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Antimicrobial and antioxidant influence of *Syzygium aromaticum* oil supplementation on minced beef quality during cold storage

Antimikrobieller und antioxidativer Einfluss von Gewürznelkenöl (Syzygium aromaticum) auf die Qualität von Rinderhackfleisch während der Kühllagerung

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Summary

The inhibition of oxidative factors and pathogenic bacteria is of importance to keep stability and microbiological safety of meat products. The potential of cold pressed clove *(Syzygium aromaticum)* oil (CPCO) supplemented at 1 %, 2 % and 4 % (w/w) to minced beef (MB) on the chemical traits and the growth of pathogens (*Salmonella* Enteritidis PT4 and *Listeria monocytogenes* Scott A) inoculated (*ca.* 4.6 log CFU/g) artificially in MB samples during cold storage (4 °C) for 15 days was investigated. Linoleic and oleic were the main fatty acids in CPCO, while α-tocopherols was the major tocol. Changes in MB parameters (pH, antioxidant potential, oxidative stability and proteins profile) were assessed during cold storage. CPCO exhibited antibacterial potential with inhibition zones of 12.7 mm and 11.8 mm against *L. monocytogenes* and *S.* Enteritidis, respectively. Supplementation with CPCO retarded the growth of total bacterial count wherein food-borne pathogens were unable to grow in CPCO-supplemented MB. After 15 days of cold storage, *L. monocytogenes* was more resistant than *S.* Enteritidis at all supplementation levels of CPCO. The shelf life of CPCO-supplemented MB was prolonged under refrigerated conditions with low microbial loads. Oxidative stability of CPCO-supplemented MB was improved while sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile was not changed. The obtained results could be applied in the development of meat products rich in bioactive phytochemicals with longer shelf life.

Keywords: Healthy oils, clove, *Listeria monocytogenes, Salmonella* Enteritidis, food-borne pathogens, ground meat

Zusammenfassung

Die Hemmung oxidativer Faktoren und pathogener Bakterien ist für die Erhaltung von Stabilität und mikrobiologischer Sicherheit von Fleischerzeugnissen von Bedeutung. Untersucht wurde das Potential von kaltgepresstem Gewürznelkenöl auf die chemischen Eigenschaften und das Wachstum von pathogenen Bakterien in Hackfleischproben. Die mit 1 %, 2 % und 4 % Gewürznelkenöl behandelten Proben wurden mit *Salmonella* Enteritidis PT4 und *Listeria monocytogenes* Scott A (ca. 4,6 log KbE/g) beimpft und bei 4 °C 15 Tage gelagert. Linolsäure und Ölsäure waren die Hauptfettsäuren des kaltgepressten Gewürznelkenöls, während α-Tocopherole das Haupttocol war. Veränderungen anderer Parameter (pH-Wert, Antioxidationspotential, Oxidationsstabilität und Proteinprofil) wurden während der Kühllagerung festgestellt. Das antibakterielle Potential des Gewürznelkenöls zeigte sich anhand von Hemmhöfen von 12,7 mm bzw. 11,8 mm gegenüber *L. monocytogenes* bzw. *S.* Enteritidis. Durch die Supplementierung des Gewürznelkenöls verzögerte sich das Wachstum der Gesamtkeimzahl, wobei die pathogenen Bakterien nicht wuchsen. Nach 15 Tagen Kühllagerung zeigte sich *L. monocytogenes* in allen Konzentrationsstufen widerstandsfähiger als *S.* Enteritidis. Die Haltbarkeit der mit Öl behandelten Hackfleischproben wurde mit einer geringen mikrobiellen Belastung unter gekühlten Bedingungen verlängert. Des Weiteren wurde die oxidative Stabilität verbessert, während das Proteinprofil der Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese (SDS-PAGE) sich nicht veränderte. Die erzielten Ergebnisse könnten bei der Entwicklung von länger haltbaren Fleischerzeugnissen angewendet werden, die reich an bioaktiven Pflanzenstoffen sind.

Schlüsselwörter: Gesunde Öle, Nelken, *Listeria monocytogenes, Salmonella* Enteritidis, Lebensmittelpathogene, Hackfleisch

Introduction

Despite the use of food additives and new preservation techniques, the problems of meat poisoning and spoilage, by microorganism's activity or by oxidation processes, during processing and storage are of great interest for food manufacture and consumers (Shan et al. 2007, Viuda-Martos et al. 2011). Meat products are microbiologically contaminated due to excessive handling such as slaughtering, processing and transporting. Minced beef (MB) is a meat product with safety considerations because it provides a suitable media for the growth of pathogens (Mahgoub et al., 2017). Food-borne pathogens are of importance for food biosafety wherein they are responsible for millions of cases of illness and hospitalizations worldwide (Buzby et al., 1996; Luther et al., 2007).

