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Evaluation of the Antimicrobial Action of Whey Protein Edible Films Incorporated with Cinnamon, Cumin and Thyme Against Spoilage Flora of Fresh Beef

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ABSTRACT

Antimicrobial packaging, besides protecting the product from external environment, inhibits or retards microorganism growth in foods minimizing direct addition of preservatives and satisfying the actual demand of consumers for healthier foods, containing less additives. The objective of this study was to evaluate the antimicrobial efficiency of Whey Protein Edible Films (WPEF) incorporated with 1-2.5% cinnamon (CI), cumin (CU) and thyme (TH) essential oils on fresh red meat during refrigerated storage for 12 days at 5°C. Strongest inhibition was observed on surfaces sliced meat onto which films containing TH essential oils extracts as a result of its greater antimicrobial activity under these conditions. In addition, WPEF films that contain a high level of TH, CI or CU essential oils, significantly reduce the number of total viable bacterial count during the 12 days storage period. The SEM analyses indicated that the incorporation of the tested plants extracts modified the structure of the films towards a less regular structure. The films incorporated with CU, CI and TH essential oils showed potential use as one hurdle technology added in the storage period among others good manufacturing practices for preservation of sliced fresh red meat.

Key words: Edible film, active packaging, fresh meat, antimicrobial, whey proteins

INTRODUCTION

Antimicrobial packaging is a form of active packaging that could extend the shelf-life of product and provides microbial safety for consumers. It acts to reduce, inhibit or retard the growth of pathogen microorganisms in packed foods and packaging material. In order to control undesirable microorganisms on food surfaces, volatile and non-volatile antimicrobial agents can be incorporated into polymers or coating or adsorbing antimicrobial onto polymer surfaces (Appendini and Hotchkiss, 2002). The film can serve as a carrier for antimicrobial compounds and/or antioxidants compounds in order to maintain high concentrations of preservatives on the food surfaces (Oussalah *et al*., 2004).

Spices are rich in phenolic compounds such as flavonoids and phenolic acids (Dadalioglu and Evrendilek, 2004). Generally, the essential oils posses antibacterial properties against food borne pathogens because it contains higher concentrations of phenolic compounds. These compounds exhibit a wide range of biological effects including antioxidant and antimicrobial properties. The mode of action is generally considered to be the disturbance of the cytoplasmic membrane,

disrupting the proton motive force, electron flow, active transport and/or coagulation of cell contents (Burt, 2004). Some spice essential oils incorporated into packaging materials can control microbial contamination in beef muscle by reducing the growth of *Escherichia coli* O157:H7 and *Pseudomonas* sp. (Oussalah *et al.*, 2004).

Whey proteins have excellent functional properties and exceptional nutritional value (Huffman, 1996; Cha and Chinnan, 2004; Bodnar *et al.*, 2007). In addition, liquid whey is produced in large quantities and its annual production increases continuously (Ozdemir and Floros, 2001). Much of this whey is not utilized and it creates serious pollution and waste disposal problems. The formation of edible films and coatings composed of whey is not only important in finding new uses for whey proteins but also in improving the microbial stability of foods particularly in the presence of preservatives in film formulations.

In contrast to the large amount of information on the use of various antimicrobial films for controlling meat pathogens, little is known about their effect on the spoilage microflora of these products. In the present study fresh beef cuts were wrapped into whey protein edible films WPEF containing essential oils of cinnamon (CI), cumin (CU) and thyme (TH) at five different levels. The effectiveness of these films against beef's spoilage flora during storage at 5°C for 12 days was studied. The impact of the above mentioned EO on the mechanical properties of the developed films was previously studied (Badr *et al.*, 2013). Since, the overall performance of the films depends on their physicochemical properties, the micro-structural properties of those films, was be tested as well.

