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Antimicrobial and antioxidant activities of edible coatings enriched with natural plant extracts: *In vitro* and *in vivo* studies

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ABSTRACT

Consumers demand less use of chemicals on minimally processed fruits and vegetables so more attention has been paid to the search for naturally occurring substances able to act as alternative antimicrobials and antioxidants.

The susceptibility of the native microflora of butternut squash and *Listeria monocytogenes* was analyzed by *in vitro* assays using (a) film-forming solutions (chitosan, carboxymethyl cellulose and casein), (b) oleoresins (olive, rosemary, onion, capsicum, cranberry, garlic, oreganum and oreganum + carvacrol 5%) and (c) film-forming solutions enriched with oleoresins. Film-forming solutions did not show significant antimicrobial properties. The oleoresins with meaningful antimicrobial activity against both squash native microflora and *L. monocytogenes* were olive and rosemary. In general, film-solutions containing 1% of different oleoresins showed limited antimicrobial effects against these indicator microorganisms. *In vitro* antioxidant properties were measured on different crude vegetable extracts. The enzyme source proved to affect peroxidase (POD) and polyphenoloxidase (PPO) susceptibility to the film-forming solutions. Most oleoresins significantly affected POD activity, regardless of the enzyme source. When the film-forming solutions were enriched with oleoresins, the latter lost, or retained their potential to reduce POD and PPO activities.

In vivo experiments were focused on the treatments offering potential antibacterial and antioxidant benefits. The use of chitosan coatings enriched with rosemary and olive oleoresins applied to butternut squash did not produce a significant antimicrobial effect, however antioxidant effects were observed during the first day, exerting POD inhibition for up to 5 d of storage. Both oleoresins and chitosan enriched with them exerted significant antioxidant activities over PPO throughout 5 d of storage. The use of chitosan enriched with rosemary and olive did not introduce deleterious effects on the sensorial acceptability of squash.

Chitosan enriched with rosemary and olive improved the antioxidant protection of the minimally processed squash offering a great advantage in the prevention of browning reactions which typically result in quality loss in fruits and vegetables.

1. Introduction

The use of edible films and coatings in food protection and preservation has recently increased since they offer several advantages over synthetic materials, such as being biodegradable and environmentally friendly (Tharanathan, 2003).

Currently, studies dealing with edible films with antimicrobial properties are on the increase. These films could prolong the shelf life and safety of foods by preventing growth of pathogenic and spoilage microorganisms as a result of their lag-phase extension and/or their growth rate reduction (Quintavalla and Vicini, 2002). Moreover, antimicrobials imbedded in films can be gradually released on the food surface, therefore, requiring smaller amounts to achieve the target shelf life (Min and Krochta, 2005).

The ability of edible films to retard moisture, oxygen, aromas and solute transport may be improved by including additives such as antioxidants, antimicrobials, colorants, flavours, fortifying nutrients and spices in film formulation (Pranoto et al., 2005). Given the fact that consumers demand less use of chemicals on minimally processed fruits and vegetables, more attention has been

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paid to the search for naturally occurring substances able to act as alternative antimicrobials and antioxidants. The addition of natural antioxidants derived from vegetable extracts as a way of increasing the shelf life of food products has become increasingly popular. It has also improved the stability of lipids and lipid-containing foods, thereby preventing sensorial and nutritional quality loss (Hemeda and Klein, 1990; Ozcan, 2003; Ponce et al., 2004; Sebranek, 2004). Along these lines, Botsoglou et al. (2002) indicated that the essential oils included in edible films could reduce water vapor permeability.

Spice oleoresins (natural plant extracts) constitute the true essence of spices in their most concentrated form, containing volatile as well as non-volatile components. Besides, they are the preferred and most convenient substitutes for raw spices in the food processing industry.

The isolated non-volatile components consist of several chemical compound groups, such as carotenoids, steroids, alkaloids, anthocyanins, glycosides, etc. This fraction can be essential for taste, colour, mouthfeel, texture and antioxidant properties of foods. Oleoresins differ from essential spice oils as they count on all the flavouring ingredients of a particular spice. They are free from bacteria and may be standardized to a desired degree of flavour strength.

When edible films are enriched with essential oils, the drying temperatures usually employed to form the edible coating are high enough to volatilize a high percentage of the aromatic components. The advantage of substituting essential oils for their corresponding food grade oleoresins could lie in the introduction of other non-volatile components, positively affecting food quality. Experiments carried out on the antimicrobial and antioxidant properties of spices and herbs and their compounds have been well documented. This issue shows interest even at present; however, the information available on their biological activity in edible films is still scarce.

This research aims to determine: (1) the antimicrobial activity of chitosan, casein and carboxymethyl cellulose films, alone as well as enriched with oleoresins, on the native microflora of butternut squash and on *Listeria monocytogenes*; (2) the antioxidant properties of these coatings; and (3) their antimicrobial and antioxidant effectiveness when applied to butternut slices (studies *in vivo*).

2. Material and methods

2.1. Film reagents

Sodium caseinate (9×10^{-3} kg kg^{-1}) and carboxymethyl cellulose were obtained from Merck (Darmstadt, Germany); and food grade glycerol from Mallinckrodt (Paris, KY, USA). Medium molecular weight chitosan was supplied by Aldrich Chemical Co. (Milwaukee, WI, USA).

2.2. Preparation of film-forming solutions

A 5% sodium caseinate aqueous solution was prepared by gradually adding sodium caseinate to distilled water and stirring continuously for 3 h at refrigerated temperature. Glycerol was included to reach a glycerol/protein ratio of 0.25 (Schou et al., 2005).

