

Efficacy of activated alginate-based nanocomposite films to control *Listeria monocytogenes* and spoilage flora in rainbow trout slice

Mehdi Alboofetileh¹ · Masoud Rezaei¹ · Hedayat Hosseini² · Mehdi Abdollahi¹

Revised: 22 August 2015 / Accepted: 2 September 2015 / Published online: 12 September 2015
© Association of Food Scientists & Technologists (India) 2015

Abstract Essential oils of clove, coriander, caraway, marjoram, cinnamon, and cumin were tested for their antilisterial activity by application of agar diffusion assay (experiment 1). Marjoram essential oil (MEO) showed the highest inhibitory effect, followed by clove and cinnamon. Subsequently, these essential oils were incorporated to alginate/clay nanocomposite films and antilisterial effectiveness of the films was studied in a model solid food system during 12 days at 10 °C (experiment 2). The results revealed that the films with MEO were more effective against *Listeria monocytogenes* in the model step. Finally, alginate-clay film incorporating 1 % MEO was applied to inoculated trout slices during refrigerated storage (4 °C) for 15 days (experiment 3). The control and the wrapped fish samples were analyzed periodically for microbiological (*L. monocytogenes*, total viable count, psychrotrophic count) and chemical (TVB-N) properties. The results

demonstrated that alginate-clay films enriched with 1 % MEO significantly delayed the growth of *L. monocytogenes* during the 15-day storage with final counts reaching 6.23 log CFU/g while the counts in control samples were significantly higher reaching 7.38 log CFU/g ($p < 0.05$). Furthermore, active films efficiently reduced total viable count and psychrotrophic count as well as TVB-N in the fish slice during refrigerated storage.

Keywords Antibacterial activity · *Listeria monocytogenes* · Fish fillet · Food packaging

Introduction

The survival and growth of microorganisms in food are one of important concerns for the current food industry. Microbial contamination of food can lead to spoilage and reduces the shelf-life of food products or cause infection and serious foodborne illnesses (Lv et al. 2011). In this regard, it has been estimated that about one-third of the world's food production is lost annually on account of microbial spoilage. Furthermore, food pathogens such bacteria, viruses and toxins produced by microorganisms are all possible contaminants of food. Among pathogenic bacteria, *L. monocytogenes* is a particular concern in the food industry.

Among various strategies used to control and eliminate the undesirable food microorganisms, chemical preservatives have been frequently utilized in food industry compared to other technologies. However, since the safety of synthetic additives has been questioned in recent years; consumers increasingly demand the use of natural products as alternative preservatives in foods (Solomakos et al. 2008). As a response to these conflicting demands, use of natural preservatives to inhibit growth of microorganism, especially serious pathogens such as *L. monocytogenes* is of great interest to the food industry.

Highlight • The antimicrobial activity of six EOs on *L. monocytogenes* was investigated.

- MMT nanoparticles and the most effective EOs were incorporated in alginate films.
- XRD, TEM and SEM images revealed proper dispersion of MMT into alginate matrix.
- Alginate/clay/MEO films showed a strong antilisterial activity in model step.
- Alginate/clay/MEO films showed a low antibacterial activity in fish slice.

✉ Masoud Rezaei
rezai_ma@modares.ac.ir

¹ Department of Seafood Processing, Faculty of Marine Sciences, Tarbiat Modares University, P.O.Box 46414-356, Noor, Iran

² Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

In this regards, functional food packaging systems using various biopolymers, including edible and biodegradable films have drawn a great attention as means of naturally controlling the shelf life and safety of food products (Mayachiew et al. 2010; Marcos et al. 2007). However, the commercial application of the antimicrobial films in the food industry has been limited due to (i) high cost of pure biopolymer materials and antimicrobials (Appendini and Hotchkiss 1997) and (ii) poor mechanical and gas barrier properties and weak water resistance of the films for commercial uses (Rhim and NG 2007).