Food scientists are always searching for value-added spices due to the rising global demand for cross-cultural cuisines and authentic ethnic. Several spices were recognized to possess health-promoting effects such as antioxidant and antimicrobial traits (Beuchat, 1994; Shan et al., 2005; El-Ghorab et al., 2010; Ramadan et al., 2013). Consumers are looking for natural preservatives for a healthy life. Natural preservatives rich in health-promoting phytochemicals are important additives for food systems. Natural antimicrobial products including spices and harbs as well as their extracts have been reported (Sitohy et al. 2012, Osman et al. 2012, Mahgoub et al. 2013, Osman et al. 2013, Sitohy et al. 2013, Hassanien et al. 2014).

Clove (*Syzygium aromaticum* L., family Myrtaceae) is utilized in food processing due to their flavor and health-promoting traits (Wenqiang et al., 2007; Ramdan et al., 2013). Clove essential oil (EO) have antimicrobial and antioxidant traits, wherein the EO is used as flavoring agents in food products (Huang et al., 2002; Mylonasa et al., 2005; Gulcin et al., 2012). Clove EO had antilisteric activity in cheese and meat (Menon and Garg, 2001; Matan et al., 2006). Moreover, clove EO exhibited antioxidant potential and inhibited the oxidation of poppy and hazelnut oils (Ozcan and Arslan, 2011). Eugenol (4-allyl-2-methoxyphenol), the major aroma compound of clove, was reported to have antimicrobial traits (Lee and Shibamoto, 2002; Miyazawa and Hisama, 2003; Mytle et al., 2006). When administered at levels lower than 1500 ppm in food products, clove EO has been approved by FDA as a ''Generally Regarded As Safe'' substance (Gulcin et al., 2012). The acceptable daily human intake of clove EO is 2.5 mg/kg body weight as approved by World Health Organization (WHO) Expert Committee on Food Additives (Kildeaa et al., 2004).

Recently, interest in edible cold pressed oils (CPO) has been considered because of consumers' desire for safe food. Cold pressing is able to retain bioactive phytochemicals with antioxidant or antimicrobial activity such as phenolic compounds, flavonoids and tocols in the oils (Simopoulos et al., 2000; Ahn et al. 2003; Parry et al. 2006; Luther et al. 2007; Lutterodt et al. 2010; Ramadan 2013; Mahgoub et al., 2017). Cold pressed clove *(Syzygium aromaticum)* oil (CPCO) was investigated for its fatty acids and tocopherols profile (Ramdan et al., 2013). Linoleic, oleic, and stearic acids were the main fatty acids in CPCO, while α-tocopherol was the main tocol. CPCO also exhibited antimicrobial activities against several strains of microorganisms.

To best of knowledge, there is no report on the protective potential of supplementing CPCO to MB. The objective of the present research was to inactivate pathogenic bacteria (*S.* Enteritidis and *L. monocytogenes*) in MB stored under cold storage (4 ºC) and monitor the quality characteristics of MB supplemented with CPCO during storage for 15 days. The obtained results are of importance to develop novel meat products rich in active molecules with a desirable shelf life.

Material and methods

Materials

CPCO and fresh beef meat were obtained from the local market (Zagazig, Egypt). Meat samples were minced in sanitized meat mincers. The samples of the minced beef (MB) were transferred to sterilized polyethylene sachets and preserved at 4 °C. *Listeria monocytogenes* Scott A and *Salmonella enterica* subsp. *enterica* serovar Enteritidis PT4 strains were kindly obtained from Prof. George John Nychas (Department of Food Science and Technology, Laboratory of Food Microbiology and Biotechnology, Agricultural University of Athens, Greece). Standards used for tocols analysis were from Merck (Darmstadt, Germany).

Analysis of fatty acids, tocols and total phenolic compounds in CPCO

CPCO was analyzed for fatty acid methyl esters (FAME) according to Arens et al. (1994). Fatty acids of CPCO were transesterified into FAME using N-trimethylsulfoniumhydroxide (Macherey-Nagel, Düren, Germany. FAME were identified on a Shimadzu GC-14A equipped with FID and C-R4AX chromatopac integrator (Kyoto, Japan). The flow rate of the carrier gas helium was 0.6 mL/min and the split value with a ratio of 1:40. A sample of 1 µL was injected on a 30 m x 0.25 mm x 0.2 µm film thickness Supelco SPTM-2380 (Bellefonte, PA, USA) capillary column. The injector and FID temperature was set at 250 °C. The initial column temperature was 100 °C programmed by 5 °C/min until 175 °C and kept 10 min at 175 °C, then 8 °C/min until 220 °C and kept 10 min at 220 °C. A comparison between the retention times of the samples with those of an authentic standard mixture (Sigma, St. Louis, MO, USA; 99 % purity specific for GLC), was made to facilitate identification.

CPCO was analyzed for tocols profile using high-performance liquid chromatography (HPLC) according to Ramadan (2013). A solution of *ca.* 250 mg of CPCO in 25 mL *n*-heptane was used for the HPLC. HPLC analysis was performed using a Merck Hitachi low-pressure gradient system, fitted with an L-6000 pump, a Merck-Hitachi F-1000 Fluorescence Spectrophotometer (detector wavelength was set at 295 nm for excitation and at 330 nm for emission) and a D-2500 integration system. CPCO sample (20 µL) was injected by a Merck 655-A40 Autosampler onto a Diol phase HPLC column 25 cm 9 4.6 mm ID (Merck, Darmstadt, Germany) at a flow rate of 1.3 mL/min. The mobile phase used was *n*-heptane: tert-butyl methyl ether (99:1, v/v).