Chitosan solution was prepared by dissolving 20 g of chitosan in 1 kg of 1% acetic acid and 1% glycerol solution. To achieve complete chitosan dispersion, the solution was stirred overnight at room temperature and centrifuged to remove impurities. It was then sterilized at 121 °C for 15 min (Park et al., 2004).

The carboxymethyl cellulose coating was prepared by solubilizing carboxymethyl cellulose powder (0.75%) in a water–ethyl alcohol mixture (31/11) at 75 °C under stirring for 15 min. Ethyl alcohol was used to reduce drying time and produce a transparent and shiny carboxymethyl cellulose coating. Then glycerol was added (1.9%) and the solution was stirred for another 10 min under the same conditions and cooled (Maftoonazad and Ramaswamy, 2005).

2.3. Oleoresins

The oleoresins used in this work were provided by Pionherb, Buenos Aires, Argentina. Food grade oleoresins were obtained by alcohol steam distillation starting from fresh vegetables. The oleoresins used were rosemary (*Rosmarinus officinalis*), oreganum (*Origanum vulgare*), olive (*Olea europea*), capsicum (*Capsicum frutescens*), garlic (*Allium sativum*), onion (*Allium cepa* L.) and cranberry (*Vaccinium oxycoccus*).

2.4. Agar diffusion method

The sensitivity of the native microflora of butternut squash (*Cucurbita moschata* Duch) and *L. monocytogenes* to film components, oleoresins and film components enriched with oleoresins was determined by the agar diffusion method. An inhibition zone assay was conducted by inoculating Brain Heart Infusion (BHI) (Britania, Buenos Aires, Argentina) agar with an overnight culture of the indicator microorganisms. Seventy μL of the different solutions were poured into agar wells (5–6 mm diameter) following the methodology described by Coma et al. (2002). Table 1 describes the experimental conditions assayed.

Two types of control were utilized: distilled water control and acidic water control (pH 5.0). The latter aimed at determining the possible role of acid pH values on the inhibition of indicator microorganisms. The dishes were incubated at 37 °C for 1–2 d and the inhibition zones were measured. The sensitivity to the different antimicrobial solutions 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 (Ponce et al., 2003). Each assay was performed in duplicate in two separate experimental runs.

Native microflora of butternut squash was prepared with 10 g of raw material macerated in 90 mL phosphate buffer solution (0.1 mol L^{-1}) with a Stomacher 400 Circulator Homogenizer (pH 7.2) in agreement with Ponce et al. (2003); and then incubated for 3 h at 37 °C. *L. monocytogenes* indicator microorganism was supplied by CERELA (Centro de Referencia de Lactobacilos, Tucumán, Argentina). This culture was kept refrigerated on tryptic soy agar (TSA) supplemented with 0.5% of yeast extract during the experiment. Before *L. monocytogenes* was used, the pathogen was cultured in BHI for 1 d at 37 °C. Immediately before each experiment, approximately 0.1 mL of culture was transferred to 90 mL of BHI at two consecutive 1 d intervals.

Table 1
Experimental conditions for the agar diffusion method

Antimicrobial solutions assayed	Indicator microorganisms
Casein solution Chitosan solution Carboxymethyl cellulose solutions Oleoresins at 1% concentration: olive, rosemary, onion, capsicum, cranberry, garlic, oreganum, oreganum + carvacrol 5% Combinations of film-forming solutions with oleoresins	Native microflora of butternut squash, *Listeria monocytogenes*

2.5. Antioxidant activity of film-forming solutions: peroxidase and polyphenoloxidase assays

Romaine lettuce, butter lettuce and butternut squash were used as raw materials for peroxidase (POD) and polyphenoloxidase (PPO) assays.

For POD assay, 10 g of each vegetable were cleaned and washed. The vegetables were chopped and homogenized with 30 mL water in a commercial blender (Multiquick, MR 5550 CA Braun, Espanola S.A., Barcelona, Spain) for 3 min. The slurry was filtered through two layers of cheesecloth and centrifuged at 10,000 × *g* for 15 min. All steps were carried out at 4 °C. The supernatant, which contained PPO activity, was used as the enzyme source for the experiment (POD crude vegetable extract) (Ponce et al., 2004). Peroxidase activity was determined spectrophotometrically at 25 °C with an UV-1601 PC UV–vis spectrometer (Shimadzu Corporation, Japan) at 470 nm using guayacol as the substrate and H_2O_2 as the hydrogen donor. The substrate mixture contained 10 mL of 1% guayacol, 10 mL of 0.3% hydrogen peroxide and 100 mL of 0.05 mol L^{-1} sodium phosphate buffer (pH 6.5). The reaction cuvette contained 2.87 mL substrate mixture, 0.1 mL POD crude vegetable extract and 0.03 mL antioxidant solution (film-forming solutions, oleoresins and film-forming solutions enriched with oleoresins, in accordance with the combinations listed in Table 1). One unit of activity was defined as a change in absorbance of 0.06 s^{-1}. Each antioxidant solution was tested for each extract in duplicate on three independent lots.

For PPO assay, 10 g of vegetables was homogenized at a 1/2 ratio with 0.5 mol L^{-1} phosphate buffer (pH 7.0) in the presence of 50 g L^{-1} polyvinylpyrrolidone (ICN Biomedicals, Inc. OH) with a commercial blender (Multiquick, MR 5550 CA Braun, Espanola S.A., Barcelona, Spain) 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 samples were divided into small aliquots and frozen; after thawing, the samples were immediately used. The substrate mixture contained 20 mmol L^{-1} catechol as substrate in 5 mmol L^{-1} sodium phosphate buffer (pH 7). The reaction cuvette contained 2.9 mL substrate mixture, 0.1 mL PPO crude vegetable extract and 0.03 mL antioxidant solution (film-forming solutions, oleoresins and film-forming solutions enriched with oleoresins, following the same combinations described in Table 1). The rate of catechol oxidation was followed at 25 °C at 400 nm for 60 and 120 s. The enzyme activity unit was defined as a 0.001 change in absorbance between 60 and 120 s under the assay conditions (Ihl et al., 2003). Each antioxidant solution was tested for each extract in duplicate on three independent lots.

