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Antimicrobial and Antioxidant Effects of Milk Protein-Based Film Containing Essential Oils for the Preservation of Whole Beef Muscle

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Milk protein-based edible films containing 1.0% (w/v) oregano, 1.0% (w/v) pimento, or 1.0% oregano−pimento (1:1) essential oils mix were applied on beef muscle slices to control the growth of pathogenic bacteria and increase the shelf life during storage at 4 °C. Meat and film were periodically tested during 7 days for microbial and biochemical analysis. The lipid oxidation potential of meat was evaluated by the determination of thiobarbituric reactive substances (TBARS). The availability of phenolic compounds from essential oils was evaluated by the determination of total phenolic compounds present in the films during storage. Antioxidant properties of films during storage were also evaluated following a modified procedure of the *N*,*N*-diethyl-*p*-phenylenediamine colorimetric method. Oregano-based films stabilized lipid oxidation in beef muscle samples, whereas pimento-based films presented the highest antioxidant activity. The application of bioactive films on meat surfaces containing 10^3 colony-forming units/cm^2 of *Escherichia coli* O157:H7 or *Pseudomonas* spp. showed that film containing oregano was the most effective against both bacteria, whereas film containing pimento oils seems to be the least effective against these two bacteria. A 0.95 log reduction of *Pseudomonas* spp. level, as compared to samples without film, was observed at the end of storage in the presence of films containing oregano extracts. A 1.12 log reduction of *E. coli* O157:H7 level was noted in samples coated with oregano-based films.

KEYWORDS: Whole muscle meat; antimicrobial; antioxidant; edible film; essential oils; lipid oxidation; phenolic compounds; *E. coli*; *Pseudomonas*

INTRODUCTION

Useful shelf life is the time required for a food to become unacceptable from a sensory, nutritional, microbiological, or safety perspective (*1*). Carved beef has a shelf life that varies between 3 and 5 days when kept at 4 °C (*2*). *Pseudomonas*, *Enterobacteriacea*, and lactic acid bacteria are responsible for meat deterioration (*3*). Depending on the region, over one-fourth to one-third of the worldwide production of meat is lost every year because of this deterioration (*4*). Moreover, meat and meat products can be contaminated by pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, and *Yersinia enterocolitica*, responsible for foodborne illnesses and deaths (*5*). Controlling the numbers and growth of pathogenic bacteria therefore remains an important objective for sectors of the meat production industry.

Spices are rich in phenolic compounds, such as flavonoids and phenolic acids, which exhibit a wide range of biological effects, including antioxidant (*6*) and antimicrobial (*7*, *8*) properties. The antimicrobial activity of phenolic antioxidants has been studied in meat and its products (*9*). Many spices and herbs and their extracts possess antimicrobial activity, which is almost invariably due to their essential oil fraction (*10*). For example, essential oil fractions of oregano and pimento are efficient against various foodborne bacteria such as *Salmonella* (*11*−*13*). Recent studies have also shown that essential oils of oregano and pimento are among the most active in this respect against strains of *E. coli* including *E. coli* O157:H7 (*14*−*17*). Spice extracts from oregano, sage, rosemary, thyme, and pimento are also reported to possess antioxidant properties comparable to or greater than those of BHA and BHT (*18*−*21*). The antioxidant properties of these extracts have been

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mostly attributed to phenolic compounds present in their essential oil fractions (*22*).

To control food contamination and quality loss, edible coating or biodegradable packaging has been recently introduced in food processing. Several applications for meat, poultry, and seafoods have been reviewed by Gennadios et al. (*23*), with particular emphasis on the reduction of lipid oxidation, weight loss, moisture loss, microbial load, and volatile flavor loss. The coating can serve as a carrier for antimicrobial compounds and/or antioxidant compounds in order to maintain high concentrations of preservatives on the food surfaces. Selected antimicrobial and antioxidant compounds including organic acids or essential oils have been incorporated in edible films and coatings to control bacterial growth during the storage of meat (*24*, *25*) and shrimp (*26*). Immobilization of organic acids in edible coatings based on calcium alginate gel or whey protein has also been used to control *L. monocytogenes* on beef tissue (*27*, *28*).

According of Papadokostaki et al. (*29*) a relationship between polymer structure and the transport of active molecules has been reported. From recent studies done in our laboratories, heat and γ-irradiation have been shown to produce cross-linking and improved physical and functional properties of edible film coatings via the production of cross-links between protein molecules (*30*−*32*). It can be hypothesized that structure modification induced by γ-irradiation could increase the capacity of edible and cross-linked films to control the release of immobilized active compounds. Also, the application of polymers on solid or semisolid foods could increase the antimicrobial and antioxidant efficiency by maintaining high concentrations of active molecules on the food surface, where microbial growth mostly occurs. The objectives of the present study were to (1) evaluate the ability of milk protein-based edible films containing 1.0% essential oils of oregano, pimento, or an oregano−pimento mixture in a ratio 1:1 (w/w) to control *Pseudomonas* spp. and *E. coli* O157:H7 growth on surface-inoculated beef muscle; (2) determine the antioxidant properties of films and the level of 2-thiobarbituric acid (TBA) production in beef muscle; and (3) evaluate the availability of phenolic compounds present in films during storage at 4 °C.