CPCO was analyzed for total phenolic content (TPC) using Folin-Ciocalteu's reagent according to Ramadan et al. (2012). Aliquots of CPCO (1 g) were dissolved in *n*-hexane (5 mL) and mixed with 10 mL methanol-water (80:20, v/v) in a glass tube for two min in a vortex. After centrifugation at 3000 rpm for 10 min, the hydroalcoholic extracts were separated from the lipid phase by using a

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Pasteur pipette then combined and concentrated *in vacuo* at 30 °C until a syrup consistency was reached. The lipidic residue was re-dissolved in 10 mL methanol: water (80:20, v/v) and the extraction was repeated twice. Hydroalcoholic extracts were re-dissolved in acetonitrile (15 mL) and the mixture was washed 3 times with *n*-hexane (15 mL each). Purified phenols in acetonitrile were concentrated in vacuo at 30 °C then dissolved in methanol for further analysis. Aliquots of phenolic extracts were evaporated to dryness under nitrogen. The residue was re-dissolved in 0.2 mL water and diluted (1:30, v/v) Folin-Ciocalteu's phenol reagent (1 mL) was added. After 3 min, 7.5 % sodium carbonate (0.8 mL) was added. After 30 min, the absorbance was measured at 765 nm using a UV-260 visible recording spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid was used for the calibration and the results of triplicate analyses are expressed as parts per million of gallic acid.

Work was carried out under subdued light conditions. Experiments were repeated at least thrice when the variation on any one was routinely less than 5 %. All experimental procedures were performed in triplicate and the mean values were given.

Minimal inhibitory concentration (MIC)

The antibacterial activity of CPCO against *L. monocytogenes* Scott A and S. Enteritidis PT4 was assayed by the Hole-Plate diffusion method according to Hammer et al. (1999) and Mahgoub et al. (2017). Each organism was cultured on 250 mL nutrient agar. The mixture was shacked and poured into sterile Petri dishes to obtain the media. The plates were left at room temperature for solidification. The wells were made in plates by sterile cork borer (6 mm in diameter) and 10 µL of the neat undiluted CPCO was placed into each well with sterile micropipette. The plates were left at room temperature prior to incubation till the CPCO diffused. The plates were incubated at 37 °C for 24 h. After incubation, the inhibition zones surrounding-hole and contact area was measured in mm (Pranoto et al. 2005). Three replications of the experiment were carried out.

Pathogens inoculation in MB

L. monocytogenes Scott A and *S.* Enteritidis PT4 strains were maintained on tryptone soy agar (TSA; Biolife, Milan, Italy) slants at 4 °C, which were sub-cultured every 30 days (Mahgoub et al., 2017). Prior to use, cultures were activated by three successive transfers into 10 mL tryptic soy broth (TSB; Biolife, Milan, Italy) at 37 °C for 24 h. Cells were harvested by centrifugation (14.000 rpm for 10 min at 4 °C), washed three times and re-suspended in Ringer's solution (Lab Merck, Bury, UK). The resulting pellet was washed once with Ringer's solution to remove residual organic material, re-centrifuged, and then re-suspended in Ringer's to a final volume of 10 mL. A final inoculum was prepared by serially diluting in Ringer's solution to reach a final level of 8 Log CFU/mL.

Experiment of storage under refrigerator conditions (4 °C)

Fresh MB (65.12 ± 0.16 g per 100 g moisture, 1.05 ± 0.11 per 100 g ash, 19.7 ± 0.23 g per 100 g protein and 10.9 ± 0.16 g per 100 g lipids) were transferred to sterilized polyethylene sachets. Analysis of protein, ash, lipids and moisture content of MB samples was performed according to AOAC (1990). Portions (400 g) of MB were placed into a sterile stomacher bags then homogenized into a sterile stomacher for 5 min at room temperature to serve as control. The MB samples were divided to four portions (one without addition of CPCO and three with addition of CPCO at 1.0 %, 2.0 % and 4.0 %, w/w). About 400 g of MB were placed into a sterile stomacher bags and inoculated with mixture strains inocula of *L. monocytogenes* (*ca.* 5 Log CFU/g) and *S.* Enteritidis (*ca.* 5 Log CFU/g). The inoculated samples were supplemented with CPCO at 1.0, 2.0 and 4.0 % (w/w), wherein control sample did not supplemented with CPCO. All stomacher bag samples were homogenized into a sterile stomacher for 5 min at room temperature to ensure proper distribution of the pathogen and CPCO. All stomacher bags with samples from all treatments were wrapped and stored under aerobic conditions at 4 °C for 15 days. Microbiological and physicochemical analyses of samples (n = 3) were carried out at different intervals of storage 0, 1, 3, 6, 12 and 15 days (Mahgoub et al., 2017).

pH value

Values of pH were recorded after different intervals of preservation (0–15 days) at 4 ± 1 °C. Five grams of each MB sample was blended with water and the pH of the suspension was measured using pH meter (pH 211 HANNA instruments Inc. Woonsocket, USA).