The application of nanotechnology and formation of nanocomposites to these biopolymer films can open new possibilities for improving not only the properties but also the cost-price-efficiency (Sorrentino et al. 2007; Azeredo 2009). Nanocomposite is material consisting of polymer matrix reinforced with nanoscale fillers having at least one dimension in the nanometer range (Rhim and NG 2007). Due to nanometer size dispersion of nanoclay in the polymer matrix, biopolymer-clay nanocomposites exhibit markedly improved packaging properties. Furthermore, in order to improve the functionality of the packaging materials natural antimicrobial agents like essential oils can be incorporated into nanocomposites films. Consequently, functional bionanocomposite films have a huge potential for application in the active food packaging industry. However, functional bionanocomposite films are in its infancy and there are few reports (Abdollahi et al. 2012; Alboofetileh et al. 2014b; Tunc and Duman 2011) on the preparation of functional bionanocomposite films. Therefore, a great deal of research on the preparation and application of bionanocomposite packaging with functional properties is expected (Rhim and NG 2007).

Thus, the objectives of this study were to (i) screen the antilisterial properties of six plant EOs in order to enrich alginate/clay nanocomposite films with the most effective EOs. (ii) screen the antilisterial efficacy of the active alginate/clay nanocomposite films in an model solid food system during 12 days at 10 °C. (iii) Finally, choose the most effective antimicrobial film and assay its antimicrobial efficacy with respect to *L. monocytogenes* and spoilage bacteria on rainbow trout fillet during refrigerated storage (4 °C) for 15 days.

Materials and methods

Materials

Sodium alginate (medium viscosity) was obtained from Sigma-Aldrich Chemical Co., USA and Na⁺-montmorillonite (MMT) was purchased from Southern Clay Products, USA. Clove (*Syzygium aromaticum*), cumin (*Cuminum cyminum*), caraway (*Bunium persicum*), marjoram (*Origanum majorana*),

cinnamon (*Cinnamomum zeylanicum*), and coriander (*Coriandrum sativum*) essential oils were acquired from Magnolia Co. (Tehran, Iran) and stored in dark container at 4 °C until used.

Bacterial strain and maintenance

Stock culture of *Listeria monocytogenes* (PTCC 1298) were obtained from Persian Type Culture Collection (Tehran, Iran). The strain was stored in BHI broth supplemented with 30 % glycerol at –20 °C until used. Subculturing was carried out every month to maintain bacterial viability. The thawed culture (0.1 mL) was transferred to 10 mL of BHI broth and grown in a shaker incubator at 37 °C for 24 h. A second transfer of 0.1 mL of culture into 10 mL of BHI broth was grown in a shaker incubator at 37 °C for 24 h to the end of the exponential phase of growth. Subsequently, this appropriately diluted culture was used for the inoculation of agar plates and fish fillets in order to obtain a target inoculum of 10², 10³ and 10⁶ CFU/cm².

Antimicrobial activity of essential oils

The antibacterial properties of the essential oils were studied using the agar diffusion method as reported by Gómez-Estaca et al. (2010). Thirty microlitre of the essential oils were poured into an agar well (with 5 mm diameter), previously created with a sterile core bore on the agar, after their plates had been seeded with 0.1 ml of inoculum by swab containing approximately 10⁶ CFU/ml of the indicated bacteria. All the strains were cultured in TSA. The plates were incubated at appropriate temperature for 48 h in the suitable incubation chamber. After incubation, the microbial growth was observed and the degree of inhibition was expressed as follows: totally inhibited, +++; partially inhibited, ++; slightly inhibited, +; no inhibition, –.

Preparation of nanocomposite film

Alginate film solution was prepared by dissolving 10 g of sodium alginate powder in 1 L distilled water to obtain a 1 % w/v alginate solution using a magnetic stirring plate at 70 °C and 1200 rpm for 30 min. Nanocomposite films were prepared according to the method reported by Abdollahi et al. (2012). A specific amount of MMT (3 % w/w on solid sodium alginate, selected from our previous study) was dispersed in 50 mL of distilled water and vigorously stirred for 24 h at room temperature. Afterward, the alginate solution was slowly added into the pretreated clay solution and the mixture was stirred for 4 h. Then, each of the tree effective essential oils found through the first step of the study, previously mixed with Tween 80 (0.25 g/g of essential oil) to help create a uniform and stable distribution in the alginate matrix, were

incorporated into the film-forming solution at several concentrations (0.5, 1.0, and 1.5 % w/v on the basis of film solution). The final solution was homogenized with Ultra-Turrax (IKA T25-Digital Ultra-Turrax, Staufen, Germany) at 9000 rpm for 2 min. The resulting film solution was degassed under vacuum for 30 min in order to remove all bubbles. Finally, the film-forming solution was cast onto Petri dishes (with 9 cm in diameter) and dried for 72 h at ambient conditions ($T=25\text{ }^{\circ}\text{C}$ and $\text{RH}=55\text{ }\pm 2\text{ }\%$) to prepare nanocomposite films. The dried films were removed from the Petri dishes and preconditioned in desiccators containing saturated magnesium nitrate solution at $25\text{ }^{\circ}\text{C}$ and 52.89 % relative humidity until used.

