in sterile distilled water and vigorously shaken at 30 °C for 30 min to obtain reasonably stable dispersions. Minimally processed broccoli was hand-sprayed with the EOs and BC solutions to a load of approximately 77 mL/m^2, and the oil solutions were allowed to remain in contact with vegetable surfaces during the 7 days of storage. In control samples, broccoli was sprayed with tap water. The BC were used in the concentrations previously detailed, but since significant inhibitory effects were only observed at higher concentrations, only these results are shown in the Results section.

2.6.2. Coating application

Broccoli florets were immersed in different solutions (chitosan or chitosan plus EO/BC) for 3 min at 20 °C. After edible coating application, broccoli florets were dried by exposure to flowing air at 30 °C and 60% relative humidity for 60 min in a controlled drying chamber (Pharma SCT, Argentina) to set a coat of the coatings on their surfaces. Fresh broccoli florets immersed in distilled water and subjected to the same drying conditions were used as control sample.

For inoculation, 100 μL of *E. coli* O157:H7 and *L. monocytogenes* bacterial suspension were added to chitosan solution (final pathogen concentration of approximately 3–4 log CFU/g). Then, broccoli florets were immersed in this solution. Control samples were: uncoated and non inoculated broccoli and uncoated samples inoculated with pathogens.

After being treated, broccoli florets (with or without the pathogen inoculation) were placed in polymeric coating bags (PD960, CRYOVAC, Argentina) of 25 μm of thickness (with an O_2 permeability of 7000 cc/m^2/d, CO_2 permeability of 20,000 cc/m^2/d, and water vapor permeability of 1 g/m^2/d), placing 3 broccoli florets per bag (approximately 60–90 g). Bags were sealed (SERVIVAC, Argentina) and stored in a refrigerated chamber at 5–7 °C for 7 days. Broccoli florets from five bags were sampled immediately (day 0) and after 2, 5 and 7 days of storage. At each storage time and for each applied treatment five bags were used for microbiological and sensory analysis. Microbial counts were performed in duplicate from two bags and sensory analysis was performed in duplicate from three bags. The assays were carried out on 3 independent experimental runs.

2.6.3. Microbiological studies

For microbiological analysis, 10 g of broccoli from each treatment bag were macerated in 90 mL of phosphate buffer solution (0.1 mol/L, pH 7.2) and were homogenized with a Stomacher 400 Circulator Homogenizer (Ponce et al., 2003). Serial dilutions (1:10) of each homogenized sample were made and surface spread in duplicate. The enumeration and differentiation of microorganisms were performed according to Ponce, Roura, Del Valle, and Fritz (2002) by using the following culture media and culture conditions: mesophilic aerobic bacteria on Plate Count Agar (PCA) incubated at 30–32 °C for 48–72 h and psycrotrophic bacteria on the same medium incubated at 5–7 °C for 5–7 d. The viable *E. coli* and *L. monocytogenes* counts were monitored as follows: 0.1 mL sample of each treatment was spread on the surface of Eosin Methylene Blue (EMB) agar plates or *Listeria* selective medium plates (soya triptein 3 g/100 mL, yeast extract 0.6 g/100 mL, and monopotassium phosphate 0.13 g/100 mL, disodium phosphate 0.96 g/100 mL, sodium piruvate 0.11 g/100 mL, acriflavine hydrochrolide 0.001 g/100 mL, nalidixic acid 0.004 g/100 mL, cycloheximide 0.001 g/100 mL). The colonies were counted after incubation at 37 °C for 24–48 h and expressed as log CFU/mL. All culture mediums were from Britania, Buenos Aires, Argentina.

2.7. Qualitative sensory evaluation

At each storage time, three individual bags of each broccoli treated samples were subjected to a panel of testers to evaluate sensory quality. This panel was comprised of nine members from the UNMdP Food Engineering Group, aged 30–50, trained for this task and with sensory evaluation experience in vegetable quality.

Evaluations were performed immediately after broccoli removal from storage conditions. The coded (3 digit) samples were presented one at a time in random order to the members who sat at a round table and made independent evaluations.

Sensory quality indices such as color, texture, brightness, floret opening, smell and browning were evaluated. The intensity of the attributes evaluated was quantified on a scale from 1 to 5 in the way described by Olarte, Sanz, Echávarri, and Ayala (2009). Color was rated using 5 = dark green, uniform color, 3 = light green and 1 = showing yellowish florets. Brightness was rated using 5 = bright, glossy surface, 3 = lighter bright and 1 = opaque surface. Texture was rated using 5 = crispy, 3 = rubbery and 1 = very soft. Florets opening was rated using 5 = very tight and firm heads, 3 = slightly loose but acceptable and 1 = very loose and limp. Smell was rated using 5 = no off-odor, 3 = slight but obvious off-odor and 1 = strong off-odor. Browning was rated using 5 = no browning, 3 = moderate browning and 1 = extreme browning. The texture was evaluated by the fracture of broccoli stems with the fingers as described by Rico et al. (2007).

The limit of acceptance was three, indicating that a score below 3 for any of the attributes evaluated was deemed to indicate end of shelf life (Rico et al., 2007).

2.8. Statistical analysis

Results reported are means (estimated by the least squares method) accompanied by their standard errors. Variance analysis

Table 1
Sensitivity of broccoli native microflora and *E. coli* to biopreservatives by the agar diffusion method.