Antioxidant tests

Five gram of each MB sample was homogenized. A volume of 10 % (w/v) homogenate was prepared in 0.05 M phosphate buffer (pH 7) and centrifuged at 12000×*g* for 60 min at 4 °C. The obtained supernatant was used for the estimation of reducing power, antiradical activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH·) and lipid oxidation.

Reducing power. The reducing power method adopted by Jung et al. (2010) was used to measure the antioxidant capacity in the CPCO-supplemented MB. A 200 µL aliquot was mixed with 500 µL sodium phosphate buffer (0.2 M, pH 6.6) and 500 µL potassium ferricyanide (1 %), and the mixture was incubated at 50 °C for 20 min. After addition of 2.5 mL of 10 %v/v trichloroacetic acid, the mixture was centrifuged (Jouan-MR 23i, France) at 2200×*g* for 10 min. The upper layer (500 µL) was mixed with 500 µL distilled water and 100 µL ferric-chloride (0.1 %). The mixture was allowed to react for 10 min followed by measuring the absorbance at 700 nm (JENWAY 6405 UV/visible spectrophotometer, UK). Values of absorbance at 700 were recorded after different intervals (0–15 days) of preservation at 4 °C. Increased absorbance of the reaction mixture indicated higher reducing power.

DPPH· radical scavenging test. Antiradical activity against DPPH· of CPCO-supplemented MB was estimated using Blois (1958) method with few modifications. A volume of 1 mL of 0.2 mM DPPH· prepared in ethanol was added to 200 µL supernatant and 800 µL distilled water. The mixture was vortexed and left to stand at room temperature for 30 min. A tube containing 1 mL of methanol and 1 mL of DPPH· solution was used as control whereas ethanol alone was used as a blank. The absorbance of the solution was measured at 517 nm (JENWAY 6405 UV/visible spectrophotometer, UK). The scavenging activity of meat samples against DPPH· was expressed as percent of control and calculated as:

$$\% \text{ Inhibition of DPPH·} = [1 - (\text{absorbance of sample/absorbance of control})] \times 100$$

Lipid oxidation assay

Lipid oxidation in the MB samples and CPCO-supplemented MB was estimated using Niehius and Samuelson (1968) method after different intervals of preservation (0–15 days) at 4 °C. A volume of 0.1 mL supernatant was treated with 2 mL of (1:1:1, v/v/v) TBA:TCA:HCl reagent (thiobarbituric acid 0.37 %, 15 % trichloroacetic acid and 0.25 N HCl). All tubes were placed in a boiling water bath for 30 min and allowed to cool. The amount formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm (JENWAY 6405 UV/visible spectrophotometer, UK) against a reagent blank. Percentage inhibition was calculated as follow:

% Inhibition of lipid oxidation =
[1 – (absorbance of sample/absorbance of control)] x 100.

Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a discontinuous buffered system according to Laemmli (1970) using 3 and 18 % stacking and principal gels, respectively. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine, and 0.1 SDS. Samples (2 g) were mixed with 20 mL of 10 % (w/v) SDS solution (85 °C). The mixture was homogenized and incubated at 85 °C in a water bath for 60 min to dissolve proteins. It was centrifuged at 5000×*g* for 10 min at room temperature to remove the undissolved debris (Mahgoub et al., 2017). An aliquot of the supernatant (20 µL) was mixed with 20 µL of SDS-sample buffer (4 % SDS, 3 % β-mercaptoethanol, 20 % glycerol, 50 mM Tris HCl, pH 6.8 and traces of bromophenol blue), heated at 96 °C for 3 min and a 10 µL aliquot from the final mixture was electrophoresed. After running at 10 mA on the stacking gel and 20 mA on the running gel, staining was performed with Coomassie Brilliant Blue R-250 dye.

Microbiological analysis

The samples of MB supplemented with CPCO (1 %, 2 % and 4.0 %, w/w) were examined after 0, 1, 3, 6, 12 and 15 days of storage at 4 °C. Samples supplemented with both CPCO and tested pathogens also were examined during the storage period. Twenty-five g of sample were added aseptically to 225 mL of sterile peptone saline diluents (1.0 g peptone, 8.5 g sodium chloride in 1 L distilled water) and homogenized in stomacher bags. Total viable count (TVC) was determined on plate count agar (PCA, Merck, 1.05463, Germany) after incubation at 30 °C for 48 h. *L. monocytogenes* was enumerated on Polymyxin-Acriflavin-Lithium Chloride-Ceftazidime-Aesculin-Mannitol agar (PALCAM, Biolife 401604, Milan, Italy) after incubation for 48 h at 35 °C. *S.* Enteritidis was counted on Xylose Lysine Deoxycholate agar (XLD) agar (Merck, 1.05287) after incubation for 24 h at 37 °C. The lowest detection limit was 2 Log CFU/g. All plates were examined for typical colony types and morphological characteristics associated with each culture medium. Presumptive colonies of *L. monocytogenes* and *S.* Enteritidis were verified by confirmation tests according to the guidelines of ISO 11290 (International Organization for Standardization 1998) and ISO 6579 (International Organization for Standardization 1991).