MATERIALS AND METHODS

Preparation of Film. Protein-based films were prepared with milk proteins according to a method developed in our laboratory (*33*−*35*). Calcium caseinate (93.0% w/w protein) and whey protein isolate (WPI, 93.0% w/w protein) were provided by New Zealand Milk Products Inc. (Santa Rosa, CA) and by Davisco Foods International Inc. (Eden Prairies, MN), respectively. Calcium caseinate and WPI were mixed in a ratio of 1:1 (w/w) and slowly solubilized at room temperature for 1 h in distilled water containing 5.0% (w/v) glycerol (Mat Laboratory, Beauport, PQ, Canada) and 0.25% (w/v) of carboxymethyl cellulose (CMC, low viscosity; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for a total protein concentration of 5.0% (w/v) in the film-forming solution. CMC was added as protein stabilizer and glycerol as plasticizer (*31*). The protein-based solution was treated by thermal treatment (90 °C for 30 min), followed by γ-irradiation treatment at a dose of 32 kGy and at a dose rate of 22.928 kGy/h, using a ^{60}Co source UC-15A (MDS-Nordion International Inc., Kanata, ON, Canada) according to the method of Vachon et al. (*32*). Heating WPI was essential for the formation of the intermolecular disulfide bonds. This process is necessary to obtain a flexible film with good mechanical properties (*32*, *36*). Irradiation of calcium caseinate was used to allow the formation of the intermolecular bityrosine bonds (*37*) to improve the functional and rheological properties of the film (*30*) and for immobilization of active compounds (*29*). Immediately after irradiation, four emulsions corresponding to four different experimental groups were prepared: without essential oils and with oregano, pimento, and a pimento−oregano mixture. For each emulsion, modified starch (Purity gum BE, National Starch and Chemical Co., Bridgewater, NJ) and l-α-phosphatidylcholine [soy lecithin, 18% (w/w) purity, Sigma-Aldrich Canada Ltd.] were mixed and solubilized at room temperature in distilled water for a final modified starch concentration of 0.5% (w/v) and a final lecithin concentration of 0.2% (w/v) in the film-forming solution. Oregano and pimento oil (Cedarome Canada Inc., Brossard, PQ, Canada) or a mixture of the two in a 1:1 (w/w) ratio were added to the starch−lecithin solutions for a final oil concentration of 1.0% (w/v) in the film-forming solution. The film-forming solution was homogenized at room temperature for 2 min using a Homogenizator Ultra-Turrax TP18/1059 (Janke & Kunkel, Staufen, Germany) at 20000 rpm. Lecithin was essential for the formation of micellae, and the presence of modified starch improved emulsion stability. Five milliliters of each film formulation was placed into small Petri dishes (50 × 11 mm) and dried under a laminar flow hood for 24 h at room temperature and 40−50% relative humidity (RH).

Preparation of Inocula. Three *Pseudomonas* strains (*P. putida* CRDA V372, *P. fragi* CRDA V378, and *P. fluorescens* V491) and *E. coli* O157:H7 strain (EDL933) were provided by Agriculture and Agri-Food Canada (Lacombe Research Station, Lacombe, AB, Canada) and FRDC (Food Research and Developement Centre, St-Hyacinthe, PQ, Canada). Strains were individually subcultured [1.0% (v/v)] in brain−heart infusion (BHI, Difco Laboratories, Detroit, MI) at 35 ± 1 °C from the stock cultures maintained at −80 ± 1 °C in BHI containing 20% glycerol. Prior to the experiment, 1 mL of each culture was incubated through two incubations of 24 h at 35 ± 1 °C in BHI broth (9 mL). After 48 h, 10 mL of *Pseudomonas* cultures was mixed and placed into a 50-mL tube. The *E. coli* O157:H7 and mixed *Pseudomonas* spp. culture were centrifuged at 1300*g* for 15 min and washed with sterile 0.85% (w/v) NaCl and then resuspended in the same NaCl solution.