For POD and PPO the reference cuvette contained only substrate mixture. For each enzyme source, a reagent blank was prepared with 30 μL deionized water instead of antioxidant solution (control sample). True negative controls were carried out using only film-forming solutions, oleoresins and film-forming solutions enriched with oleoresins with no vegetable extract.

2.6. Studies in vivo

Before film application, squash slices were washed by immersion in tap water for 60 s and then drained. Afterwards, squash slices were immersed in the film-forming solutions for 180 s at 20 °C and then drained. Two types of controls were used: (1) fresh raw squash slices with no treatment (fresh control samples) and (2) fresh raw squash slices immersed in distilled water and subjected to the same drying conditions (dry control samples). After film application, squash slices were dried by exposure to flowing air at 30 °C with 40–50% relative humidity for 50 min.

For microbiological studies, diced squash (25 g) was macerated in PO_4K_3 buffer solution (pH 7.2) with a homogenizer (Stomacher 400 Circulator Homogenizer). The enumeration and differentiation of mesophilic aerobic bacteria were performed on PCA (plate count agar) after 48 h at 35 °C (ICMSF, 1983; Mossel and Moreno García, 1985). Microbial counts were conducted in duplicate on six independent lots.

Peroxidase and polyphenoloxidase activities were determined just as described in Section 2.5, and were performed in duplicate on three independent lots.

2.7. Sensory evaluation

Butternut squash slices were rated by the panelist (members of our laboratory with experience in sensory evaluation of fresh vegetables) for sensorial acceptability (OVQ) and aroma. Samples were scored on a 5-point scale, in which 5 = very good and 1 = poor. This assay was performed in duplicate in two separate experimental runs.

2.8. Statistical analysis

For *in vitro* studies, differences in antimicrobial and antioxidant properties between film-forming solutions with and without oleoresins were calculated by analysis of variance (one way ANOVA) using a statistical package (MATLAB). Whenever differences were significant, a 99% confidence level was used. For *in vivo* studies, a *t*-Student test was employed to calculate the differences in antimicrobial and antioxidant properties between edible films with and without oleoresins. Whenever differences were significant, a 95% confidence interval was used (Volk, 1980).

3. Results and discussion

3.1. In vitro assays: antibacterial and antioxidant properties

Table 2 shows the susceptibility of the native microflora of butternut squash and *L. monocytogenes* to film-forming solutions (chitosan, carboxymethyl cellulose and casein) and to oleoresins (olive, rosemary, onion, capsicum, cranberry, garlic, oreganum and oreganum + carvacrol 5%) as determined by the agar diffusion method.

Table 2
Inhibition halos observed with different antimicrobial agents over squash native microflora and *Listeria monocytogenes*

	Inhibition halos (mm)	
	Butternut squash native microflora	*Listeria monocytogenes*
Water control	<8	<8
Acid water control (pH 5.0)	<8	<8
Chitosan film	8.75 ± 2.16	<8
CMC film	<8	<8
Casein film	<8	<8
Olive	16.25 ± 1.30	13.50 ± 0.50
Rosemary	30.00 ± 4.96	19.00 ± 1.00
Onion	<8	<8
Capsicum	<8	10.50 ± 0.50
Cranberry	<8	<8
Garlic	<8	<8
Oreganum	<8	<8
Oreganum + carvacrol (5%)	31.00 ± 2.16	<8

The sensitivity to the different antimicrobial agents was classified by the diameter of the inhibition halos as follows: not sensitive to diameters less than 8 mm; sensitive to diameters 9–14 mm; very sensitive to diameters 15–19 mm; and extremely sensitive to diameters larger than 20 mm. Each assay was performed by duplicate in two separate experimental runs.

To override a possible inhibitory effect of the acetic acid used during chitosan solution preparation, an acidic water control (pH 5.0; 25 mL L^{-1}) was used. Native microflora and *L. monocytogenes* were not sensitive to this acid water. Pure edible coatings solutions and pure oleoresins served as control to determine the potential antimicrobial and antioxidant effects of these solutions *per se*. The film-forming solutions did not show significant antimicrobial properties on squash native microflora and *L. monocytogenes*. There are two possible explanations for this. On the one hand, the inocula in this experiment were approximately 10^6 to 10^7 CFU per Petri dish. Zivanovic et al. (2005) found similar results with pure chitosan film, thereby indicating that the high number of bacteria may exceed chitosan inhibition activity. On the other hand, the limitations film diffusion is presented in agar medium (Coma et al., 2002). These authors also detected limited inhibitory activity of chitosan-forming solution against *L. monocytogenes* (Coma et al., 2002).

The only oleoresins with meaningful antimicrobial activity against both squash native microflora and *L. monocytogenes* were olive and rosemary. Capsicum presented activity against *L. monocytogenes* and oreganum + carvacrol against the native microflora. Previous studies revealed (Ponce et al., 2003; Moreira et al., 2005) low susceptibility of Swiss chard native microflora and *E. coli* to oreganum essential oil. Carvacrol is the major active component of oreganum (Burt, 2004). A high antimicrobial effect against squash native microflora was observed when the oreganum oleoresin was supplemented with 5% carvacrol (Table 2). From the viewpoint of Lambert et al. (2001), carvacrol makes the cell membrane permeable, disintegrating the outer membrane of Gram-negative bacteria.