Statistical analysis

Data were statistically analyzed using ANOVA variance analysis through the general linear models (GLM) procedure of the statistical analysis system software (SAS version 9.1, SAS Institute, 2003). Least significant differences were used to separate means at $p < 0.05$. The model included treatment, storage time and their interaction as fixed effects. Differences between effects were assessed by the Duncan test ($p < 0.05$).

Results and discussion

Fatty acids, tocols and phenolics content of CPCO

Seven FAME were detected in CPCO including C10:0 (2.76 %), C16:0 (8.60 %), C16:1 (0.35 %), C18:0 (6.71 %), C18:1*n*-9 (39.5 %), C18:2*n*-6 (40.1 %) and C18:3 (2.01 %). From the results, it could be stated that in CPCO, linoleic acid was the main FAME followed by oleic acid (comprising together about 80 % of the total FAME), wherein palmitic and stearic acids were the main saturated fatty acids. High levels of monounsaturated and polyunsaturated fatty acids in CPCO makes the oil a value-added material for nutritional applications. Amounts of α-, β-, γ- and δ-tocopherols in CPCO were 11912, 40, 3344 and 149 mg/kg oil, respectively. Furthermore, the levels of α-, β-, γ- and δ-tocotrienols were 888, 44, 68 and 7598 mg/kg oil, respectively. α-Tocopherol constituted about 50 % of the total estimated tocols followed by δ-tocotrienol and γ-tocopherol. γ-Tocopherol found in high levels in linseed, corn and rapeseed oils (Schwarz et al., 2008). α-Tocopherol is the most effective antioxidant of tocol isomers, while β-tocopherol has about 50 % of the antioxidant potential of α-tocopherol and the γ-tocopherol has about 10–35 % (CAC, 2001; Kallio et al., 2002). Amounts of tocols measured in CPCO might contribute to the oil oxidative stability. On the other hand, total phenolic content in CPCO was 4.42 mg GAE/g. it well stated that phenolics are associated with the antioxidant potential of plant extracts.

Effect of CPCO supplementation on the pH values, stability and protein profile of MB

pH values of CPCO-supplemented MB samples compared to control MB during storage at 4 °C for 15 days are tabulated in Table 1. CPCO-supplemented MB retarded greatly the changes in pH. After 15 days of storage, pH levels reached the highest value in the control MB (6.89) compared to lower values in case of CPCO-enriched MB at 1 %, 2 % and 4 % (6.21, 6.09 and 5.97, respectively). pH values of control MB increased significantly ($p<0.05$) during 15 days of storage from 5.54 to 6.89. Other researchers reported the increase in the pH levels in MB stored under chill conditions which linked to the growth of bacteria especially pseudomonads (Agunbiade et al. 2010). The effect of nisin and NaCl on the survival of *L. monocytogenes* added to buffalo mince was investigated, whereinthe pH values in the supplemented groups were lower than in the control (Pawar et al. 2000).

The antioxidant activity of CPCO in MB was tested using reducing power test and the results in different CPCO-supplemented MB are presented in Table 2. In the control MB sample, the antioxidant traits increased from 0.46 at zero time to 0.78, 1.46 and 1.95 after supplementation with 1 %, 2 % and 4 % CPCO, respectively. This increase in antioxidant activity might be due to CPCO enrichment. Compared to samples at zero time, the increase in storage period to 15 days reduced the antioxidant potential slightly in CPCO-enriched BM. For example, the antioxidant potential in BM supplemented with 4 % CPCO decreased from 1.95 at zero-day to 1.78 after 15 days. At the end of the storage period, the antioxidant

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activity in MB supplemented with 2 % CPCO decreased from 1.64 at zero-day to 1.51. Lipid oxidation is the main cause of food deterioration that leads to rancidity and shortening of the product shelf life (Philanto 2006; Balasundram, et al., 2006). Moreover, lipid oxidation products could react with protein leading to protein oxidation (Parkin and Damodaran 2003).

Antioxidants are added to meat products to inhibit lipid oxidation wherein α-tocopherol is usually used as an antioxidant agent in meat products, but its antioxidative impact depends mainly on the variety and part of meat (Yamauchi et al., 1977; Endo et al., 2015). DPPH· antiradical assay is a simple method to evaluate the antioxidant potential of bioactive compounds. Changes in the antiradical potential (DPPH· assay) of CPCO-enriched MB samples are presented in Table 3. In general, CPCO-supplemented MB samples were more stable compared to control sample. The results stated a variation in the percentage inhibition of DPPH· in CPCO-supplemented MB (1 %, 2 % and 4 % CPCO), wherein the DPPH· percentage of inhibition recorded 35.5 %, 58.3 and 76.3 %, respectively, compared to control at zero time (23.4 %). This fast increase in antiradical potential could be due to supplementation with CPCO. In case of control MB, the inhibition of oxidation decreased from 23.4 % (at zero-day) to 13.0 % (after 12 days). After 15 days of cold storage, the percentage inhibition of DPPH· for CPCO-supplemented MB (1 %, 2 %, and 4 %) decreased from 35.3 % to 27.3 %, from 58.3 % to 43.2 % and from 76.3 % to 65.5 %, respectively.