MATERIALS AND METHODS

Whey protein isolate was obtained from Davisco Foods International Inc. (BiPRO®, Le Sueur, MN, USA). Glycerol, NaOH, Brain Heart Infusion agar (BHI) and Brain Heart Infusion broth (BHI) were purchased from Sigma Chemical Co., St. Louis, MO, USA, plate count agar (MO91, Himedia, India), Ringer solution (Sigma Chemical Co., St. Louis, MO, USA). Cinnamon powder, Cumin powder and Thyme were purchased from local market in Cairo, Egypt.

Essential oil extraction: Cinnamon, cumin and thyme essential oils were obtained by steam-distillation for 3 h using a Clevenger-type apparatus according to the method of Dadalioglu and Evrendilek (2004). The obtained Essential Oil EO was then removed and stored at 4°C until use.

Film preparation: The whey protein isolate WPI films were formed with the modification of the method described by Kim and Ustunol (2001). Whey protein isolate (5% wt/vol) was dissolved in distilled water and glycerol (5% w/v) was added. The pH was adjusted to 8.0 with 2 N NaOH. Then, solutions were heated to 90± 2°C while being stirred continuously for 30 min. Solutions were cooled to room temperature for 1.5 h. Vacuum was applied for 30 min to remove dissolved air in the solutions. The films forming solutions (30 mL) were casted on 18.5 cm circular glass surfaces and then dried overnight at 35°C and 45±5% RH.

Film conditioning: Protein and polysaccharide films, being hydrophilic, are susceptible to moisture absorption and due to the fact that most of their properties are affected by Relative

Humidity (RH) and temperature, preconditioning was necessary to standardize all films by keeping them in an environmental chamber at 50±3% RH and a temperature of 23±2°C for 48 h before the tests were carried out (ASTM, 2000).

Micro-structural analysis of the developed films: Film samples were examined for surface and cross-section characteristics using SEM (JXA-840A, JEOL Ltd. Tokyo, Japan). Samples were affixed to aluminum stubs with double-sided cellophane tape and coated with a layer of gold prior to imaging (Wang *et al*., 2010).

Meat sample packing and storage: Freshly cut beef was purchased from a local retail store. The meat was divided in small pieces (2.1×2.5×1 cm) and these were wrapped in cross-shaped antibacterial cinnamon, cumin and thyme films prepared in concentrations 0, 1, 1.5, 2 and 2.5% (mg g^{-1} film) that covered the entire meat surface. Unwrapped Beef samples were served as controls. The meat samples were placed into a sterile petri dish covered with plastic film and stored in high precision (5±0.2°C) low-temperature incubators (model D-30938 Burguedel; Geselischaft for labor technik (GFL), Germany). All samples were evaluated periodically for microbiological quality at 0, 2, 4, 6, 8, 10 and 12 days (Zinoviadou *et al*., 2010).

Microbiological analyses for meat samples: Beef samples (5 g) were aseptically removed from the plastic film, added to 45 mL of sterile Ringer solution and homogenized for 2 min at room temperature. In the case of the samples wrapped with antimicrobial films, the film was carefully removed and added in the ringer solution to wash off the bacteria that could be attached to its surface. Decimal dilutions in Ringer solution were prepared and 0.1 mL samples of appropriate dilutions were poured or spread to plate count agar for Total Viable Count (TVC) and incubated at 25°C for 72 h. The storage experiments for the beef cuts were performed twice and duplicate samples for each treatment were analyzed for their microflora at each time.

Statistical analysis: Results obtained in the present study were analyzed using SPSS software (SPSS Statistics Version 17.0, Chicago, USA). Data was reported as mean and standard deviation. All assays were carried out in duplicate unless otherwise stated.

RESULTS AND DISCUSSION

Micro-structural analysis of the developed films: Scanning Electron Microscope (SEM) is widely used to analyze the micro-structure of edible films and to correlate the properties of films with the morphological structure (Chen *et al*., 2009; Garcia *et al*., 2009; Rhim *et al*., 2006). Also in the present study, the surface and cross-section morphology of WPI films were analyzed using SEM. The surface and cross-sections of scanning electron microscope for films formed from whey protein isolate only and film incorporated with EO of cinnamon, cumin and thyme were tested Fig. 1. The SEM images revealed that there are significant structural differences among the samples. The surface area of films without adding essential oils shows film structures characterized by homogeneous, interpenetrating polymer network (sample 1). Incorporating essential oils are generally present as discrete oil droplets dispersed within the continuous matrix of the predominant phase of the films (samples 3, 5, 7). General speaking the surface area of the films shows a smooth and compact structure but there is no visible pores and may be used as an edible film wrap structure.