Essential oils extracted from spices and herbs are generally recognized as containing active antimicrobial compounds. Eugenol, carvacrol and thymol are phenolic compounds in cinnamon, cloves, sage and oregano with antimicrobial activity (Ponce et al., 2003; Draughon, 2004; Friedman et al., 2004; Burt, 2004). Allicin is a garlic oil component that inhibits Gram (−) as well as Gram (+) bacteria growth. Sulfur-containing compounds found in onions, leaks and chives are also antimicrobial components. Benkeblia (2004) found that garlic and onion extracts exhibited marked antibacterial activity against fungi and Gram (+) pathogens. However, in this study, onion and garlic oleoresins did not present inhibitory activity on squash native microflora and *L. monocytogenes* (Table 2). This different behavior could be explained on different grounds. To begin with, Benkeblia used high oil concentrations (5–50%) while, in this work, the concentration did not exceed 1%. Besides, Benkeblia employed essential oils, which are purer forms of the active principles if compared to the corresponding oleoresins. Finally, other factors such as the method used to extract the essential oils from plant material, inoculum volume and its growth phase, the culture medium, the media pH and the incubation time and temperature, can yield different results (Burt, 2004).

The combined effect of the film-forming solutions with 1% oleoresins on squash native microflora and *L. monocytogenes*, as determined by the agar diffusion method, was analyzed. Only combinations producing inhibition halos with diameters larger than 8 mm are displayed in Table 3. Squash native microflora was sensitive to chitosan enriched with olive, rosemary and capsicum and to CMC enriched with rosemary. *L. monocytogenes* was sensitive to carboxymethyl cellulose plus rosemary and very sensitive to chitosan enriched with rosemary. The combinations of casein with the various oleoresins had inhibitory activity neither against the native microflora of squash nor against *L. monocytogenes*. In general, film-solutions containing 1% of different oleoresins showed limited antimicrobial effects against the native microflora of butternut squash and *L. monocytogenes*. Seydim and Sarikus (2006) reported that edible films prepared from whey protein with oreganum, rosemary and garlic essential oils at 1% concentrations did not yield antibacterial activity. They also reported that when concentrations were raised to 4% levels, antimicrobial activity against different food pathogens, including *L. monocytogenes*, was observed. Also, chemical interactions between amino groups in edible films and carboxyl groups in oleoresins could block the active antibacterial sites (Pranoto et al., 2005). Moreover, the high levels of inoculums assayed (10^9 to 10^{10} CFU kg^{-1}) could exceed the inhibition activity of antibacterial compounds.

Table 3
Inhibition halos observed with edible coatings enriched with natural oleoresins on squash native microflora and *Listeria monocytogenes*

	Inhibition halos (mm)	
	Squash native microflora	*Listeria monocytogenes*
Chitosan film + olive 1%	8.85 ± 1.50	<8
Chitosan film + rosemary 1%	8.65 ± 0.70	15.00 ± 0.60
Chitosan film + capsicum 1%	10.00 ± 0.28	<8
CMC film + rosemary 1%	8.75 ± 1.92	9.50 ± 0.50

The sensitivity to the different antimicrobial agents was classified by the diameter of the inhibition halos as follows: not sensitive to diameters less than 8 mm; sensitive to diameters 9–14 mm; very sensitive to diameters 15–19 mm; and extremely sensitive to diameters larger than 20 mm. Each assay was performed by duplicate in two separate experimental runs. CMC, carboxymethyl cellulose.

The antioxidant properties of the film-forming solutions and the oleoresins were determined analyzing POD and PPO activities in crude vegetable extracts from butternut squash, romaine lettuce and butter lettuce. Table 4 shows the activities of these enzymes in crude vegetable extracts in the presence of film-forming solutions and oleoresins. The indigenous activity of these enzymes provided notable differences among the different vegetable crude extracts. Hemeda and Klein (1990) and Ponce et al. (2004) also reported differences in POD activity of different crude vegetable extracts.

The source of the enzyme proved to affect POD and PPO susceptibility to the film-forming solutions. The activity of the PPO obtained from romaine lettuce was not significantly affected by the edible coatings. Yet its activity was strongly influenced by chitosan and casein films when it was extracted from butter lettuce. Finally, a pro-oxidant behavior was observed in the activity of the PPO obtained from butternut squash when exposed to the chitosan solution. POD activity was only affected by chitosan solution (reductions in activity ca. 50%) when the enzyme was obtained from butter and romaine lettuce.

Zhang and Quantick (1997) and Jiang and Li (2001) reported that the application of 2% chitosan solution was effective in delaying browning in litchi and longan fruits stored at low temperature. Jiang et al. (2005) stated that the chitosan film delayed the increase in PPO activity in cold-stored litchi fruit.

Most oleoresins at 1% concentration significantly affected ($p < 0.01$) POD activity, regardless of the enzyme source (Table 4). Rosemary, onion, capsicum, cranberry and garlic oleoresins exerted the highest antioxidant activity on the three vegetable extracts. Even though olive, oreganum and oreganum + 5% carvacrol showed a significant antioxidant effect on POD from the two lettuce varieties, the effect on the enzyme from butternut squash was not meaningful. On the other hand, the effect of oleoresins on PPO activity was not significant. Only rosemary, olive and cranberry oleoresins brought about a considerable reduction in the activity of PPO obtained from butter lettuce. Spices have high polyphenol content with apparent antioxidant properties. Spice extracts from oreganum, sage, rosemary, garlic, thyme and capsicum enjoy antioxidant properties (Seydim and Sarikus, 2006). Auroma et al. (1996) assessed the antioxidant action of rosemary and provencal herbs extracts, and proposed the use of vegetable extracts as both food antioxidants and prophylactic agents for human diseases Auroma (1997).