Lipid oxidation inhibition in MB samples supplemented with different levels of CPCO (1 %, 2 % and 4 %, w/w) compared to control is presented in Table 4. During the cold storage experiment, there was a gradual decrease in the percentages of lipid oxidation inhibition for all samples. However, control sample exhibited much decrease in lipid oxidation inhibition (10.07 %) than CPCO-enriched MB samples at 1 %, 2 % and 4 % (16.9 %, 20.0 % and 22.0 %, respectively) after 15 days of storage at 4 °C.

Electrophoretic profile of CPCO-supplemented MB samples (Fig. 1) with different CPCO levels (1 %, 2 % and 4 %, w/w) during storage for 0–15 day at 4 °C did not deviate from the control MB sample. Supplementation with different levels of CPCO did not change the number and intensity of the protein bands and the overall electrophoretic patterns in MB components [actin (AC), paramyosin (PM) and myosin heavy chains (MHC)]. It could be observed that the most intense band was MHC and actin was the second most intense. The study of the functionality of meat proteins emerges as an important issue to take into account to elucidate the impact of additives incorporated into meat products and the mechanisms involved in the improvement of sensorial and physical traits (Pighin, 2012). Meat proteins have a wide range of size between 20 kDa to 3,000 kDa. Among them, high molecular weight proteins display an important role in conferring meat functional traits (Warris, 2000). Myosin and actin are major proteins of the skeletal sarcomere and the most important myofibrillar proteins interacting with each other in the muscular tissue. Myosin (approx. 540 kDa) and actin (approx. 43 kDa) are the most contractile proteins of the thick and thin filaments, respectively (Clark et al., 2002; Pighin, 2012).

Antimicrobial effect of CPCO supplementation on pathogenic bacteria in MB

The antibacterial activity of CPCO against *L. monocytogenes* and *S.* Enteritidis was tested using the Hole-Plate diffusion test. CPCO exhibited antibacterial traits against both pathogenic bacteria with inhibition zones of 12.7 ± 0.23 and 11.8 ± 0.31 mm against *L. monocytogenes* and *S.* Enteritidis, respectively. Clove EO, containing mainly

TABLE 1: *Changes in pH values of MB supplemented with different levels (1 %, 2 % and 4 %, w/w) of CPCO during storage at 4 °C for different periods compared to control MB.*

Preservation time (day)	pH			
	Control	1 % CPCO	2 % CPCO	4 % CPCO
0	5.54 ± 0.000	5.54 ± 0.000	5.54 ± 0.000	5.54 ± 0.000
1	5.56 ± 0.012	5.54 ± 0.008	5.53 ± 0.006	5.53 ± 0.008
3	5.78 ± 0.000	5.72 ± 0.015	5.61 ± 0.000	5.58 ± 0.011
6	6.12 ± 0.011	5.81 ± 0.000	5.73 ± 0.013	5.69 ± 0.000
12	6.75 ± 0.000	6.02 ± 0.005	5.96 ± 0.007	5.87 ± 0.013
15	6.89 ± 0.009	6.21 ± 0.000	6.09 ± 0.000	5.97 ± 0.000

TABLE 2: *Antioxidant capacity measured by reducing power assay (OD_{700}) of MB supplemented with 1 %, 2 % and 4 % (w/w) of CPCO during storage at 4 °C for different periods compared to control MB.*

Preservation time (day)	Reducing power (OD_{700})			
	Control	1 % CPCO	2 % CPCO	4 % CPCO
0	0.46 ± 0.000	0.78 ± 0.006	1.64 ± 0.000	1.95 ± 0.005
1	0.45 ± 0.002	0.82 ± 0.009	1.68 ± 0.006	1.95 ± 0.000
3	0.39 ± 0.004	0.81 ± 0.005	1.66 ± 0.000	1.92 ± 0.004
6	0.28 ± 0.005	0.74 ± 0.004	1.61 ± 0.003	1.86 ± 0.000
12	0.24 ± 0.008	0.69 ± 0.007	1.58 ± 0.007	1.82 ± 0.005
15	0.22 ± 0.003	0.61 ± 0.000	1.51 ± 0.006	1.78 ± 0.000

TABLE 3: *DPPH· free radical scavenging activity of MB supplemented with different levels (1 %, 2 % and 4 %, w/w) of CPCO during storage at 4 °C for different periods compared to control MB.*