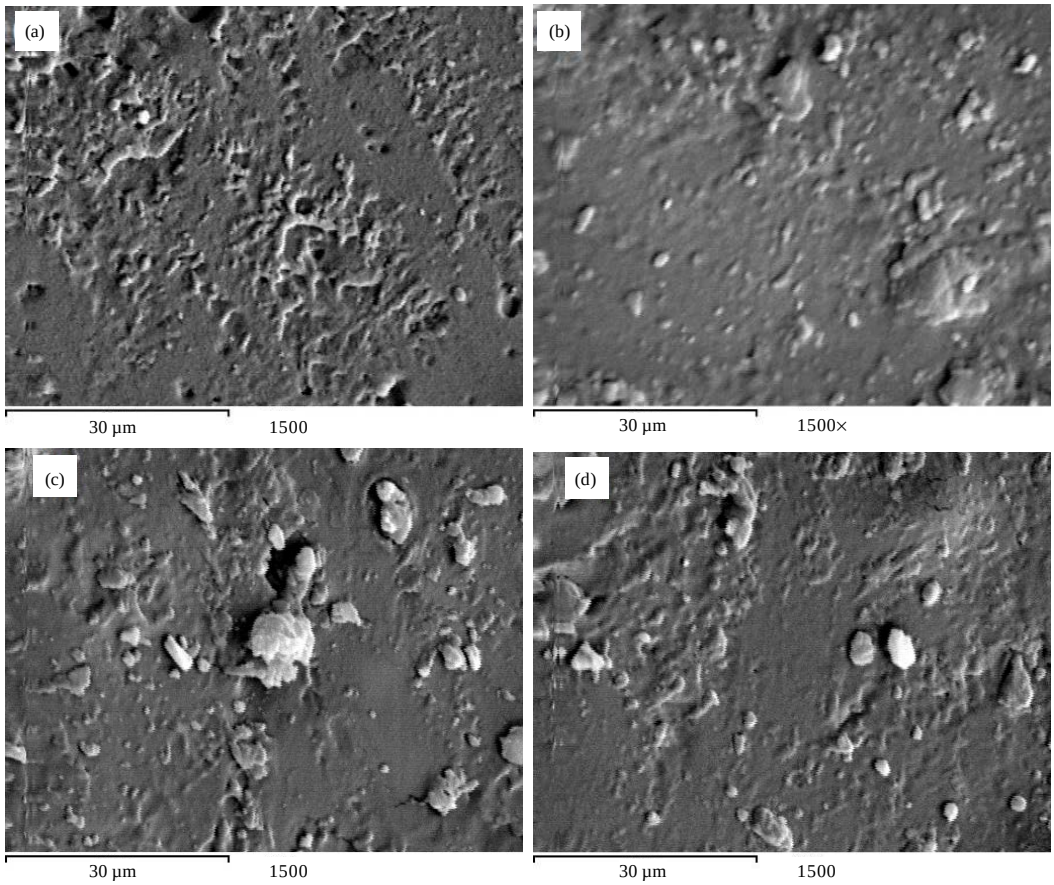

Fig. 1(a-d): Scanning Electron Microscope (SEM) micrographs of surface-section from WPI films to (a) Sample 1: Control, (b) Sample 3 (1% cinnamon), (c) Sample 5 (1% cumin) and (d) Sample 7 (1% thyme)