Biopreservatives	Inhibition zone diameter (mm)[a]	
	E. coli ATCC 43895	Native microflora
Melaleuca alternifolia	18.0 ± 1.0[a]	17.8 ± 1.2[a]
Rosmarinus officinalis	19.0 ± 2.3[a]	16.9 ± 2.0[a]
Pomegranate	18.8 ± 0.9[a]	19.5 ± 1.8[a]
Resveratrol	17.5 ± 1.1[a]	16.6 ± 1.5[a]
Pollen	16.5 ± 0.9[a]	17.0 ± 1.2[a]
Propolis	16.0 ± 1.0[a]	16.2 ± 1.5[a]
Syzygium aromaticum	9.8 ± 1.5[b]	10.5 ± 1.2[b]
Aloe vera	10.0 ± 1.3[b]	8.5 ± 1.0[b]
Origanum vulgare	11.5 ± 0.2[b]	10.9 ± 0.8[b]
Calendula	10.1 ± 1.8[b]	8.3 ± 1.0[b]
Citrus limonum	9.3 ± 1.0[b]	8.9 ± 1.2[b]

[a] The diameter of the filter paper discs (6 mm) is included. The sensitivity to the different antimicrobial agents was classified by the diameter of the inhibition halos as: not sensitive, diameters less than 8 mm; sensitive, diameters 9–14 mm; very sensitive, diameters 15–19 mm; and extremely sensitive, diameters larger than 20 mm. Each assay was performed in duplicate on three separate experimental runs. Values followed by the same lowercase letters in the same column were not significantly different ($P > 0.05$).

(ANOVA) was applied to the data using a statistical package (MATLAB). Differences among samples were determined by the Tukey–Kramer multiple comparison test. Wherever differences are reported as significant, a 95% confidence level was used (Khuel, 2001, p. 37).

3. Results and discussion

3.1. In vitro assay

Antimicrobial inhibition zones for essential oils (EOs) and bioactive compounds (BC) against the native microflora of broccoli and *E. coli* are shown in Table 1. The agar Diffusion Method was used to determine the susceptibility to 11 different biopreservatives. It was demonstrated that the negative control DMSO showed no inhibition halos, which ensures that the solvent did not affect the inhibitory activity of pomegranate and resveratrol. The results showed that tea tree, rosemary, pomegranate, resveratrol, pollen and propolis produced 16–20 mm in diameter inhibition zones, thus becoming the EO and BC with the highest inhibitory effects. Therefore, the native microflora and *E. coli* resulted very sensitive to these biopreservatives. Elgayyar, Draughon, Golden, and Mount (2001) reported similar results for rosemary on *E. coli*, showing inhibition zones ranging from 23 to 30 mm in diameter.

Moreover, *E. coli* and the native microflora of broccoli showed a lower susceptibility to oregano, calendula, lemon, clove and aloe vera, with 8–11 mm diameter inhibition halos.