Preservation time (day)	% Inhibition of DPPH·			
	Control	1 % CPCO	2 % CPCO	4 % CPCO
0	23.43 ± 0.20	35.58 ± 0.17	58.39 ± 0.09	76.38 ± 0.21
1	22.87 ± 0.40	36.48 ± 0.12	57.38 ± 0.12	78.39 ± 0.13
3	20.20 ± 0.20	35.22 ± 0.15	56.48 ± 0.23	77.41 ± 0.17
6	17.73 ± 0.19	32.12 ± 0.16	56.49 ± 0.48	75.39 ± 0.18
12	13.09 ± 0.21	30.30 ± 0.11	53.28 ± 0.19	73.39 ± 0.28
15	12.63 ± 0.23	27.39 ± 0.15	43.22 ± 0.17	65.56 ± 0.38

TABLE 4: *Percentages of lipid oxidation inhibition in MB supplemented with CPCO (1 %, 2 % and 4 %, w/w) during storage at 4 °C for different periods compared to control MB.*

Preservation time (day)	Lipid oxidation inhibition (%)			
	Control	1 % CPCO	2 % CPCO	4 % CPCO
0	28.37 ± 0.08	28.37 ± 0.08	28.37 ± 0.08	28.37 ± 0.08
1	27.12 ± 0.12	26.38 ± 0.00	27.84 ± 0.18	27.71 ± 0.00
3	26.73 ± 0.16	25.03 ± 0.06	26.10 ± 0.21	26.98 ± 0.19
6	16.24 ± 0.21	22.00 ± 0.23	24.89 ± 0.25	26.82 ± 0.16
12	12.21 ± 0.18	18.80 ± 0.27	22.28 ± 0.09	24.45 ± 0.11
15	10.07 ± 0.11	16.93 ± 0.19	20.02 ± 0.00	22.07 ± 0.14

eugenol, was effective in inhibition of the growth of *L. monocytogenes, E. coli, S.* Enteritidis and *S. aureus* in different agar mediums (Cressy et al., 2003; Mytle et al., 2006). Other *in vitro* and *in situ* studies on the antimicrobial potential of edible films incorporated with clove EO and black cumin oil against health-related pathogens or food spoilers have been reported (Burt, 2004; Fernández-Pan et al., 2012; Mahgoub et al., 2013; Hassanien et al., 2014).

The addition of CPCO conferred important *in situ* antimicrobial properties to MB stored at 4 °C. The total viable count (TVC), *S.* Enteritidis and *L. monocytogenes* in MB supplemented with different levels of CPCO (1 %, 2 % and 4 %, w/w) during storage at 4 °C for 15 days is shown in Figure 2. MB samples have not been subjected to any treatment to reduce its initial microbial load to avoid interference with the natural ecosystem of the samples and minimize any potential impact of decontamination on the interactions between the spoilage flora and the pathogens. In the control experiment (without CPCO), TVC and *L. monocytogenes* were unaffected by storage, wherein TVC and pathogens were grown gradually in MB from 5.7 and 4.8 to 8.8 and 6.7 Log CFU/g (maximum increases were 3.1 and 1.9 Log CFU/g), respectively. However, *S.* Enteritidis was influenced by cold storage and this bacterium was not significantly grow (4.6 to 5.0 Log CFU/g). On the other hand, *L.monocytogenes* and *S.* Enteritidis were significantly ($p < 0.05$) influenced in CPCO-supplemented MB and the pathogens were decreased from 4.8 and 4.6 to 3.30 and 3.5 Log CFU/g (maximum decreases were 1.5 and 1.1 Log CFU/g), respectively. In the control MB and CPCO-supplemented MB, the final pH values did not exceed 6.8. This pH value is suitable for growing of these pathogens in meat samples. However, *L. monocytogenes* Scott A and *S.* Enteritidis PT4 strains were influenced by CPCO supplementation at 4 % for 15 days, but no significant ($p > 0.05$) effect was measured for 1 % or 2 % (w/w) supplementation levels of CPCO. *L. monocytogenes* was more survival than *S.* Enteritidis in MB stored at 4 °C. The antimicrobial potential of natural extracts, especially *Syzygium aromaticum,* is linked with their total phenolics (Cai and Wu, et al., 1996; Ahn et al. 2003; Alzoreky and Nakahara, 2003; Allahghdri, et al., 2010). Thus, oils or extracts rich in bioactive phtochemcials might serve as natural antimicrobial factors (Arora and Kaur 1999; Luther et al., 2007; Ramadan, 2013). Phenolic compounds and other natural antioxidants are powerful active compounds expressing high antioxidant and antimicrobial traits (Ahn et al., 2003). These activities are due to their redox potential that play a great role in quenching reactive oxygen species and chelating metals, especially iron and copper cations as well as neutralizing free radicals (Bettaieb et al., 2010).