Table 4
Effects of antioxidant agents (edible coatings and oleoresins) on percent relative peroxidase and polyphenoloxidase activities of crude vegetable extracts, expressed in AU $kg^{-1} \times 10^6$

	Peroxidase activity			Polyphenoloxidase activity		
	Butternut squash	Butter lettuce	Romaine lettuce	Butternut squash	Butter lettuce	Romaine lettuce
Crude extracts	3.81 ± 0.10	1.40 ± 0.60	2.12 ± 0.05	0.66 ± 0.15	1.48 ± 0.04	3.61 ± 0.12
Chitosan	4.52 ± 0.06 (0)	0.73 ± 0.08 (48)	0.74 ± 0.06 (65)	1.94 ± 0.12 (0)	0.78 ± 0.06 (47)	3.61 ± 0.10 (0)
Casein	3.72 ± 0.01 (2)	1.32 ± 0.06 (5)	2.29 ± 0.05 (0)	0.71 ± 0.10 (0)	0.77 ± 0.04 (48)	3.39 ± 0.09 (6)
CMC	3.76 ± 0.06 (2)	1.28 ± 0.02 (8)	1.76 ± 0.03 (17)	0.35 ± 0.10 (47)	1.87 ± 0.02 (0)	3.32 ± 0.07 (8)
Rosemary	0.03 ± 0.01 (100)	0.06 ± 0.01 (100)	0.05 ± 0.00 (100)	0.34 ± 0.08 (48)	0.03 ± 0.01 (100)	3.32 ± 0.14 (8)
Olive	3.53 ± 0.05 (8)	0.60 ± 0.07 (57)	0.70 ± 0.07 (67)	0.55 ± 0.13 (17)	0.41 ± 0.03 (73)	3.40 ± 0.25 (6)
Onion	0.26 ± 0.03 (93)	0.06 ± 0.00 (100)	0.29 ± 0.03 (86)	0.58 ± 0.16 (10)	0.88 ± 0.00 (40)	3.44 ± 0.20 (5)
Capsicum	1.72 ± 0.44 (55)	0.08 ± 0.02 (100)	0.52 ± 0.05 (75)	0.53 ± 0.11 (20)	0.85 ± 0.21 (42)	2.98 ± 0.85 (18)
Cranberry	0.60 ± 0.06 (85)	0.16 ± 0.02 (89)	1.18 ± 0.11 (44)	0.54 ± 0.10 (19)	0.61 ± 0.13 (59)	3.40 ± 0.15 (6)
Garlic	1.76 ± 0.05 (54)	0.02 ± 0.01 (100)	1.10 ± 0.10 (48)	0.52 ± 0.13 (21)	1.22 ± 0.10 (17)	3.61 ± 0.08 (0)
Oreganum	2.62 ± 0.16 (31)	0.07 ± 0.00 (100)	0.16 ± 0.01 (92)	0.56 ± 0.15 (15)	1.55 ± 0.52 (0)	3.55 ± 0.19 (2)
Oreganum + carvacrol (5%)	3.08 ± 0.02 (20)	0.49 ± 0.25 (100)	0.70 ± 0.07 (67)	0.56 ± 0.10 (15)	1.73 ± 0.05 (0)	3.27 ± 0.07 (10)

Number in bracket: percentage of activity reduction. Each antioxidant solution was tested for each extract in duplicate on three independent lots.

The effect of chitosan enriched with the oleoresins on POD and PPO activities is presented in Table 5. Once again, the effect of the chitosan solution combined with oleoresins was dependent on the source for the enzymes. The POD from butternut squash was most affected by chitosan enriched with rosemary, olive and cranberry oleoresins in descending order. The POD from butter lettuce was affected by the combinations with olive, garlic and oreganum + carvacrol. Finally, the romaine lettuce POD was most affected by the combination with rosemary, olive, cranberry and oreganum + carvacrol oleoresins, although other combinations also resulted in reduced POD activity.

The combinations of chitosan with olive, garlic and capsicum resulted in significant reductions in the activity of PPO obtained from butternut squash (Table 5). None of the combinations of chitosan with oleoresins enhanced the reduction of PPO activity from butter and romaine lettuce extracts. The combination of chitosan and rosemary oleoresin was less effective in the reduction of PPO activity from butter lettuce than chitosan or rosemary oleoresins by themselves. Possible interactions between functional chemical groups of rosemary and chitosan could account for the loss of antioxidant properties (Pranoto et al., 2005). Generally speaking, when the film-forming solutions were added to the oleoresins, the latter lost, or at their best, retained their potential to reduce POD and PPO activities.