Sample Preparation. Beef from the semimembranosus (inside round) (15 kg) was purchased at a local grocery store (IGA, Laval, PQ, Canada) and transported to INRS-Institut Armand Frappier−Canadian Irradiation Center (CIC) under refrigerated conditions in an ice-filled cooler. Meat was cut into 225 pieces (150 pieces for microbiological analysis and 75 pieces for biochemical analysis) of equal thickness (7.5 mm) and diameter (47 mm) using a pastry cutter. The 225 beef muscle pieces (10 pieces of beef/package) were placed in 0.5 mm metallized polyester/2 mm EVA copolymer sterile bags (305 mm × 210 mm, Winpak, St-Léonard, PQ, Canada) and sealed under vacuum. The packages were stored overnight at −80 ± 1 °C until sterilization treatment by irradiation. The irradiation treatment was done at a dose of 25 kGy and a dose rate of 22.928 kGy/h, using a ^{60}Co source UC-15A (MDS-Nordion International Inc.). After irradiation, 150 pieces of beef muscle were inoculated with either *E. coli* O157:H7 or the *Pseudomonas* spp. mixture to obtain a final concentration of 10^3 colony-forming units (CFU)/cm^2 of meat. Each beef muscle sample was inoculated with a volume of 50 μL, which was spread over all of the upper surface of meat. The 75 pieces of meat for the biochemical analysis were not inoculated. Samples of meat was divided into five separate groups: (i) the control group without film (M); (ii) a protein-based film coating without essential oils (C); (iii) a protein-based film coating containing oregano oil (O); (iv) a protein-based film coating containing pimento oil (P); and (v) a protein-based film coating containing oregano oil−pimento oil in a 1:1 (w/w) ratio (P/O). Meat samples of each group were placed individually into sterile Petri dishes (50 × 11 mm) and, except for the control, all samples were covered on either side with one of the corresponding films. Petri dishes were sealed hermetically and stored at 4 ± 1 °C; samples were tested periodically (days 4, 5, 6, and 7) for microbiological and biochemical analysis. Day 1 corresponded to the day of sample preparation.

Bacterial Enumeration. Each meat sample was homogenized for 2 min in 77 mL of sterile peptone water [0.1% (w/v), Difco Laboratories] using a Lab-blender 400 stomacher (Seward Medical, London, U.K.). From this homogenate, serial dilutions were prepared, and appropriate ones were spread plated on sterile Petri plates containing McConkey and BHI agar (Difco Laboratories) for *E. coli* O157:H7 and *Pseudomonas* spp., respectively. Then, BHI plates were incubated

Table 1. Effect of Film Composition on *Pseudomonas* Species during Storage at 4 °C

	log CFU/cm^2 [a,b]				
sample[c]	day 1	day 4	day 5	day 6	day 7
M	3.02 ± 0.20 aA	3.03 ± 0.30 cdA	3.20 ± 0.19 cAB	3.55 ± 0.22 cC	3.33 ± 0.11 dB
C	aA	3.09 ± 0.23 dA	3.57 ± 0.27 dB	3.62 ± 0.13 cB	3.67 ± 0.10 eB
O	aB	2.53 ± 0.21 abA	2.52 ± 0.16 aA	2.44 ± 0.11 aA	2.38 ± 0.09 aA
P	aB	2.77 ± 0.28 bcA	2.96 ± 0.34 bcAB	2.93 ± 0.23 bAB	3.04 ± 0.14 cB
P/O	aC	2.45 ± 0.12 aA	2.91 ± 0.27 bBC	2.78 ± 0.17 bB	2.87 ± 0.12 bBC

[a] Means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$). [c] M, meat without film; C, films without essential oil; O, films containing oregano essential oil; P, films containing pimento essential oil; P/O, films containing oregano and pimento essential oils.

for 24 h and McConkey plates for 48 h at 35 ± 1 °C, and results were expressed as log CFU per cm^2 of meat.

Lipid Oxidation of Meat. The lipid oxidation potential of meat was evaluated by the determination of thiobarbituric acid reactive substances (TBARS). A 10-g portion of each meat sample was blended with 50 mL of distilled−deionized water and 10 mL of trichloroacetic acid (15%, final concentration; TCA, Sigma-Aldrich Canada Ltd.) using a Lab-blender 400 stomacher (Laboratory Equipment) for 2 min. The homogenate was centrifuged at 5900*g* for 5 min, and the supernatant was filtered through a Whatman 0.45 μm filter paper (Whatman International Ltd., Maidstone, U.K.). Two milliliters of 0.06 M thiobarbituric acid (TBA, Sigma-Aldrich Canada Ltd.) was added to 8 mL of the filtrate. The solution was mixed using a vortex for 15 s and placed into an 80 °C water bath for 90 min and then cooled on ice. Absorbance was read at 520 nm on a DMS 200 spectrophotometer (Varian Techtron Pty. Ltd., Mulgrade, Australia), and the concentration of malonaldehyde was calculated on the basis of a standard curve obtained using serial dilutions of a solution of 1,1,3,3-tetramethoxypropane [97% (v/v) TMP; Sigma-Aldrich Canada Ltd.]. The TBA values were expressed as milligrams of malonaldehyde per kilogram of meat.

Phenolic Compound Availability. To evaluate the availability of phenolic compounds in the packaging, film samples (1 g each) were cut using a bistoury; the obtained thin strips were placed in 50 mL of acetone solution and kept under agitation with a magnetic stir bar at room temperature for 30 min. Then the extract was filtered through a Whatman 0.45 μm filter paper (Whatman International Ltd.). Total phenolic compound content in each film extract was determined according to the Folin−Ciocalteu procedure (*38*), and results were expressed as milligrams of gallic acid equivalent (GAE) per milliliter of extract.