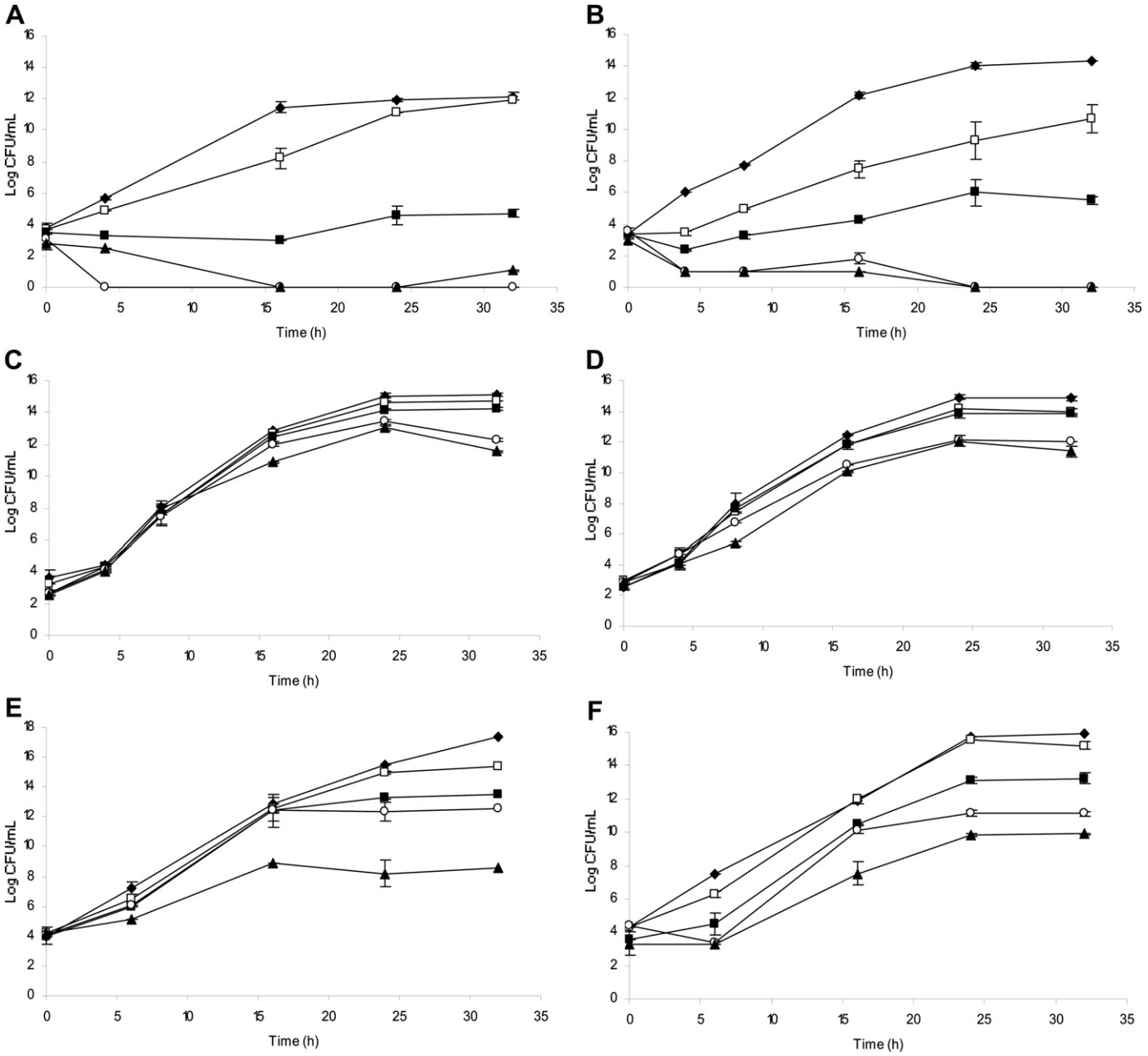

Fig. 1. Inhibitory effects of essential oils (EOs) and bioactive compounds (BC) on *E. coli* (left column graphs) and *L. monocytogenes* (right column graphs). (A–B correspond to tea tree: (◆) control (□) 0.5 μL/mL (■) 1 μL/mL (○) 2 μL/mL (▲) 4 μL/mL); (C–D resveratrol: (◆) control (□) 60 μg/mL (■) 80 μg/mL (○) 100 μg/mL (▲) 120 μg/mL); and (E–F propolis: (◆) control (□) 2 μL/mL (■) 4 μL/mL (○) 10 μL/mL (▲) 20 μL/mL).

The effects of EOs and BC on *E. coli* and *L. monocytogenes* survival were determined by Microdilution agar plate method. These results are presented in Fig. 1(A–F). The use of tea tree (Fig. 1A–B) and rosemary (data not shown) indicated that *E. coli* and *L. monocytogenes* were significantly sensitive to these biopreservatives. Tea tree and rosemary essential oils showed bacteriostatic effect on pathogen counts at low concentrations (0.5–1 μL/mL). At the end of storage, significant reductions were observed in these counts, being the antibacterial activity of tea tree and rosemary more significant on *L. monocytogenes* counts (5 log decrease with 0.5 μL/mL of EO) (Fig. 1B), compared to *E. coli* (3 order log with 1 μL/mL of EO) (Fig. 1A). Besides, tea tree and rosemary at higher concentrations than 2 μL/mL showed a bactericidal effect against *E. coli* and *L. monocytogenes* during storage (Fig. 1A–B).

Furthermore, *E. coli* and *L. monocytogenes* were less sensitive to resveratrol (Fig. 1C–D) and pomegranate. These BC applied at low concentrations (60–100 μg/mL) did not show significant antimicrobial effects on both pathogens. *E. coli* and *Listeria* growth was significantly ($P < 0.05$) sensitive to pomegranate (data not shown) and resveratrol (Fig. 1C–D) applied at higher concentrations with reductions of approximately 3–4 order log in pathogen counts. It was demonstrated that the maximum concentration of DMSO used in this assay to dissolve pomegranate and resveratrol had no effect on the growth of *L. monocytogenes* and *E. coli* (data not shown). In addition, low concentrations (4–10 μL/mL) of propolis were able to produce significant reductions in *E. coli* and *L. monocytogenes* counts. At the end of storage the highest concentration of propolis (20 μL/mL) reduced the pathogens counts in 5–7 order log (Fig. 1E–F). Compared to propolis, higher concentrations were required to produce bacteriostatic effect on *L. monocytogenes* and *E. coli* when pollen was applied (20 and 40 μL/mL respectively; data not shown). In general, for all bioactive compounds tested, the antimicrobial activity was found to be concentration dependent.

The results presented by *in vitro* assay revealed the potential of EOs and BC analyzed as natural preservatives to use on minimally processed broccoli.

Coma, Martial-Gros, Garreau, Copinet, and Deschamps (2002) reported a poor inhibitory activity of the CH solution in agar medium; this happens due to the fact that only the microorganisms in direct contact with the active sites of the polymer are inhibited because chitosan cannot diffuse through the adjacent agar media. Due to the low diffusivity of chitosan biopolymer in agar diffusion method, we determined the antimicrobial activity of CH coating-forming solutions by tube-assay method. Table 2 shows the inhibitory effects exerted by CH solutions at 0 h and after 24 h of incubation at 37 °C. The native microflora of broccoli was strongly inhibited ($P < 0.05$) by acetic acid (2 mL/100 mL) and by CH solutions. It is well known that CH shows its antibacterial activity only in an acidic medium, which is usually ascribed to the poor solubility of this biopolymer at high pH (Liu, Wang, & Sun, 2004). These authors reported that antimicrobial activity might be the effect of dissolved chitosan in acidic media, such as acetic acid (Devlieghere et al., 2004).

Table 2
Effects of acetic acid and chitosan film forming solution on the native microflora of broccoli (OD at 610 nm).

Time (h)	Control	Chitosan	Acetic acid
0	0.225	0.295	0.415
24	1.689	0.436	0.130

Each assay was performed in duplicate on three separate experimental runs.