3.2. *In vivo assays: antibacterial and antioxidant properties*

In view of the results obtained from the *in vitro* assays, *in vivo* experiments were focused on the treatments offering potential antibacterial and antioxidant benefits. Table 6 shows changes in mesophilic bacterial counts in butternut slices coated with edible films enriched with rosemary and olive during refrigerated storage. Reductions in bacterial counts were measured as $\log N_0 - \log N_w$, where N_0 represent samples with no oleoresins (in CFU kg^{-1}), and N_w represent samples with oleoresins (in CFU kg^{-1}). For dry control samples, initial reference values were 5910 ± 820; for chitosan-coated samples, they were 5720 ± 980 CFU kg^{-1}; for samples coated with casein, 5930 ± 810 CFU kg^{-1}; and for CMC-coated samples, 6170 ± 120 CFU kg^{-1}. These results reveal that the application of these edible films would produce no significant initial bactericidal effect ($p < 0.05$). An initial reduction in total bacterial counts was observed in all samples treated with rosemary and olive oleoresins. No combinations of film–oleoresins conferred significant advantages regarding the antibacterial properties observed when oleoresins were applied to dry control samples. A possible explanation could be that the oleoresins antibacterial compounds would be dispersed in the coating and, thus, bacterial cells would be less exposed to them. Antimicrobial agents may selectively and gradually migrate from the film to the food surface, producing their antimicrobial effects during storage. Actually the antimicrobial activity of the oleoresins coatings increased during the first 2 d of storage in all coating/oleoresin pairings except olive in CMC. After 2 d of storage, the antimicrobial activity decreased.

Chitosan and olive and rosemary oleoresins presented, by themselves, antibacterial activity in *in vitro* assays. Still, when the combination of chitosan and these oleoresins was employed in both *in vitro* and *in vivo* assays, the antibacterial activity did not increase, ruling out any additive or synergic effects. In fact, the antibacterial activities of olive and rosemary oleoresins were reduced when they were applied to the coating material. These results are prob-

Table 5
Effects of edible coatings enriched with oleoresins on percent relative peroxidase and polyphenoloxidase activities of crude vegetable extracts, expressed in AU $kg^{-1} \times 10^6$

Films	Peroxidase activity			Polyphenoloxidase activity		
	Butternut squash	Butter lettuce	Romaine lettuce	Butternut squash	Butter lettuce	Romaine lettuce
Crude extracts	2.14 ± 0.26	1.99 ± 0.13	1.68 ± 0.60	0.60 ± 0.19	2.25 ± 0.05	4.14 ± 1.30
Chitosan	2.57 ± 0.32 (0)	2.05 ± 0.10 (0)	1.17 ± 0.34 (30)	1.58 ± 0.46 (0)	1.20 ± 0.17 (47)	7.52 ± 3.47 (0)
Chitosan + rosemary	0.15 ± 0.02 (100)	1.98 ± 0.15 (0)	0.05 ± 0.00 (100)	0.91 ± 0.58 (0)	1.60 ± 0.12 (29)	5.16 ± 1.43 (0)
Chitosan + olive	0.43 ± 0.06 (80)	0.02 ± 0.00 (100)	0.72 ± 0.32 (57)	0.01 ± 0.00 (100)	1.14 ± 0.33 (49)	3.94 ± 1.19 (0)
Chitosan + onion	2.03 ± 0.28 (5)	1.99 ± 0.05 (0)	1.02 ± 0.42 (39)	0.28 ± 0.06 (52)	1.75 ± 0.14 (22)	4.69 ± 0.40 (0)
Chitosan + capsicum	1.72 ± 0.54 (20)	1.47 ± 0.08 (26)	0.87 ± 0.10 (48)	0.07 ± 0.01 (87)	2.07 ± 0.13 (8)	4.11 ± 2.79 (0)
Chitosan + cranberry	0.86 ± 0.21 (60)	1.27 ± 0.06 (39)	0.71 ± 0.25 (57)	0.33 ± 0.05 (44)	1.48 ± 0.24 (34)	6.87 ± 1.08 (0)
Chitosan + garlic	2.44 ± 0.11 (0)	0.20 ± 0.10 (90)	1.28 ± 0.39 (24)	0.05 ± 0.00 (91)	2.03 ± 0.18 (10)	4.79 ± 0.23 (0)
Chitosan + oreganum	2.07 ± 0.49 (3)	1.47 ± 0.09 (26)	0.90 ± 0.48 (46)	0.59 ± 0.25 (1)	3.08 ± 0.71 (0)	4.01 ± 1.38 (0)
Chitosan + oreganum–carvacrol (5%)	2.35 ± 0.28 (0)	0.95 ± 0.13 (52)	0.71 ± 0.49 (57)	0.28 ± 0.04 (52)	2.63 ± 0.17 (0)	4.44 ± 0.41 (0)

Number in bracket: percentage of activity reduction. Each antioxidant solution was tested for each extract in duplicate on three independent lots.

Table 6
Changes in mesophilic bacterial counts in butternut slices coated with edible films enriched with rosemary and olive during refrigerated storage

Days of storage	Dry control sample		Chitosan		Casein		CMC	
	Rosemary	Olive	Rosemary	Olive	Rosemary	Olive	Rosemary	Olive
0	0.558	0.826	0.684	1.118	0.678	0.435	0.530	0.708
1	1.637	2.394	0.539	n.d.	0.929	1.055	n.d.	n.d.
2	1.389	1.245	1.139	1.356	1.311	1.753	1.043	0.592
3	−0.160	−0.050	0.252	0.010	0.062	−0.800	0.532	0.221
5	−0.170	−0.340	0.015	0.050	−0.393	−0.225	0.037	0.057

Bacterial reductions were measured as $\log N_0 - \log N_w$, where N_0 represent CFU kg^{-1} in samples without oleoresins and N_w represent CFU kg^{-1} in samples treated with oleoresins. Microbial counts were performed in duplicate on six independent lots.

ably due to the dispersion effect of the active compounds and the interactions among these compounds.