Antioxidant Properties of Films. Antioxidant properties were evaluated by their antiradical capacity following a modified procedure of the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (*39*), as reported by Le Tien et al. (*40*). A piece of film weighing 25 mg was added in a cell containing 3 mL of 0.15 M NaCl and subjected to an electrolysis for 1 min (continuous current, 400 V, 10 mA) using a generator (model 1000/500, Bio-Rad, Mississauga, ON, Canada). After electrolysis, 200 μL was sampled and added to 2 mL of DPD solution (25 mg/mL; Sigma-Aldrich Canada Ltd.). The oxidative species released during the electrolysis reacted instantaneously with DPD, producing a red color, and the intensity of the color was measured at 515 nm using a DMS 200 spectrophotometer (Varian Techtron Pty. Ltd.). The colorimetric reaction was calibrated with ascorbic acid. The antioxidant capacity describes the film capacity to inhibit the accumulation of oxidative species (able oxidize DPD) such as free radicals and consequently to prevent the appearance of the red color at 515 nm. The scavenging percentage is calculated according to the equation

$$\text{scavenging (\%)} = 100 - [(OD_{sample}/OD_{control}) \times 100]$$

where $OD_{control}$ represents the OD of the electrolyzed solution in the absence of film. In fact, OD is directly related to the degree of oxidation of DPD reagent by the oxidative species. Thus, films able to reduce completely the level of reactive oxidative species will have a 100% scavenging capacity.

Experimental Design and Statistical Analysis. Experiments for microbiological analysis were done using a 3 × 5 × 2 × 5 factorial design [3 replicates, 5 treatments (control, film without essential oils, oregano, pimento, and oregano−pimento mix of essential oils), 2 bacteria (*Pseudomonas* spp. and *E. coli*), and 5 storage periods (1, 4, 5, 6, and 7 days)].

Biochemical analyses were done using a 3 × 5 × 5 factorial design [3 replicates, 5 treatments (control, film without essential oils, oregano, pimento, and oregano−pimento mix of essential oils), and 5 storage periods (1, 4, 5, 6, and 7 days)].

Analysis of variance and Duncan's multiple-range tests were employed to perform statistical analysis on all results. Differences between means were considered to be significant when $p \leq 0.05$. StatPackets Statistical Analysis software (Walonick Associates, Inc., Minneapolis, MN) was used for the analysis. For each measurement, three samples in each replication were tested.

RESULTS AND DISCUSSION

Microbiology. Effects of films on *Pseudomonas* spp. and *E. coli* O157:H7 counts during storage at 4 °C are presented in **Tables 1** and **2**. Results showed that the use of film containing essential oils reduced significantly ($p \leq 0.05$) the microorganism level in meat as compared to meat samples coated with C film and M samples during 7 days of storage. Moreover, results showed that O film was the most effective against the growth of both bacteria. P/O-based films were significantly ($p \leq 0.05$) more efficient against the growth of *E. coli* O157:H7 than P-based film. However, P/O- and P-based films have a similar effectiveness against the growth of *Pseudomonas* spp. Results also showed that the application of C films enhanced the growth of both bacteria on meat.

Results on *Pseudomonas* content presented in **Table 1** showed that after 4 days of storage, the application of O-, P-, and P/O-based films showed significant 0.50, 0.25, and 0.60 log decreases ($p \leq 0.05$), respectively. At day 4, *Pseudomonas* contents were, respectively, 2.53 ± 0.21, 2.77 ± 0.28, and 2.45 ± 0.12 log CFU/cm^2 in meat coated with O-, P-, and P/O-based films. However, the *Pseudomonas* content was stable in M and C meat samples during the 4 first days, showing levels of 3.03 ± 0.30 and 3.09 ± 0.29 log CFU/cm^2, respectively. Also, the level of microorganisms in meat coated with O-based film was stable until the end of the storage. Between days 4 and 7, the content of *Pseudomonas* increased significantly ($p \leq 0.05$) in M, C, P, and P/O samples. At the end of storage the contents of *Pseudomonas* in M, C, P, and P/O samples were, respectively, 3.33 ± 0.11, 3.67 ± 0.10, 3.04 ± 0.14, and 2.87 ± 0.12 log CFU/cm^2 as compared to 2.38 ± 0.09 log CFU/cm^2 in O samples. These results showed a 0.95 log reduction in O samples after 7 days of storage as compared to 0.30 and 0.45 log reductions in P and P/O samples, respectively.

The determination of *E. coli* O157:H7 counts is presented in **Table 2**. Results showed that the content of *E. coli* O157:H7 was stable in M and P samples during the 4 first days of storage, showing levels of 2.80 ± 0.18 and 2.73 ± 0.21 log CFU/cm^2,

Table 2. Effect of Film Composition on *E. coli* O157:H7 during Storage at 4 °C

	log CFU/cm^2 [a,b]				
sample[c]	day 1	day 4	day 5	day 6	day 7
M	2.82 ± 0.26 aA	2.80 ± 0.18 bA	3.03 ± 0.33 cA	3.56 ± 0.20 bB	3.52 ± 0.30 cB
C	aA	3.50 ± 0.24 cB	4.29 ± 0.26 dC	4.77 ± 0.32 cD	5.02 ± 0.40 dD
O	aB	2.40 ± 0.02 aA	2.44 ± 0.11 aA	2.40 ± 0.10 aA	2.40 ± 0.13 aA
P	aA	2.73 ± 0.21 bA	2.72 ± 0.22 bA	3.47 ± 0.36 bB	3.58 ± 0.21 cB
P/O	aCD	2.42 ± 0.09 aA	2.52 ± 0.16 aAB	2.66 ± 0.37 aBC	2.92 ± 0.32 bD