3.2. In vivo assay

The antimicrobial effects of EO and BC on broccoli native microflora are shown in Fig. 2. When tea tree and rosemary EOs were applied at 5, 10 and 6 and 12 μL/mL, respectively, they did not produce any inhibitory effects. In a similar way, pomegranate and resveratrol (at 30–100 μg/mL), pollen and propolis (at 3–10 μL/mL) did not show any effects on mesophilic and psychrotrophic bacteria. Therefore, these results are not shown in this work. Fig. 2(A–C) shows the effects of tea tree and rosemary (at 15 μL/mL), pomegranate and resveratrol (at 120 μg/mL) and pollen and propolis (at 12 μL/mL) on the mesophilic

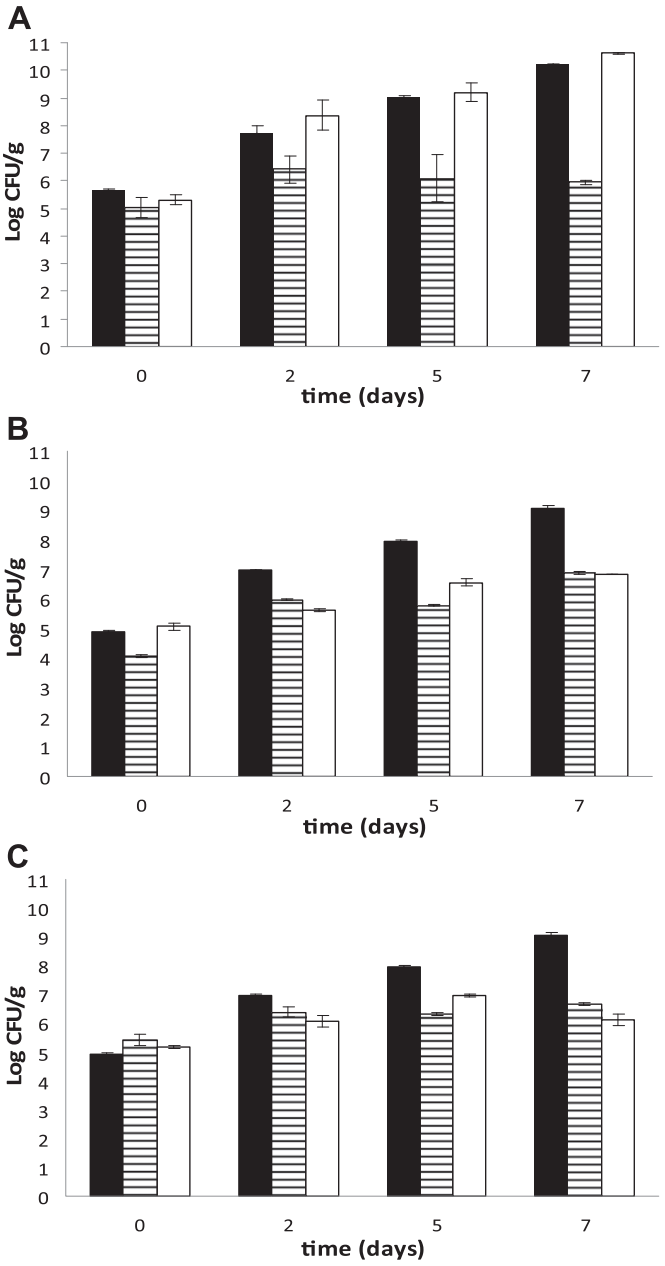

Fig. 2. Mesophilic bacteria counts in minimally processed broccoli, treated with essential oils and bioactive compounds, during refrigerated storage. (A): (■) control sample (⊟) sample treated with tea tree (□) sample treated with rosemary. (B): (■) control sample (⊟) sample treated with pomegranate (□) sample treated with resveratrol. (C): (■) control sample (⊟) sample treated with pollen (□) sample treated with propolis. Data are the mean ± s.d. of 12 determinations ($n = 12$).

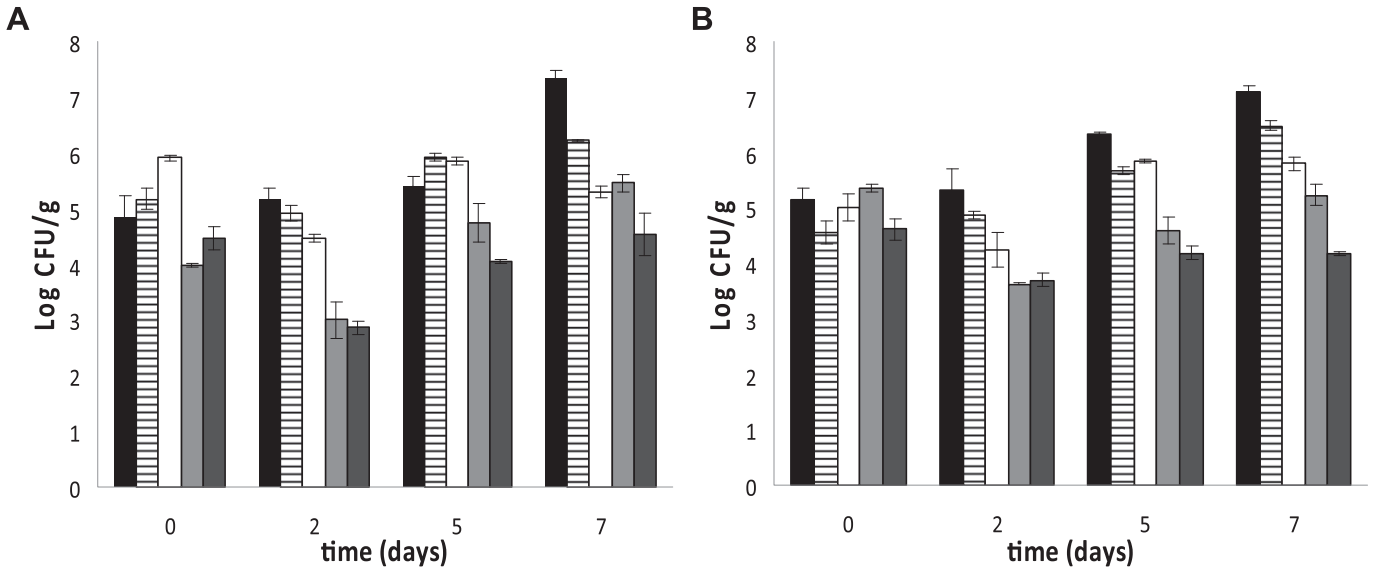

Fig. 3. Antimicrobial activities of CH film-forming solutions and acetic acid against (A) mesophilics and (B) psychrotrophics bacteria in minimally processed broccoli. (■) control (⊟) acetic acid (□) chitosan 5 g/L (▩) chitosan 10 g/L (■) chitosan 20 g/L.