Micrographs of the cross section of the prepared films show aligned protein fibrils embedded in a continuous material, arrangements of oriented ribbon-like structures of whey Fig. 2. Sample 2 shows multiple pores and glossy, while poor formation would reduce structural strength of a film, pores would adversely affect the barrier properties. The glossy side of films is thought to consist of deposited protein from the casting solution during the drying process. The heterogeneity of cast films plasticized with fatty acids has been observed by several researchers. Garcia *et al.* (2009) showed that the surface morphologies of films was influenced by the preparation method, indicating that the incorporation of plant extracts was an influencing factor, while film forming characteristics were not affected significantly. Zinoviadou *et al.* (2010) indicated that whey protein isolate films can sustain their structural integrity at the high water activity (a_w) of beef surface and serve as effective carriers of antimicrobial agents. The development of films, with a uniform and compact layer, can be an important achievement towards the improvement of various film properties, such as their permeability to gases. The search for homogeneous structures thus becomes a target of the research involving edible films.

Effect of WPI films incorporated with EO of cinnamon, cumin and thyme on spoilage flora of meat samples: In the present study, fresh beef cuts were wrapped in WPI films

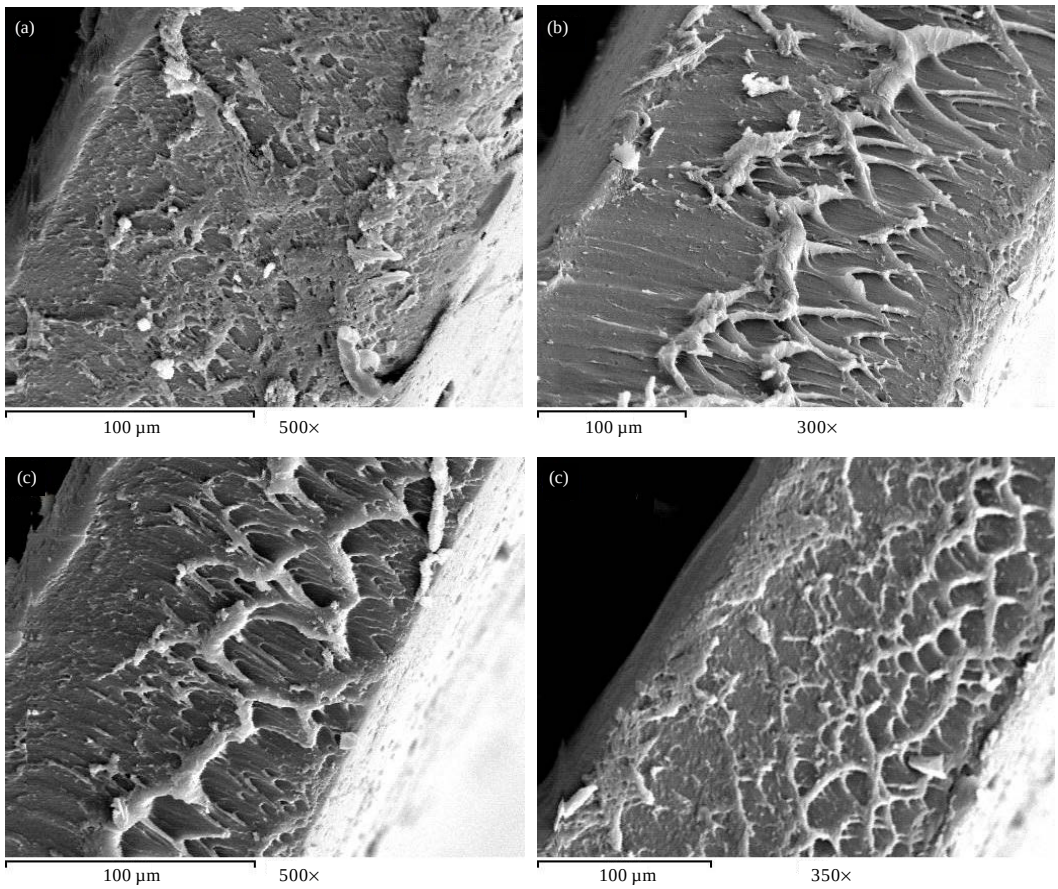

Fig. 2(a-d): Scanning Electron Microscope (SEM) micrographs of cross-section from WPI films to (a) Sample 1: Control, (b) Sample 3 (1% cinnamon), (c) Sample 5 (1% cumin) and (d) Sample 7 (1% thyme)