[a] Means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$). [c] M, meat without film; C, films without essential oil; O, films containing oregano essential oil; P, films containing pimento essential oil; P/O, films containing oregano and pimento essential oils.

respectively. During the same period, a significant 0.68 log increase ($p \leq 0.05$) was noted in C samples, reaching a value of 3.50 ± 0.24 log CFU/cm^2, whereas significant decreases ($p \leq 0.05$) of 0.40 and 0.38 log were, respectively, observed in O and P/O samples, reaching respective values of 2.40 ± 0.02 and 2.42 ± 0.09 log CFU/cm^2. Between days 4 and 7, the content of *E. coli* O157:H7 in O samples was stable, whereas a significant increase ($p \leq 0.05$) was observed in all other samples. The *E. coli* O157:H7 counts in M, C, P, and P/O samples at day 7 were, respectively, 3.52 ± 0.30, 5.02 ± 0.40, 3.58 ± 0.21, and 2.92 ± 0.32 log CFU/cm^2. These results showed that O and P/O films were able to reduce by 1.12 and 0.60 log, respectively, the content of *E. coli* O157:H7 during the 7 days of storage. However, P film did not have any effect ($p > 0.05$) on the content of *E. coli* O157:H7 during storage. At the end of the storage period, the level of *E. coli* O157:H7 in C samples was significantly higher ($p \leq 0.05$) compared to all other samples.

Our study showed that the incorporation of essential oils into the cross-linked coating film formulation significantly decreased ($p \leq 0.05$) the level of *E. coli* O157:H7 and *Pseudomonas* spp. contents on meat samples. However, our data suggest also that both bacteria seem to use milk protein-based film in the absence of essential oils as a substrate to sustain their growth. Bagamboula et al. (*41*) have observed that, when oregano and pimento extracts were added at a concentration of 1% (w/v) in MH agar, these spice extracts inhibited the growth of five strains such as *E. coli* LMG 8223. Also, Burt and Reinders (*16*) have shown in vitro results in agreement with those obtained in our study, where oregano essential oil is more active against the strain *E. coli* O157:H7 than pimento essential oil. In addition, our results showed that an oil concentration of 1% (w/v) was effective against *Pseudomonas* spp. and *E. coli* O157:H7 growth. Bagamboula et al. (*41*) showed that the minimal inhibitory concentration (MIC) of oregano was 0.5% (w/v) for five bacterial strains (*Shigella sonnei* strain 6, *Shigella sonnei* CIP 82.49, *Shigella flexneri* strain1, *Shigella flexneri* CIP 82.48, and *E. coli* LMG 8223). These results are in agreement with those obtained by Paster et al. (*11*), who found that below 0.25%, the oregano essential oil was ineffective or very slightly effective against five tested bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Clostridium sporogenes*). However, Gangrade et al. (*42*) examined the antimicrobial properties of the different essential oils in their pure state at four dilutions (1:10, 1:100, 1:1000, and 1:10000) against *E. coli*. They found that essential oils had a significant inhibitory effect ($p \leq 0.05$) against the bacterial strain for all dilutions tested.

Chemical analysis of essential oils provided by the manufacturer has indicated that the major compounds identified in oregano essential oil are phenolic monoterpenes, that is, carvacrol (61.75%) and thymol (4.32%), and nonoterpenic hydrocarbons such as *p*-cymene (10.73%), γ-terpinene (7.42%), caryophyllene (3.20%), and myrcene (1.94%). Tested pimento essential oil is rich in phenolic monoterpenes, that is, eugenol (74.40%) and methyleugenol (5.98%), and monoterpenic hydrocarbon caryophyllene (6.81%).

It is known that phenolic compounds such as carvacrol, thymol, and eugenol exhibit a major antimicrobial effect (*43*−*50*). According to Farag et al. (*51*), the antimicrobial activities of essential oils are predominantly related to their main components. Diluted by 100-fold, the essential oils inhibited the tested meat spoilage organisms, and a relationship between the inhibitory effect of essentials oils and the presence of eugenol was found (*52*). Kim et al. (*48*) found that eugenol possesses a potent inhibitory activity against five bacterial strains including *E. coli* O157:H7 and noted a dose-related increase of the inhibition zone against all tested microorganisms. The antimicrobial potential of oregano essential oils is related to their high phenolic contents, particularly carvacrol and thymol (*12*). According to Helander et al. (*13*), carvacrol and thymol are able to inhibit *E. coli* O157:H7 at a concentration between 1 and 3 mm by disintegration of the outer membrane and by the release of outer membrane-associated materials from the cells to the external medium. Moreover, according to these authors, these components decrease the intracellular ATP pool of *E. coli* O157:H7 and also increase the extracellular ATP pool, indicating a disruptive action on the cytoplasmic membrane. Besides, substances with similar properties, such as eugenol and carvacrol, may have additive or cumulative effects. Güllüce et al. (*53*) have reported that the combination of these compounds showed a synergistic antimicrobial effect on microorganisms. The synergistic effect of these chemical compounds could also account for the activity of the essential oils (*54*). Our results, however, failed to demonstrate a synergistic antimicrobial effect of oregano oil in combination with pimento oil.