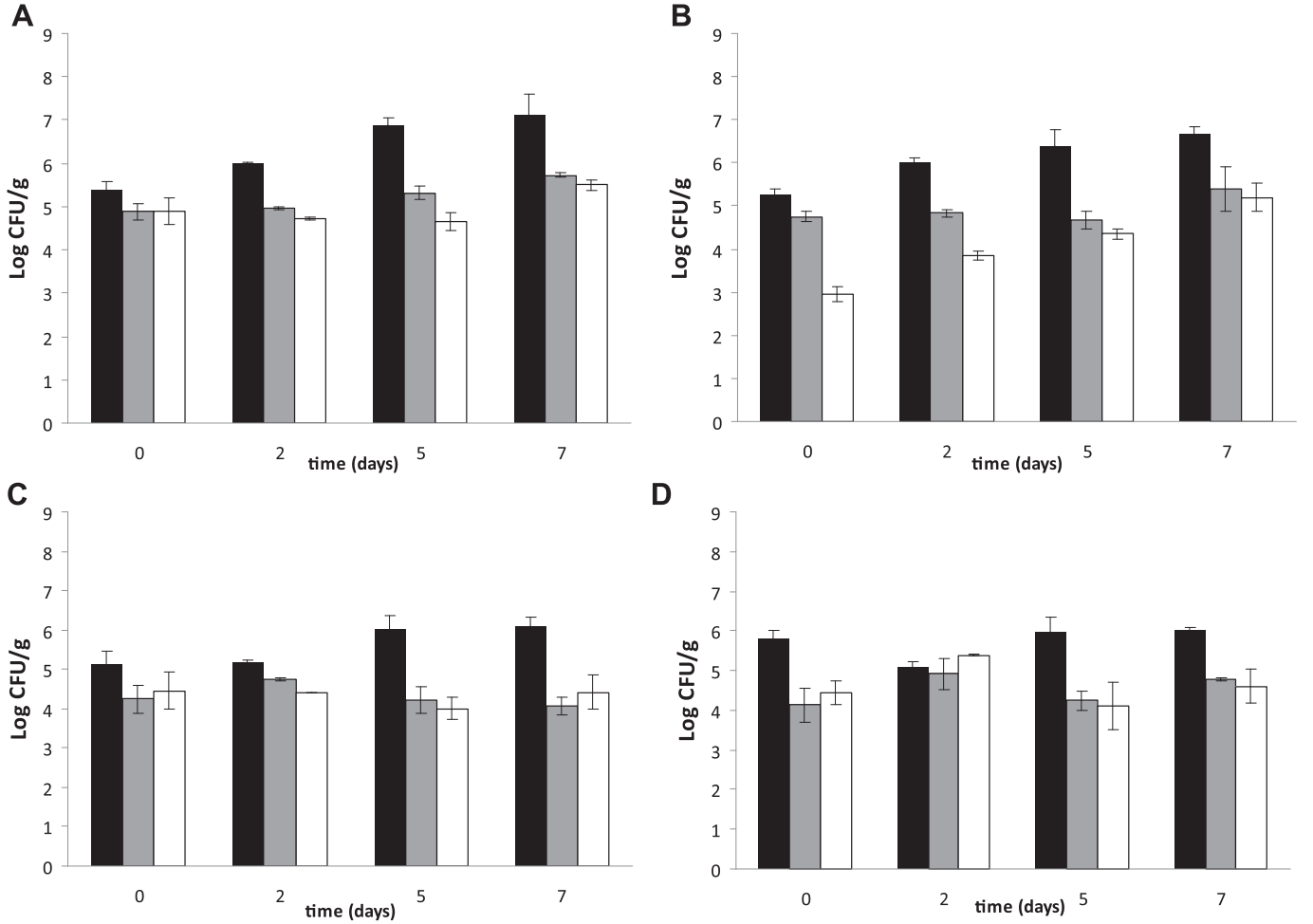

Fig. 4. Microbial counts in minimally processed broccoli treated with chitosan edible coating alone and enriched with tea tree EO, during refrigerated storage. (A) mesophilics; (B) psychrotrophics bacteria; (C) *E. coli* and (D) *L. monocytogenes*. (■) control (▩) chitosan (□) chitosan + tea tree. Data are the mean ± s.d. of 12 determinations ($n = 12$).

bacteria counts, during refrigerated storage of treated broccoli. Immediately after all BC were applied, there was not a significant ($P > 0.05$) antimicrobial effect. Between days 2 and 7 of storage, it was observed a significant bacteriostatic effect of tea tree, pomegranate, resveratrol, pollen and propolis (with reductions of 2.0–5.0 log CFU/g) on mesophilic (Fig. 2A–C) and psychrotrophic (data not shown) bacteria, compared to control sample. This difference was maintained until the end of storage. Moreover, broccoli samples treated with rosemary EO (15 μL/mL) did not show any significant inhibitory effect on mesophilic and psychrotrophic bacteria during the entire storage period (Fig. 2A).