containing cinnamon (CI), cumin (CU) and thyme (TH) essential oils at four different concentrations ranging from 1-2.5%. The effectiveness of the films against the beef's spoilage flora (total viable count) during storage at 5°C was investigated. Tables 1-3 showed that the presence of whey protein film alone did not affect the growth of any of the bacteria studied, while films that contain high levels of CI, CU or TH essential oils can reduce the number of total viable bacterial count during the 12 days of the storage. The activity of the antibacterial films varied where, thyme showed the highest activity against spoilage flora of meat samples. By the day 6, the use of 2.5% of TH essential oils reduced the bacterial growth to 4.210 log CFU cm^{-2} compared to the control which was 8.876 log CFU cm^{-2}.

It has been previously suggested that the bacterial growth can use milk protein based films as a substrate for growth (Oussalah *et al.*, 2004). Under the high water content conditions of the meat surface, the contacting films are highly hydrated and probably do not exhibit high O_2 barrier properties. Consequently, no differences in the microflora profile were observed using the antimicrobial-free film. On the contrary, the total viable count population increased from 3-~11 log CFU cm^{-2} by the end of the study for samples wrapped in antimicrobial-free film. Same results were reported by Guo *et al.* (2014) where *L. monocytogenes* population in sliced turkey deli meat was reduced significantly when wrapped in antimicrobial containing edible film.

Table 1: Effect of the concentration of cinnamon oil in the WPI film on total viable count of spoilage flora at 5°C for different storage days (0-12)

			WPI with cinnamon oil (%)			
	Control	WPI only	1	1.5	2	2.5
Storage days	(log CFU cm^{-2})					
0	3.04±0.94	3.00±0.72	3.04±0.57	3.04±0.92	3.04±0.58	3.04±0.71
2	3.90±0.98	3.70±1.03	3.50±0.80	3.25±0.13	3.56±1.01	3.45±0.43
4	6.96±0.12	6.69±0.79	6.59±0.72	6.00±0.08	4.11±0.37	3.96±0.62
6	8.88±0.63	8.59±1.28	8.25±0.62	7.87±0.92	5.85±0.58	4.24±0.97
8	9.87±0.49	9.52±0.44	9.11±0.75	8.77±0.17	6.35±0.64	5.58±1.07
10	10.22±1.00	10.02±0.13	9.75±0.48	9.10±1.00	7.05±0.48	6.05±0.98
12	11.05±0.69	11.00±0.19	10.86±0.97	10.02±0.62	8.97±0.17	7.92±0.09

Data were the mean value±SD, standard deviation was at least two replicate experiments

Table 2: Effect of the concentration of cumin oil in the WPI film on total viable count of spoilage flora at 5°C for different storage days (0-12)

			WPI with cinnamon oil (%)			
	Control	WPI only	1	1.5	2	2.5
Storage days	(log CFU cm^{-2})					
0	3.04±0.11	3.00±0.20	3.04±0.18	3.04±0.71	3.04±0.37	3.04±0.18
2	3.90±0.09	3.70±0.13	3.91±0.25	3.57±0.62	3.36±0.21	3.32±0.37
4	6.96±0.07	6.69±0.21	5.27±0.44	4.89±0.48	4.30±0.33	4.15±0.58
6	8.88±0.09	8.59±0.28	6.86±0.37	5.96±0.25	5.05±0.25	5.01±0.34
8	9.87±0.12	9.52±0.09	8.53±0.97	7.55±0.57	6.33±0.46	5.86±0.64
10	10.22±0.13	10.02±0.08	9.10±0.16	8.39±0.34	7.28±0.53	6.13±0.26
12	11.05±0.08	11.00±1.01	10.06±0.29	9.42±0.53	7.87±0.28	6.87±0.44

Data were the mean value±SD, standard deviation was at least two replicate experiments