Durango et al. (2006) reported that starch and chitosan coatings had low initial control action on mesophilic bacteria in carrot slices but, after 15 d of storage, the coatings achieved 1.34 log cycle reductions in microbial populations. These results cannot be readily compared with those in the present work since the authors rinsed their samples with chlorinated water (active chlorine of 3 mg L^{-1}) and stored them in sanitized nylon bags (active chlorine of 0.2 g L^{-1}) prior to applying the film. The initial microbial counts were only 10^4 to 10^5 CFU kg^{-1}.

The antioxidant properties of chitosan edible film enriched with rosemary, onion, cranberry, garlic and capsicum on butternut squash slices during refrigerated storage are listed in Table 7. The selection of the edible coating and the oleoresins was implemented on the basis of its antioxidant effects observed during *in vitro* assays on butternut squash extract. Large differences in the initial indigenous activity of POD and PPO enzymes between different butternut squash lots were observed. Initial activities ranged between 0.072 and 2.52 activity units (AU kg^{-1}) for POD and 0.074–0.44 activity units (AU kg^{-1}) for PPO. The great initial variability could be explained by biological differences such as soluble phenol content, differences in raw material texture leading to differential leaching of soluble phenolic compounds from cut surfaces during washing, and differences in the migration rate through cut surfaces due to presence of isozymes, among others (Choi and Sapers, 1994). Because of the initial variability found in POD and PPO activities, the percentage of reductions for each individual lot was recorded and expressed as the arithmetical mean of three independent lots (Table 7).

When oleoresins were dispersed in water and applied to butternut slices, no initial antioxidant action was detected. However, some reduction in POD activity was observed with storage time. Greater antioxidant effects on POD were obtained in slices treated with chitosan, but oleoresins did not enhance the antioxidant power exerted by chitosan alone (Table 7). The antioxidant effects of chitosan coating enriched with oleoresins on POD activity increased during the first day, exerting POD inhibition for up to 5 d of storage. Regarding capsicum oleoresin, the antioxidant power lasted only the first day of storage. Comparing the chitosan antioxidant properties *in vitro* and *in vivo*, two different behaviors were observed. In *in vitro* studies, chitosan was applied to a butternut homogenized extract as a film-forming solution, and, in such form, it did not exert any antioxidant effect over POD and PPO enzymes (Table 4). However, in *in vivo* studies, when chitosan was used as a coating and applied directly to squash slices, it enhanced its antioxidant properties over POD enzyme (Table 7). This result would indicate that chitosan, as a coating, interacts with components in the food surface, enhancing its antioxidant properties. When chitosan enriched with oleoresins was applied as coatings to butternut slices, the active antioxidant principles of oleoresins were slowly released into the food surface, and, therefore, enhanced its effects with storage time (Table 7). Applications of chitosan coating have been reported to form an ideal coating on fruits, and thus to maintain the quality and delay the browning of harvested fruits and vegetables (Pen and Jiang, 2003).

Both oleoresins and chitosan enriched with oleoresins exerted significant antioxidant activities over PPO throughout 5 d of storage (Table 7).

If edible coatings enriched with oleoresins are to be used as natural biopreservatives in minimally processed vegetables, they should not introduce deleterious effects on the sensory attributes of the products. The overall visual quality (OVQ) was evaluated on raw and cooked squash slices. Results obtained from fresh control, dry control, and samples coated with edible films enriched with rosemary and olive indicated that the edible coatings were imper-

Table 7
Percentage of reductions of peroxidase and polyphenoloxidase activities in butternut slices coated with water and chitosan films enriched with oleoresins during refrigerated storage

Oleoresins	Peroxidase activity (% of reduction)					Polyphenoloxidase activity (% of reduction)				
	0[a]	1[a]	2[a]	3[a]	5[a]	0[a]	1[a]	2[a]	3[a]	5[a]
Rosemary	0	53	48	15	45	42	65	24	57	15
Onion	20	60	55	58	64	87	66	60	54	73
Cranberry	0	51	50	55	76	74	61	86	73	73
Garlic	14	31	28	25	30	77	70	62	88	49
Capsicum	27	35	30	24	71	97	89	88	24	40
Chitosan	71	52	52	48	62	0	0	0	0	7
Chitosan + rosemary	47	87	58	40	57	80	47	98	94	42
Chitosan + onion	40	91	76	52	58	75	63	61	98	74
Chitosan + cranberry	61	86	64	89	87	78	61	73	64	17
Chitosan + garlic	58	84	53	80	66	56	48	69	96	33
Chitosan + capsicum	30	83	0	27	17	76	74	76	94	14

POD and PPO activity values represent the percentage of reductions for each individual lot respect crude vegetable extract values and were expressed as the arithmetical mean of three independent lots. Initial indigenous activity of POD and PPO ranged between 0.0720–2.502 and 0.074–0.445 activity units (AU kg^{-1}), respectively.

[a] Days of storage.

ceptible and the oleoresins, at the concentration used (1%), did not produce residual aroma. The panelist found no differences in OVQ among the different samples throughout the storage time. The use of edible coatings enriched with rosemary and olive did not introduce deleterious effects on the sensorial acceptability of minimally processed squash.

Even though the use of chitosan coatings enriched with oleoresins applied to butternut squash did not produce a significant antimicrobial effect, it improved the antioxidant protection of the minimally processed squash offering a great advantage in the prevention of browning reactions which typically result in quality loss in fruits and vegetables. Yet, further improvements are necessary to develop a more successful application of edible coatings enriched with oleoresins on squash slices. Careful sanitizing wash of raw materials to reduce initial counts (lower than 10^6 CFU kg^{-1}) together with the use of optimal oleoresin concentrations (higher than 2%) to increase antimicrobial and antioxidant effectiveness without affecting sensorial acceptability could be concrete steps to such improvement.