The effect of chitosan coating on the growth of broccoli native microflora is shown in Fig. 3. Pure CH coatings were applied in different concentrations (5, 10 and 20 g/L) by dipping the broccoli florets, with the aim of analyzing its effects on the native microflora and determining the optimal concentration of CH to use combined with BC compounds. A control sample dipped in acetic acid solution (1 mL/100 mL) was included, because CH solutions were prepared by dissolving in acetic acid (as the CH is not soluble in aqueous phase). The objective was to determine if the inhibitory effects were not only due to the acid concentration used in the chitosan coating solution. Fig. 3 shows that pure CH coating (10 and 20 g/L) produced a significant reduction ($P < 0.05$) in mesophilic and psychrotrophic bacteria counts (2.5–3.5 log CFU/g) compared to control samples, between days 2 and 7 of storage. In addition, acetic acid solution and CH coating at low concentrations (5 g/L) did not produce significant reductions in the microbial counts during the first 5 days of storage. But at day 7, a significant reduction was observed in mesophilic and psychrotrophic counts (1.5 and 1.0 log, respectively). Durango, Soares, and Andrade (2006) carried out a research using chitosan coating on minimally processed carrots and reported similar reductions in mesophilic and psychrotrophic counts. Coma et al. (2002) and Kim, Min, Kimmel, Coosey and Park, (2011) tested antimicrobial effects of chitosan coating against *L. monocytogenes* and *E. coli* and they found that two bacteria were completely inhibited.

Incorporating antimicrobial agents, such as essential oils and bioactive compounds, into chitosan edible coatings can improve its antimicrobial efficiency, as the diffusion of the oil compounds would compensate the non-migrated antimicrobial power of CH (Aider, 2010; Fiedman & Juneja, 2010). Since rosemary EO applied alone did not show any significant inhibitory effect on mesophilic (Fig. 2A) and psychrotrophic bacteria during the entire storage period, this EO was not used combined with CH. Therefore, the bioactive compounds added to CH coating were tea tree, pomegranate, resveratrol, pollen and propolis. Fig. 4(A–D) shows the effect of CH coatings alone and enriched with tea tree against broccoli native microflora growth, and on *E. coli* and *L. monocytogenes* survival. Chitosan coating with and without tea tree exerted a bacteriostatic effect on mesophilic and psychrotrophic bacteria counts. In broccoli treated samples, bacteria counts were 2 order log lower compared to control sample up to day 2 of storage (Fig. 4A and B). In the same way, broccoli samples inoculated with *E. coli* O157:H7 and *L. monocytogenes* and treated with CH alone and CH plus tea tree showed a significant reduction ($P > 0.05$) on pathogen counts, between day 5 and 7 of storage (Fig. 4C and D).

Fig. 5(A–D) shows the effect of CH coatings alone and enriched with resveratrol against broccoli native microflora growth and *E. coli* and *L. monocytogenes* survival. Fig. 5(A and B) shows that CH