Table 3: Effect of the concentration of thyme oil in the WPI film on total viable count of spoilage flora at 5°C for different storage days (0-12)

			WPI with cinnamon oil (%)			
	Control	WPI only	1	1.5	2	2.5
Storage days	(log CFU cm^{-2})					
0	3.04±0.10	3.00±0.12	3.04±0.11	3.04±0.16	3.04±0.25	3.04±0.10
2	3.90±0.09	3.70±0.06	3.79±0.16	3.69±0.07	3.53±0.11	3.49±0.32
4	6.96±0.18	6.69±0.14	5.89±0.01	4.86±0.12	4.56±0.16	3.83±0.06
6	8.88±0.08	8.59±0.09	7.02±0.09	5.65±0.06	5.21±0.06	4.21±0.14
8	9.87±0.14	9.52±0.11	8.94±0.18	6.43±0.14	6.19±0.16	4.86±0.29
10	10.22±0.16	10.02±0.25	9.09±0.08	7.75±0.09	7.30±0.19	5.09±0.36
12	11.05±0.07	11.00±0.11	10.15±0.14	9.16±0.11	7.84±0.25	5.55±0.22

Data were the mean value±S.D, standard deviation was at least two replicate experiments

The use of films containing the highest level of TH oil (2.5% w/w in the film forming solution) resulted in a highly reduction of the total viable count TVC population compared to the use of cinnamon and cumin oils during the entire storage period Fig. 3. The TVC population of the samples wrapped in the films with the high thyme oil content at day 6 was 4.210 log CFU cm^{-2},