Lipid Oxidation. Results of the TBARS concentration in meat, which represents the malonaldehyde concentration, are presented in **Table 3**. The initial concentration of TBARS was 1.19 ± 0.16 mg/kg at day 1 of storage. The concentration was stable in M samples during 5 days of storage, followed by a significant increase ($p \leq 0.05$) to reach a value of 1.77 ± 0.05 mg/kg at day 6. At day 4, no significant difference ($p > 0.05$) was observed between all treated samples and M samples except for O samples, where the TBARS content was significantly ($p \leq 0.05$) higher with a value of 1.94 ± 0.15 mg/kg. The TBARS values in C, P, and P/O samples were, respectively, 1.54 ± 0.19, 1.64 ± 0.21, and 1.65 ± 0.21 mg/kg at day 4 as compared to 1.26 ± 0.37 mg/kg in M samples. However, the TBARS concentration in O samples remained stable from day 4 to day 7. During the same period of time, the TBARS content increased significantly ($p \leq 0.05$) in M, P, and P/O samples. The TBARS content in C samples increased significantly ($p \leq 0.05$) between days 4 and 5 to reach a value of 1.98 ± 0.15 mg/kg, followed

Table 3. Concentration of TBARS in Meat Samples with and without Film during Storage at 4 °C

	concn of TBARS[a,b] (mg of malonaldehyde/kg of meat)				
sample[c]	day 1	day 4	day 5	day 6	day 7
M	1.19 ± 0.16 aA	1.26 ± 0.37 aA	1.18 ± 0.10 aA	1.77 ± 0.05 aB	1.60 ± 0.24 aB
C	aA	1.54 ± 0.19 abAB	1.98 ± 0.15 bC	1.92 ± 0.12 abC	2.02 ± 0.12 aC
O	aA	1.94 ± 0.15 bB	1.93 ± 0.09 bB	2.04 ± 0.20 abB	1.76 ± 0.18 aB
P	aA	1.64 ± 0.21 abAB	2.16 ± 0.16 bC	2.57 ± 0.21 cCD	2.81 ± 0.30 bD
P/O	aA	1.65 ± 0.21 abAB	2.18 ± 0.28 bBC	2.32 ± 0.30 bcC	2.67 ± 0.28 bC

[a] Means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$). [c] M, meat without film; C, films without essential oil; O, films containing oregano essential oil; P, films containing pimento essential oil; P/O, films containing oregano and pimento essential oils.

Table 4. Availability of Total Phenolic Compounds in Films Containing Essential Oils during Storage at 4 °C

	total phenolic compounds[a,b] (mg of EAG/g of film)						
film[c]	day 1	day 2	day 3	day 4	day 5	day 6	day 7
O	2.08 ± 0.05 aA	5.31 ± 0.37 aB	6.02 ± 0.91 aBC	7.02 ± 0.23 aC	6.39 ± 0.64 aBC	6.21 ± 0.52 aBC	6.60 ± 0.62 aC
P	4.46 ± 0.07 cA	10.02 ± 1.08 bBC	11.29 ± 1.01 cCD	11.90 ± 0.34 cD	10.39 ± 0.44 bBC	9.39 ± 0.77 bB	10.39 ± 0.28 bBC
P/O	2.92 ± 0.12 bA	6.42 ± 0.66 aA	8.32 ± 0.67 bC	10.22 ± 0.50 bD	9.66 ± 0.94 bD	7.27 ± 0.71 aBC	7.53 ± 0.71 aBC

[a] Means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$). [c] O, films containing oregano essential oil; P, films containing pimento essential oil; P/O, films containing oregano and pimento essential oils.

by a stabilization until the end of storage. At day 7, no significant difference ($p > 0.05$) was noted between M, C, and O samples. Moreover, TBARS values were significantly ($p \leq 0.05$) higher in P (2.81 ± 0.30 mg/kg) and P/O (2.67 ± 0.28 mg/kg) samples than in M (1.60 ± 0.24 mg/kg), C (2.02 ± 0.12 mg/kg), and O (1.76 ± 0.18 mg/kg) samples.