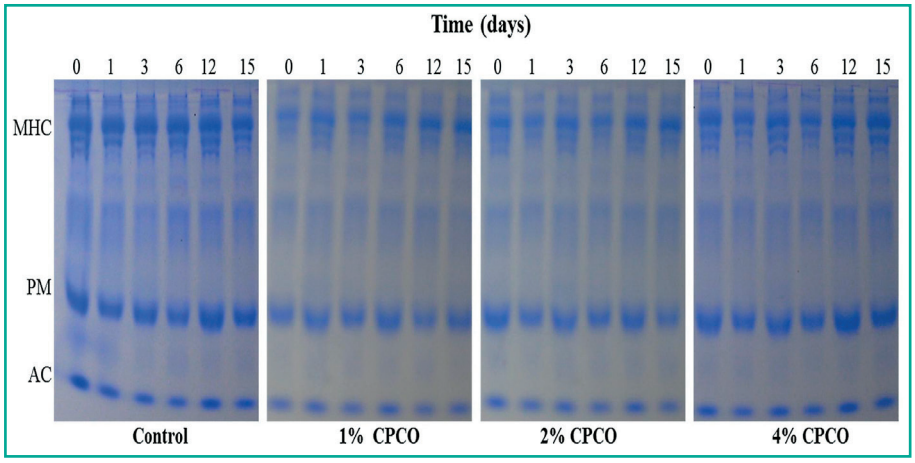

FIGURE 1: *SDS-PAGE electrophoretic patterns of control MB and MB supplemented with CPCO at different concentrations (1 %, 2 % and 4 %, w/w) during storage at 4 °C for 0–15 days (MHC: myosin heavy chains; AC: actin; PM: paramyosin).*

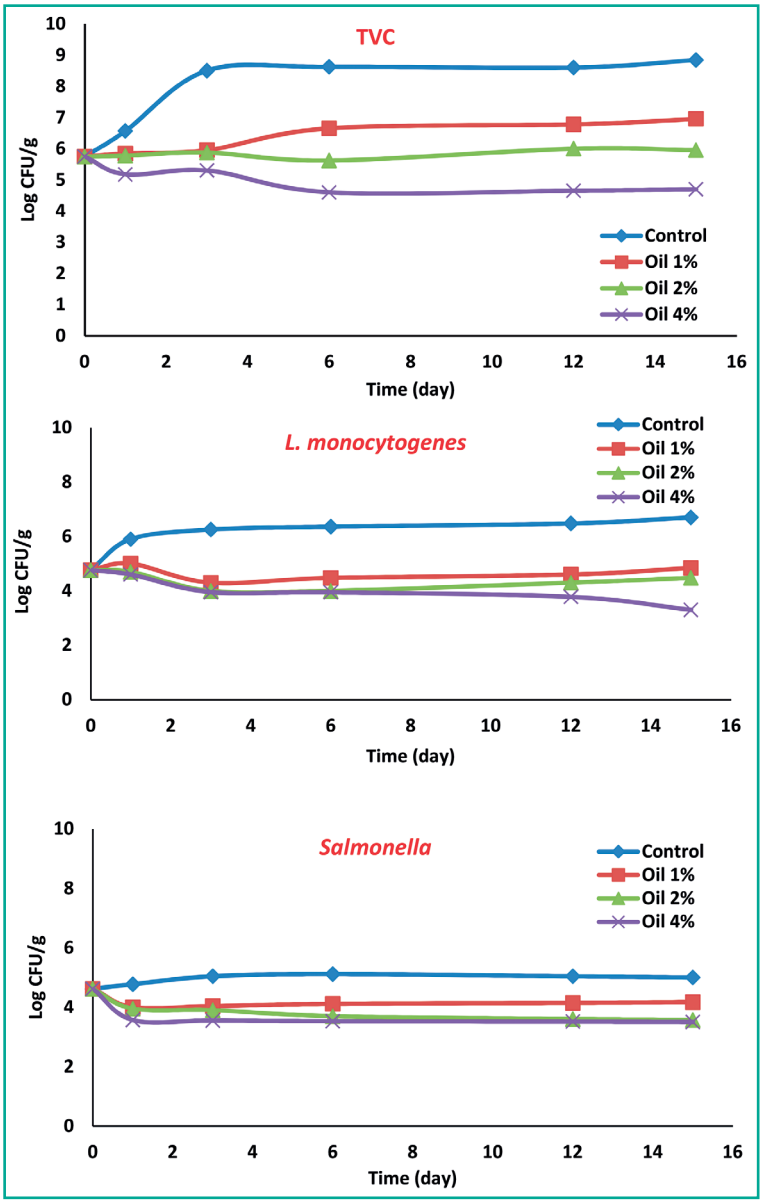

FIGURE 2: *Survival of L. monocytogenes Scott A and S. Enteritidis PT4 in MB supplemented with CPCO during storage at 4 °C for 15 days.*

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Conclusions

The interest of applying natural antioxidants in food products is increased. Food rich in phytochemicals became the consumer approach. CPCO is rich in lipid-soluble bioactives and antioxidants. In our study, CPCO supplementation improved stability and microbiological traits of supplemented BM. The shelf life of CPCO-enriched BM was prolonged with lower microbial loads during cold storage. Our results suggest that CPCO might be utilized in some technological fields as a natural ingredient in food, nutraceutical and/or pharmaceutical products.

Conflict of interest

The authors declare that they have no conflict of interest.

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