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References

Auroma, A., 1997. Extracts as antioxidant prophylactic agents. Inform 8, 1236–1242.
Auroma, O., Spencer, J., Rossi, R., Aeschbach, R., Kahn, A., Mahmood, N., Munoz, A., Murcia, A., Butler, J., Halliwell, B., 1996. An evaluation of the antioxidant and the antiviral action of extracts of rosemary and provencal herbs. Food Chem. Toxicol. 34, 449–456.
Benkeblia, N., 2004. Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). LWT 37 (2), 263–268.
Botsoglou, N., Christaki, E., Fletouris, D., Florou-Paneri, P., Spais, A., 2002. The effect of dietary oregano essential oils on lipid oxidation in raw and cooked chicken during refrigerated storage. Meat Sci. 62, 259–265.
Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food Microbiol. 94, 223–253.
Choi, S., Sapers, G., 1994. Effects of washing on polyphenols and polyphenol oxidase in mushroom. J. Agric. Food Chem. 12, 219–222.
Coma, V., Martial-Gros, A., Garreau, S., Copinet, A., Salin, F., Deschamps, A., 2002. Edible antimicrobial films based on chitosan matrix. J. Food Sci. 67, 1162–1169.
Draughon, A., 2004. Use of botanicals as biopreservatives in foods. Food Technol. 58 (2), 20–28.
Durango, A., Soares, N., Andrade, N., 2006. Microbiological evaluation of an edible antimicrobial coating on minimally processed carrots. Food Control 17, 336–341.
Friedman, M., Henika, R., Mandrell, R., 2004. Bactericidal activities of plant essential oils and some of their isolated constituents against *C. jejuni*, *E. coli*, *L. monocytogenes* and *S. enterica*. J. Food Protect. 65 (10), 1545–1560.
Hemeda, H., Klein, B., 1990. Effects of naturally occurring antioxidants on peroxidase activity of vegetable extracts. J. Food Sci. 55 (1), 184–192.
ICMSF, 1983. Microorganismos de los alimentos. Acribia S.A., Zaragoza, España, pp. 105–280.
Ihl, M., Aravena, L., Scheuermann, E., Uquiche, E., Bifani, V., 2003. Effect of immersion solutions on shelf life of minimally processed lettuce. LWT 36, 591–599.
Jiang, Y., Li, Y., 2001. Effects of chitosan coating on postharvest life and quality of longan fruit. Food Chem. 73, 139–143.
Jiang, Y., Li, Y., Jiang, W., 2005. Effects of chitosan on shelf life of cold-stored litchi fruit at ambient temperature. LWT 38, 757–761.
Lambert, R., Skandamis, P., Coote, P., Nychas, G., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91, 453–462.
Maftoonazad, N., Ramaswamy, H., 2005. Postharvest shelf-life extension of avocados using methyl cellulose-based coating. LWT 38, 617–624.
Min, S., Krochta, J., 2005. Inhibition of *Penicillium* commune by edible whey protein films incorporating lactoferrin, lacto-ferrin hydrolysate, and lactoperoxidase systems. J. Food Sci. 70 (2), 87–94.
Moreira, M., Ponce, A., del Valle, C., Roura, S., 2005. Inhibitory parameters of essential oils to reduce a foodborne pathogen. LWT 38, 565–570.
Mossel, D., Moreno García, B., 1985. Microbiología de alimentos. Acribia S.A., Zaragoza, España, pp. 214–272.
Ozcan, M., 2003. Antioxidant activities of rosemary, sage and sumac extract and their combinations on stability of natural peanut oil. J. Med. Food. 6 (3), 267–270.
Park, S., Daeschel, M., Zhao, Y., 2004. Functional properties of antimicrobial lysozyme–chitosan composite films. J. Food Sci. 69 (8), M215–M221.
Pen, L., Jiang, Y., 2003. Effects of chitosan coating on shelf life and quality of fresh-cut Chinese water chestnut. LWT 36, 359–364.
Ponce, A., Fritz, R., Del Valle, C., Roura, S., 2003. Antimicrobial activity of essential oils on the native microflora of organic Swiss chard. LWT 36, 679–684.
Ponce, A., del Valle, C., Roura, S., 2004. Shelf life of leafy vegetables treated with natural essential oils. J. Food Sci. 69 (1), 50–56.
Pranoto, Y., Rakshit, S., Salokhe, V., 2005. Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. LWT 38, 859–865.
Quintavalla, S., Vicini, L., 2002. Antimicrobial food packaging in meta industry. Meat Sci. 62, 373–380.
Schou, M., Longares, A., Montesinos-Herrero, C., Monahan, F., O'Riordan, D., O'Sullivan, M., 2005. Properties of edible sodium caseinate films and their application as food wrapping. LWT 38, 605–610.
Sebranek, J., 2004. Antioxidant effectiveness of natural rosemary extract in pork sausage. IOWA State University Animal Industry Report.
Seydim, A., Sarikus, G., 2006. Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. Food Res. Int. 39, 639–644.
Tharanathan, R., 2003. Biodegradable films and composite coatings: past, present and future. Trends Food Sci. Technol. 14, 71–78.
Volk, W., 1980. Applied Statistics for Engineers, Correlation-regression. McGraw-Hill, Inc., New York.
Zhang, D., Quantick, P., 1997. Effects of chitosan coatings on enzymatic browning and decay during postharvest storage of litchi (*Litchi chinensis* Sonn.) fruit. Postharvest Biol. Technol. 12, 195–202.
Zivanovic, S., Chi, S., Draughon, A., 2005. Antimicrobial activity of chitosan films enriched with essential oils. J. Food Sci. 70 (1), 45–51.