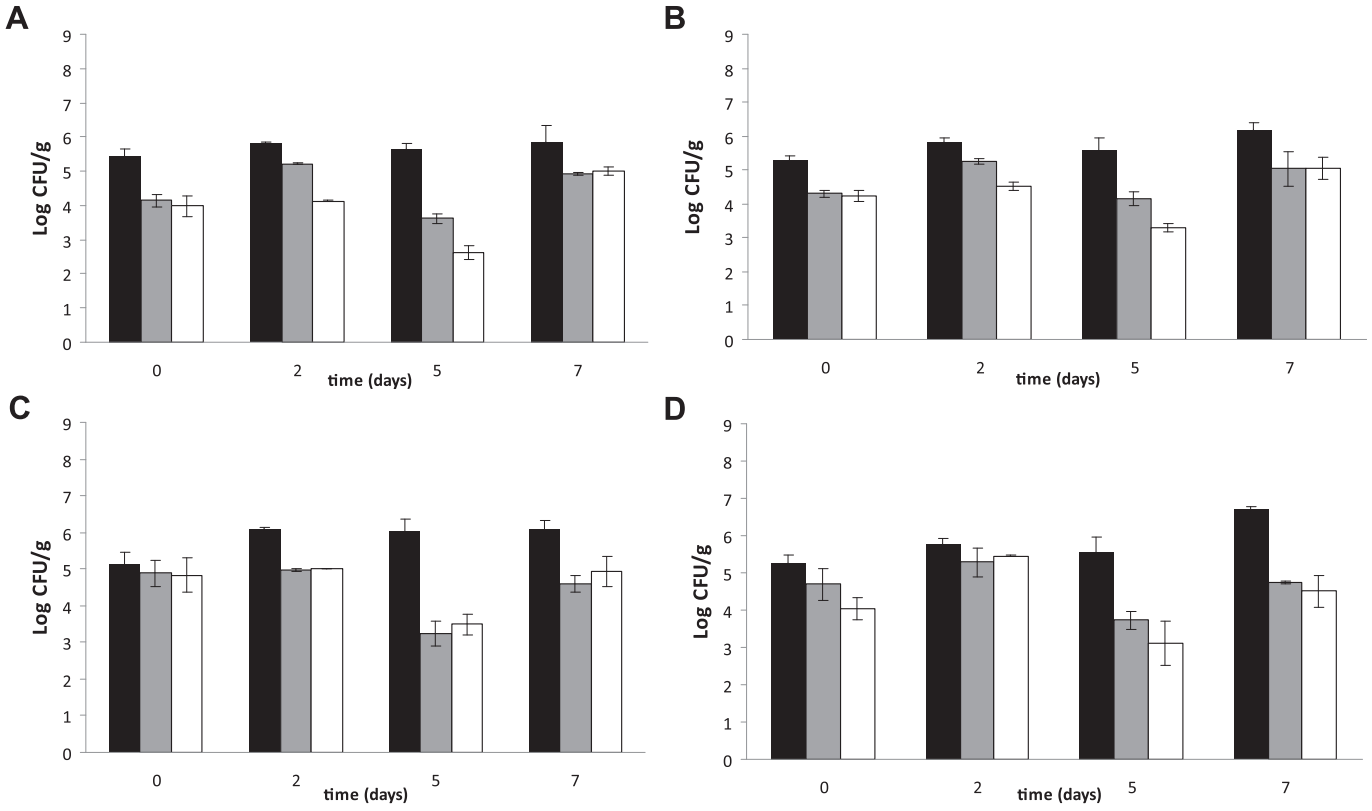

Fig. 5. Microbial counts in minimally processed broccoli, treated with chitosan edible coating alone and plus resveratrol, during refrigerated storage. (A) mesophilics; (B) psychrotrophics bacteria; (C) *E. coli* and (D) *L. monocytogenes*. (■) control (▒) chitosan (□) chitosan + resveratrol. Data are the mean ± s.d. of 12 determinations ($n = 12$).

coating produces a significant reduction ($P < 0.05$) in mesophilic and psychrotrophic bacteria counts, compared to control samples, during the entire storage period. A similar reduction in the counts was observed with the CH coatings plus resveratrol. In the same way, CH coatings alone and enriched with pomegranate produced similar reductions on mesophilic and psychrotrophic bacteria counts (data not shown). Up to day 5 of storage, the inhibitory effects of CH coating alone and enriched with resveratrol, on *E. coli* and *L. monocytogenes* counts (1.2–1.5 log CFU/g) were very significant ($P < 0.05$) (Fig. 5C and D). On the contrary, broccoli samples inoculated with *E. coli* O157:H7 and treated with CH alone and CH plus pomegranate did not show a significant reduction ($P > 0.05$) on pathogen counts, during the entire storage period. Furthermore, broccoli samples coated with CH plus pomegranate and inoculated with *L. monocytogenes* showed a significant reduction ($P < 0.05$) in the counts (1.5–1.7 log CFU/g), between days 2 and 5 of storage (data not shown).

Fig. 6(A–D) shows the effects of Chitosan coatings alone and enriched with propolis against broccoli native microflora growth and *E. coli* and *L. monocytogenes* survival. Fig. 6(A and B) shows the effects of CH edible coating alone and enriched with propolis on mesophilic and psychrotrophic bacteria counts. It was observed that chitosan produced a significant reduction (1.5–2.0 log CFU/g) ($P < 0.05$) in treated samples, compared to control samples, from day 2 of storage. Moreover, broccoli samples coated with CH plus propolis and inoculated with *E. coli* O157:H7 and *L. monocytogenes* showed a significant reduction ($P < 0.05$) in pathogen counts (1.0–2.0 log CFU/g), between days 2 and 5 of storage (Fig. 6C and D).

When broccoli florets were treated with CH coating alone and enriched with pollen, a slight reduction was observed ($P > 0.05$) in mesophilic and psychrotrophic bacteria counts compared to control samples, between days 2 and 5. Up to the end of storage, CH coating enriched with pollen exerted a significant inhibitory effect ($P < 0.05$) in mesophilic and psychrotrophic bacteria counts (2.0–2.5 log CFU/g), compared to control samples (data not shown). In addition, broccoli samples inoculated with *E. coli* O157:H7 and treated with CH plus pollen presented a significant reduction ($P < 0.05$) on pathogen counts during the storage. The inhibitory effect exerted by CH plus pollen on broccoli inoculated with *L. monocytogenes* was more significant, compared to the effect observed on *E. coli*. A significant reduction in *L. monocytogenes* counts (2.0–2.5 log CFU/g) was observed, between days 4 and 7 of storage (data not shown).

The reductions in mesophilic and psychrotrophic bacteria counts of broccoli exerted by CH coating alone and enriched with EO and BC were considerable when compared to other methods applied to reduce the microbial load in foods. In accordance with our results, Durango et al. (2006) reported a satisfactory performance of chitosan coatings applied to carrot, in controlling