These results showed that the incorporation of essential oils in the films did not improve the protection of the meat samples against lipid oxidation. P- and P/O-based films increased significantly ($p \leq 0.05$) lipid oxidation in beef muscle samples, despite the fact that these oils contained compounds which were identified as potential antioxidants. These results seem to be apparently inconsistent with previous studies. Lee and Anh (*55*) showed that phenolic compounds, such as gallate and sesamol, were effective antioxidants in reducing TBARS in turkey breast during 5 days of storage. Nam et al. (*56*) observed a similar trend in ground beef storage at 4 °C during 7 days. Phenolic compounds such as quercetin were also effective in preventing lipid oxidation in both raw and cooked turkey during 7 days of storage (*57*). Nevertheless, it is known that pimento oil is rich in phenolic compounds such as eugenol and that these compounds are unstable. The degradation pathway of some phenolic compounds could generate phenolic aldehydes. These aldehydes could provoke a similar reaction to malonaldehyde reaction when lipid oxidation of meat was evaluated by the determination of TBARS. It would probably result in an increase of TBARS values, which would mask the antioxidant effect of essential oils. This hypothesis could explain also the difference between our results and those presented in the literature. Indeed, all previous authors used phenolic compounds at concentrations of 0.01% (w/v) in meat homogenates as compared to 1% in our study. The results indicated that C- and O-based films stabilized lipid oxidation in beef muscle samples. The results also showed that the ability of oregano oil to inhibit lipid peroxidation is more important than that of pimento oil, which is richest in phenolic content. A similar trend was observed by Martinez-Tome et al. (*58*). Results obtained with C film are probably related to the presence of some active compounds showing scavenging properties in film. Effectively, according to Le Tien et al. (*40*) milk proteins and CMC present in film formulations possess good antiradical properties. These authors showed that whey protein and CMC were the most effective compounds.

Availability of Phenolic Compounds. The availability of total phenolic compounds in O-, P-, and P/O-based films was determined during the whole storage period at 4 °C (**Table 4**). Initial concentrations of phenolic compounds were 2.08 ± 0.05, 4.46 ± 0.07, and 2.92 ± 0.12 mg/g in O, P, and P/O films, respectively. Between days 1 and 4, a significant increase ($p \leq 0.05$) of phenolic compound availability was noted in all films evaluated. At day 4, total phenolic concentrations were 7.02 ± 0.23, 11.90 ± 0.34, and 10.22 ± 0.50 mg/g in O-, P-, and P/O-based films, respectively. From day 4 to day 7 a significant decrease ($p \leq 0.05$) of the phenolic compound availability was observed in P- and P/O-based film to reach respective levels of 10.39 ± 0.28 and 7.53 ± 0.71 mg/g at day 7. However, during the same period of time, the phenolic compound availability in O-based films remained stable until the end of the storage time, with a value of 6.60 ± 0.62 mg/g at day 7. The results also indicated that at day 1 the phenolic compound availability in P-based films is twice (4.46 ± 0.07 versus 2.08 ± 0.05 mg/g) that of O-based films. However, the average release rates of phenolic compounds between days 1 and 4 are, respectively, 1.64, 2.48, and 2.43 mg/g/day for O-, P-, and P/O-based films. At days 4 and 7, the phenolic compound availabilities in the P-based films are, respectively, 1.70 (11.90 ± 0.34 versus 7.02 ± 0.23 mg/g) and 1.57 (10.39 ± 0.28 versus 6.60 ± 0.62 mg/g) times higher than the phenolic compound availability in the O-based films. According to these results, it seems that the phenolic compound migration in oregano-based films is higher than that in pimento-based films. Chemical analysis of essential oils provided by the manufacturer has shown that tested pimento and oregano essential oils have, respectively, average concentrations of phenolic monoterpenes of 80 and 68% (w/w). The major phenolic monoterpenes of these essentials oils (eugenol and methyleugenol in pimento and carvacrol and thymol in oregano) have comparable molecular weights. On the other hand, the hydrophobicity of these compounds, which is related to their structure, could have an influence on their migration (*59*, *60*). According to Zhao and Singh (*61*), eugenol is a highly

Table 5. Antiradical Properties of the Different Films during Storage at 4 °C

	antiradical properties[a,b] (USP/g of film)			
film[c]	day 1	day 4	day 5	day 7
C	58.78 ± 3.45 aA	80.22 ± 7.64 aB	80.20 ± 8.13 aB	74.98 ± 7.51 aB
O	70.60 ± 5.56 aA	89.33 ± 8.92 abB	88.37 ± 8.78 aB	84.37 ± 8.59 aAB
P	132.46 ± 11.33 cB	125.28 ± 11.48 cB	128.11 ± 12.34 bB	109.37 ± 10.42 bA
P/O	111.22 ± 9.21 bB	114.81 ± 10.07 bcB	121.45 ± 11.95 bB	98.77 ± 9.01 abA

[a] Means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$). [c] C, control films without essential oil; O, films containing oregano essential oil; P, films containing pimento essential oil; P/O, films containing oregano and pimento essential oils.