Sample characterization

X-ray diffraction (XRD) analysis

The structure of nanoparticle and its nanocomposites was studied by XRD measurement. XRD patterns were taken with a Philips X'Pert MPD Diffractometer (Eindhoven, Netherlands), with Co Ka radiation at a wavelength of 1.544 nm, at 40 kV and 30 mA. Alginate/clay films were scanned over the range of diffraction angle $2\theta=1\text{--}12^{\circ}$, with a scan speed of $1^{\circ}/\text{min}$ at room temperature.

Transmission electron microscopy (TEM)

TEM was used to investigate the nanostructure of SA/MMT composite films. The film samples were studied with Transmission Electron Microscope (TEM) Philips CM300-FEG (Philips, Eindhoven, Netherlands), operating at an acceleration voltage of 100 kV. The specimens were sliced in liquid nitrogen with the Reichert-Jung Ultracut E microtome (Leica Microsystems, Wetzlar, Germany).

Scanning electron microscopy (SEM)

Surface morphologies of the alginate and alginate/ clay nanocomposite films were investigated by scanning electron microscopy using a Philips XL 30 scanning electron microscope (Philips, Eindhoven, Netherlands) under high vacuum condition and at an accelerating voltage of 20.0 kV. Prior to examination, the sample films were mounted on the specimen holder with aluminium tape and then sputtered with gold in a BAL-TEC SCD 005 sputter coater (BAL-TEC AG, Balzers, Liechtenstein) to enhance surface conductivity.

Antilisterial effectiveness of films in model step

In order to test the antilisterial effectiveness of the active nanocomposite-based films in model step, the method reported by Sánchez-González et al. (2011) was used. Briefly,

Tryptone Soy Agar medium with 3 % NaCl (Sigma-Aldrich, Germany) was used as a model solid food system (TSA-NaCl). Aliquots (20 g) of TSA-NaCl were poured into Petri dishes (8.5 cm diameter). After the culture medium solidified, aliquots (0.1 ml) of the properly diluted culture were inoculated on the surface of TSA-NaCl. Then, different test films (containing or without antimicrobial substances) of the same diameter as the Petri dishes were placed on the inoculated surface. Inoculated uncoated TSA-NaCl were used as control. Plates were then covered with plastic bags to avoid dehydration and stored at $10\text{ }^{\circ}\text{C}$ for 12 days. Microbial counts on TSA-NaCl plates were examined immediately following the inoculation and periodically during the storage period. To this end, the agar was removed aseptically from the Petri dishes and placed in a sterile glass container. 100 ml of 0.9 % NaCl solution was added to each container and homogenized for 3 min which resulted in a very homogeneous system. Serial dilutions were made and then poured onto TSA. Plates were incubated at $37\text{ }^{\circ}\text{C}$ for 48 h before colonies were counted. All tests were done in triplicate.

Film effectiveness against *L. monocytogenes* and spoilage flora on rainbow trout fillet during chilled storage

Fish sample preparation

Live rainbow trout (*Oncorhynchus mykiss*) was obtained from an aquaculture farm and were transferred to the laboratory within 60 min after harvesting, packed in sealed foamed polystyrene boxes containing flaked ice. They were then washed with tap water, filleted, skinned and cut into $3.0\times 3.0\text{ cm}$ weighing $25\pm 1\text{ g}$. The samples were placed onto a piece of sterile aluminum foil and *L. monocytogenes* suspensions ($125\text{ }\mu\text{l}$) were randomly inoculated at five different spots selected on the fish slices and the samples were left undisturbed for 10 min for absorption on each side. Fish slices were then flipped and inoculated on the other side. The inoculation level was approximately $3\text{ log}_{10}\text{ CFU/g}$. A control, inoculated sample without wrapping, was also prepared. The procedure was carried out in a laminar flow hood to avoid contamination. After inoculation, the fish slices were covered with the different films and packaged in nylon/polyethylene pouches and sealed. The samples were stored at $4\text{ }^{\circ}\text{C}$ for 15 days and the populations of *L. monocytogenes* and spoilage bacteria in the samples were determined immediately following inoculation and periodically at 3-day intervals during the storage period. All tests were done in triplicate.