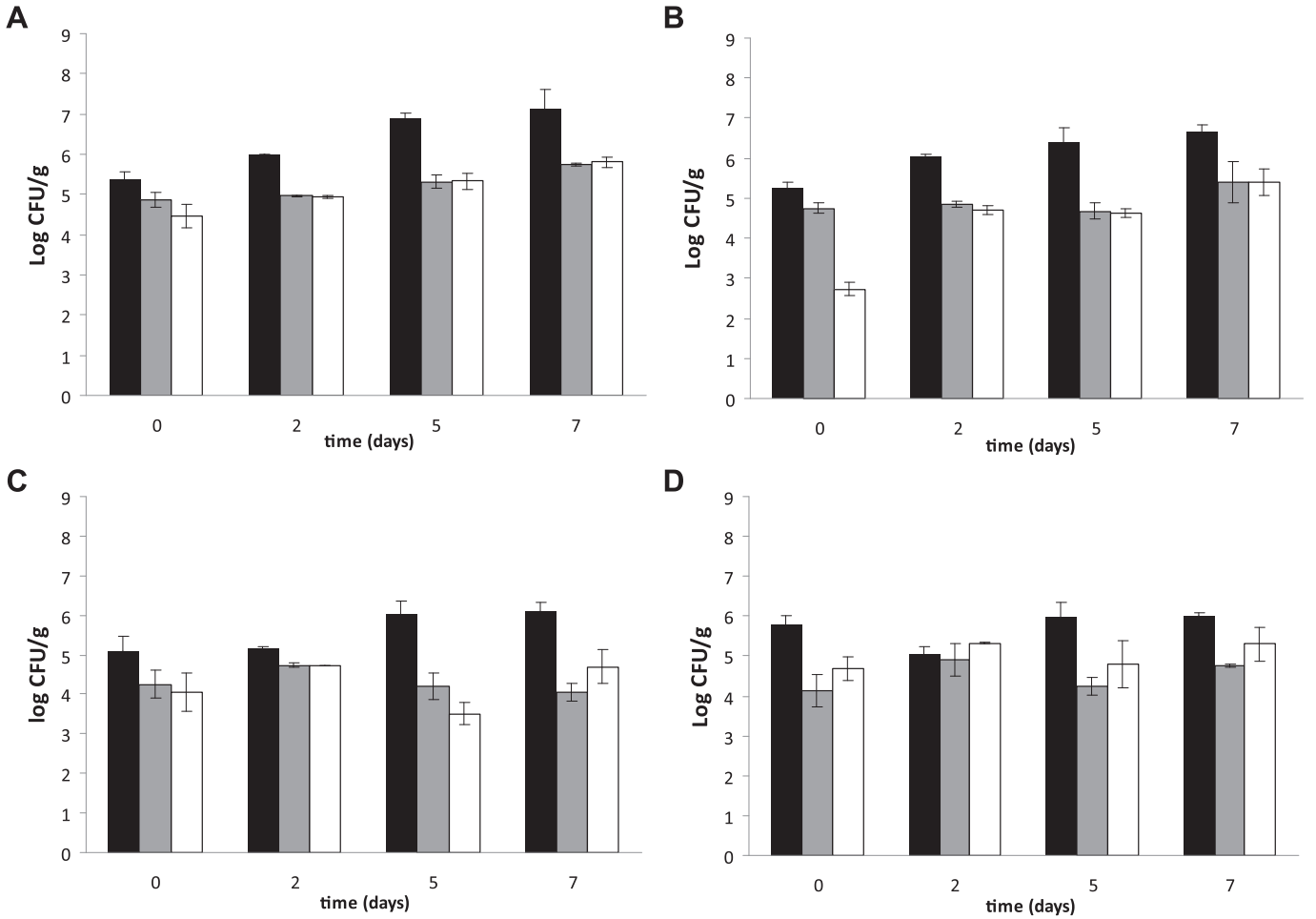

Fig. 6. Microbial counts in minimally processed broccoli, treated with chitosan edible coating alone and enriched with propolis, during refrigerated storage. (A) mesophilics; (B) psychrotrophics bacteria; (C) *E. coli* and (D) *L. monocytogenes*. (■) control (■) chitosan (□) chitosan + propolis. Data are the mean ± s.d. of 12 determinations ($n = 12$).

mesophilic aerobes, with a reduction of 1.3 log CFU/g at the end of storage.

According to Rojas-Grau et al. (2007) and to Pranoto, Rakshit, and Salokhe (2005), the use of edible coatings in minimally processed fruit and vegetables, has earned increased interest because coatings can serve as carriers for a wide range of food additives, including anti oxidant and antimicrobial agents that can extend the shelf life and reduce the pathogen growth on food surfaces.

The antimicrobial effects of EOs and BC obtained by "*in vitro*" assay were more significant compared to the inhibitory effects obtained when the biopreservatives were added to CH coatings and applied by immersion of broccoli florets. In this work, as in Dawson, Carl, Acton, and Han (2002), a higher concentration of these compounds were necessary to obtain similar inhibitory effects because its effectiveness decreased when it was applied to a coating.

3.3. Qualitative sensory evaluation

Since the results of sensory analysis of broccoli samples treated with CH coating enriched with different EO (tea tree) and BC (pomegranate, resveratrol, pollen and propolis) were similar, we described the results obtained with CH enriched with tea tree (at 15 μL/mL), as an example. It was observed that after 7 days of storage, the application of CH coating alone and enriched with tea tree allows the samples to present higher color and brightness scores than control samples. The application of CH coating alone and enriched with BC did not affect the texture and inhibited the florets opening, being this an important quality improvement for broccoli. There were no significant differences in the flavor between treated and untreated samples. Moderate enzymatic browning was present in control sample. In this sense, CH coating alone and plus BC was effective in the inhibition of the enzymatic browning along storage. In accordance with our results, Dutta et al. (2009) reported that if edible coatings are to be used as natural biopreservatives in minimally processed broccoli, they should not introduce deleterious effects on the sensory attributes of the products.

4. Conclusions

The incorporation of EO and BC to edible coatings as natural bactericides might be an interesting option. In this study, the results obtained by "*in vivo*" assay have shown that CH coating plus EO/BC have significant antibacterial properties.

The use of antimicrobial coating consisting of CH and CH enriched with BC, applied as coatings produced by vegetable immersion in the coating-forming solutions was a good alternative for controlling the microorganisms present in minimally processed broccoli. CH and CH plus EO/BC significantly inhibited the growth of mesophilic and psychrotrophic bacteria, and also controlled *E. coli* and *L. monocytogenes* survival. In general, the inhibitory effects exerted by CH plus BC/EO on broccoli inoculated with *L. monocytogenes* were more significant, respect to *E. coli*.

The application of CH coatings alone and enriched with EO/BC did not introduce deleterious effects on the sensory attributes of minimally processed broccoli.

Based on the concept of hurdle technologies, the use of such coatings enriched with biopreservatives in combination with other barriers such as hygienic processing conditions and adequate storage temperatures may contribute to improve the safety in minimally processed vegetables.

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