Table 6. Antiradical Properties Related to the Available Phenolic Compounds in Films Containing Essential Oils during Storage at 4°C

	antiradical properties[a,b] (USP/mg of phenolic compounds)			
film[c]	day 1	day 4	day 5	day 7
O	33.94 ± 2.67 abB	12.73 ± 1.27 aA	13.83 ± 1.37 aA	12.78 ± 1.30 abA
P	29.70 ± 2.54 aB	10.53 ± 0.96 aA	12.33 ± 1.19 aA	10.53 ± 1.00 aA
P/O	38.09 ± 3.15 bB	11.23 ± 0.99 aA	12.57 ± 1.24 aA	13.12 ± 1.20 bA

[a] Means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$). [c] O, films containing oregano essential oil; P, films containing pimento essential oil; P/O, films containing oregano and pimento essential oils.

hydrophobic compound. Indeed, the increase of the phenolic compound availability in all films at day 4 is probably due to the increase of film hydration obtained in the presence of meat, making easier the extraction of phenolic compounds. This hypothesis is consistent with the fact that the high level of film hydration could increase the diffusion rate of phenolic compounds from the film to the meat and could, consequently, decrease the phenolic compound availability in the films.

Antiradical Properties of Films. The antiradical properties of films are presented in **Table 5**. The antiradical properties of C-, O-, P-, and P/O-based films were, respectively, 58.78 ± 3.45, 70.60 ± 5.56, 132.46 ± 11.33, and 111.22 ± 9.21 USP/g at day 1. The antiradical properties increased significantly ($p \leq 0.05$) in C- and O-based films to reach respective values of 80.22 ± 7.64 and 89.33 ± 8.92 USP/g after 4 days of storage, followed by a stablization, and respective values of 74.98 ± 7.51 and 84.37 ± 8.59 USP/g were reached at day 7. On the other hand, the antiradical properties remained stable in P- and P/O-based films from day 1 to day 6 of storage, followed by a significant decrease ($p \leq 0.05$) at day 7 to reach respective values of 109.37 ± 10.42 and 98.77 ± 9.01 USP/g. The antiradical properties reported on the basis of phenolic compound availability in films are presented **Table 6**. The antiradical properties of O-, P-, and P/O-based films were, respectively, 33.94 ± 2.67, 29.70 ± 2.54, and 38.09 ± 3.15 USP/mg at day 1. The antiradical properties decreased significantly ($p \leq 0.05$) in O-, P-, and P/O-based films to reach respective values of 12.73 ± 1.27, 10.53 ± 0.96, and 11.23 ± 0.99 USP/mg at day 4, followed by a stablization, and respective values of 12.78 ± 1.30, 10.53 ± 1.00, and 13.12 ± 1.20 USP/mg were reached at day 7. This result suggests a release of phenolic compounds between days 4 and 7.

The results of **Table 5** showed that the pimento essential oil addition permitted a significant increase ($p \leq 0.05$) of the antiradical properties. On the other hand, no significant ($p > 0.05$) difference was observed between C and O during the whole storage period. According to Radonic and Milos (*62*), the antiradical properties of essential oils were related to their chemical composition of free volatile compounds. Results showed that antiradical properties of O-based films was significantly ($p \leq 0.05$) lower than those of P- and P/O-based films, although oregano oil contained compounds with greater antiradical properties than those contained in pimento oil (*58*). However, **Table 6** showed no significant difference ($p > 0.05$) between the O- and P-based films during the whole storage time. In fact, the higher antiradical properties of pimento as compared to oregano oils could be explained by the higher concentration of phenolic compounds present in them. According to Teisseidre and Waterhouse (*63*), the antiradical activity of pimento oil was essentially due to the presence of eugenol. Bostsoglou et al. (*64*) showed that oregano oil increased the oxidative stability of breast and thigh turkey meat during frozen storage. A high radical scavenging property has been demonstrated for the main components of oregano essential oil (carvacrol, γ-terpinene, thymol, *p*-cymene) (*65*, *66*). However, other compounds could contribute to the antiradical properties of oregano oil. Indeed, Lagouri and Boskou (*67*) showed that α-, β-, γ-, and δ-tocopherol were found to be present in all tested species of oregano. According to the same authors, total tocopherol content ranged from 288 to 672 ppm, and these compounds have powerful antioxidant properties. However, it is important to stress that a high antiradical activity protects not only the meat but also the bacteria by reacting with the free radicals that would otherwise react with the bacteria. Chiasson (*68*, *69*) showed that the bactericidal action of carvacrol against *E. coli* ATCC 25922 in ground beef was eliminated or reduced with the addition of another compound with high antiradical properties, such as ascorbic acid.

In summary, the incorporation of essential oils into milk protein-based edible film applied onto muscle meat helps to reduce microbial load and increase antioxidative activity, during 7 days of storage. These results support also the hypothesis that the inhibition of *E. coli* O157:H7 and *Pseudomonas* spp. growth by essential oils depends on the nature of the phenolic compounds. The phenolic compound concentration could also play an important role in the antimicrobial and antioxidant activities that they confer to essential oils used in this study. Oregano-based films exhibited the most effective antimicrobial property, whereas pimento-based films presented the highest antioxidant activity. Also, the films allow a progressive release of phenolic compounds during storage. After 7 days, the availability of the phenolic compounds in films being always

significant, the films remain effective. The use of edible films containing essential oils as a preservation method of meat is promising. However, the formulation and the mechanical properties must be improved to control the moisture content of the film and the diffusion rate of the essential oil from the film to the meat surface during storage. Other works are in progress to improve the physicochemical properties of films.

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