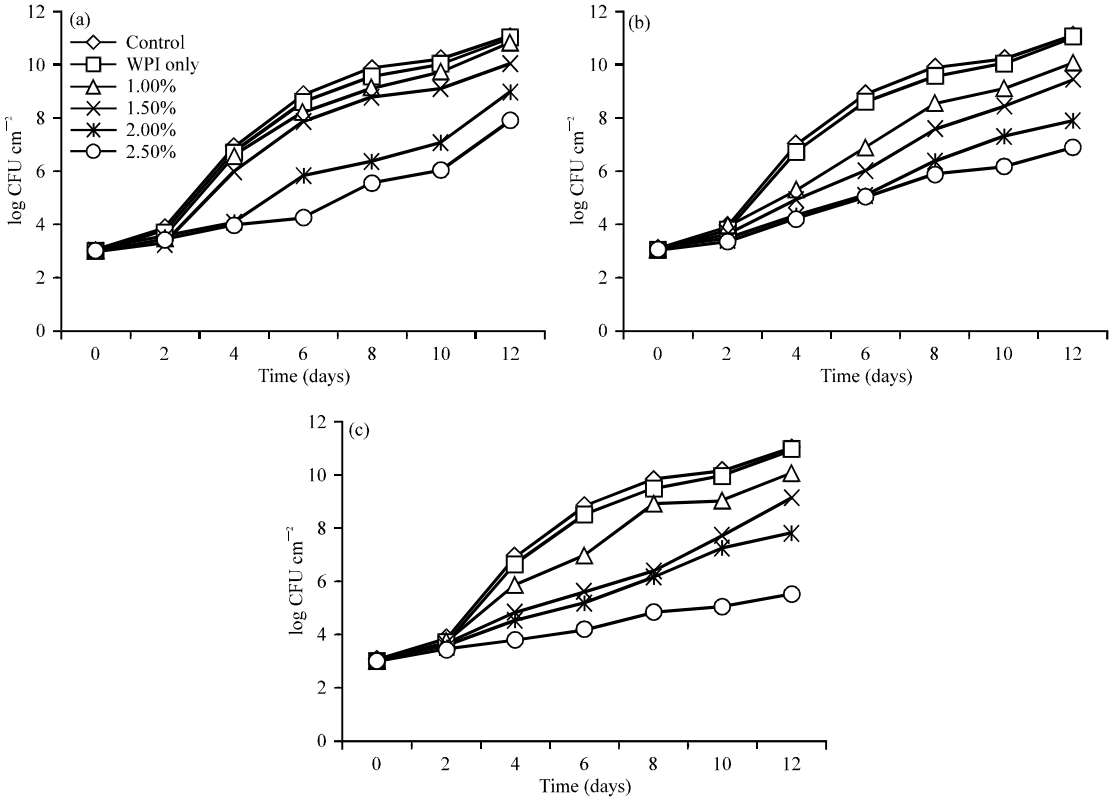

Fig. 3(a-c): Effect of whey films incorporated with essential oils of (a) Cinnamon, (b) Cumin and (c) Thyme on total viable count of fresh meat during storage for 12 days at 5°C

while for the control it was 8.876 log CFU cm^{-2}, while in day 12, it was 5.547 log CFU cm^{-2} and the control was 11.052 log CFU cm^{-2}. In sample wrapped in the films with the high cinnamon oil content, the TVC population at day 6 was 4.241 log CFU cm^{-2}, while for the control it was 8.876 log CFU cm^{-2}, while in day 12 was 7.924 log CFU cm^{-2} and the control was 11.052 log CFU cm^{-2}. Also, The TVC population of the samples wrapped in the films with the high cumin oil content at day 6 was 5.005 log CFU cm^{-2}, while for the control it was 8.876 log CFU cm^{-2}, while in day 12 was 6.867 log CFU cm^{-2} and the control was 11.052 log CFU cm^{-2}.

Since, microbial loads higher than 10^7 CFU cm^{-2} are usually associated with off-odors (Ercolini *et al.*, 2006), it may be suggested that the use of WPEF containing 2.5% w/w of cinnamon, cumin and thyme essential oil could double the shelf life of fresh beef stored under refrigerated conditions. However, the hydrophobic constituents of the essential oils are capable of gaining access to the periplasm of the bacteria through the proteins of the outer membrane as demonstrated by Confocal Scanning Laser Microscopy (Lambert *et al.*, 2001). The increase in membrane permeability provokes a release of the cell constituents, a decrease in ATP production in the cells and a decrease of the intracellular pH (Oussalah *et al.*, 2006).

As previously demonstrated for food borne pathogens, antimicrobial compounds might be more effective in reducing the level of bacteria when incorporated in a biopolymer film applied on the product surface than when the antimicrobial is directly applied to the surface via., spraying or dipping (Kristo *et al.*, 2008). Alginate and milk protein films containing 1.0% oregano oil were effective against food borne pathogens inoculated on beef (Oussalah *et al.*, 2004, 2006;

Terjung *et al*., 2014). Alginate-apple puree films containing low concentration of oregano oil (0.1% v/w) have been tested against *E. coli* on agar and a large inhibitory zone was found demonstrating high antimicrobial activity (Rojas-Grau *et al*., 2007).

In general, it can be said that a higher concentration of essential oil required achieving the same antibacterial effect in food as in vitro; in this context, it has been suggested that the greatest availability of nutrients in food, compared to laboratory media may enable bacteria to repair damaged cells (Gutierrez *et al*., 2008). Food composition can also affect the migration mechanism of the antimicrobial agent into the food structure. For example, the active compounds of the essential oils are highly hydrophobic substances and thus their diffusion into the product could be affected by the presence of fat. Studies on the use of antimicrobial films on ham (15% fat) and bologna (25% fat) found that the availability of essential oils in alginate based films was lower in the case of bologna, pointing to the significance of the affinity between the antimicrobial agents and the product matrix (Oussalah *et al*., 2006).

CONCLUSION

The activity of antibacterial whey protein edible films treated with various concentrations of CN, CU, TH essential oils ranging from 1-2.5% on the growth of total viable count on meat sample during the storage period (12 days) under refrigerated conditions were evaluated. The results revealed that the use of WPEF containing 2.5% w/w of cinnamon, cumin and thyme essential oil could double the shelf life of fresh beef stored under refrigerated conditions. The film microstructures were dependent on the type and proportions of EO added to the films and impart changes on their internal structure.

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