Microbiological analysis of fish samples

Fish slices were examined for the levels of the inoculum, as well as total viable count, psychrotrophic bacteria immediately after inoculation and again following 3, 6, 9, 12, 15 day of

refrigerated storage. Inoculated fish slices were added to appropriate volume of 0.9 % sterile saline solution and homogenized for 2 min. Ten-fold serial dilutions were made using 0.9 % sterile saline solution. Counts of *L. monocytogenes* were determined by an overlay method to enhance recovery of injured cells described by Neetoo et al. (2010). Briefly, the serial dilutions were spread-plated on solidified TSA plus 0.6 % yeast extract (TSAYE) plates and the plates were incubated at 37 °C for 3 h. Then, approximately 7 ml of Modified Oxford Medium (Quelab co.) at 45 °C was overlaid on the TSAYE plates. The plates were incubated at 37 °C and small black colonies with black haloes on the plates were counted after 48 h. Total viable count (TVC) was determined by the pour plate method, using plate count agar (Merck, Germany) with incubation at 37 °C for 2 days. Plate count agar was also used for psychrotrophic count and plates were incubated at 7 °C for 10 days. All counts were expressed as log₁₀ cfu/g and performed in duplicate (Ojagh et al. 2010).

Determination of total volatile basic nitrogen (TVB-N)

Total volatile base nitrogen (TVB-N) was evaluated according to the method as reported by Pezeshk et al. (2011). The microdiffusion procedure was performed by distillation after the addition of MgO (2 g) to minced fish sample (10 g) and was mixed with distilled water. The distillate was collected in a flask containing a 2 % aqueous solution of boric acid (25 mL) and a mixed indicator produced from dissolution of 0.1 g of methylene blue and 0.1 g methyl red to 100 mL of ethanol. Finally, the boric acid solution was titrated with a 0.1 N sulfuric acid solution. The TVB-N value (mg N per 100 g fish muscle) was determined according to the consumption of sulfuric acid.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's one-way multiple comparisons were used to determine differences in the populations of *L. monocytogenes*, spoilage bacteria on trout filleted samples. Significant differences were considered at the 95 % confidence level ($P < 0.05$).

Results and discussion

XRD analysis of alginate nanocomposites

X-ray diffraction of the MMT and alginate-based nanocomposite films is shown in Fig. 1. Results showed that the pattern of pure SA has a characteristic crystallinity peak at $2\theta = 5.56^\circ$. The data reported in Fig. 1 show lower intensity for this characteristic crystallinity peak for composite films, which means the crystallinity of alginate is reduced by the addition of the

MMT. The results are in agreement with previously reported results of Lavorgna et al. (2010) and Abdollahi et al. (2012). As in the XRD patterns of the present study, MMT had a characteristic peak at $2\theta = 8.56^\circ$ ($d_{001} = 10.43 \text{ \AA}$). With the addition of MMT to the polymer matrix, the reflection peak of MMT disappeared, indicating formation of an exfoliated structure, which was disordered and not detectable by XRD. Furthermore, Fig. 1 shows that the structure of nanocomposites is not affected by the presence of MO. These results are in good agreement with those obtained by Abdollahi et al. (2012) that incorporated the rosemary essential oil in chitosan-MMT nanocomposite films.

Transmission electron microscopy (TEM)

The TEM image of SA-based nanocomposite film containing 3 % of MMT is shown in Fig. 2. The dark lines in the TEM image correspond to MMT platelets and the gap between two adjacent lines is the d-spacing. The TEM image shows the good dispersion of MMT in the SA matrix. The TEM results correspond well with the XRD patterns.

Scanning electron microscopy (SEM)

A microstructural study of the films gives relevant information about the arrangement of the components, allowing for a better discussion of results. Microstructure of alginate and alginate/clay films was tested with scanning electron microscopy as shown in Fig. 3. The surface of the control film was smooth and homogeneous without pores or cracks (Fig. 3a). After adding 3 % MMT, SEM images indicated high degree of dispersion of clay in the polymeric matrix. After the addition

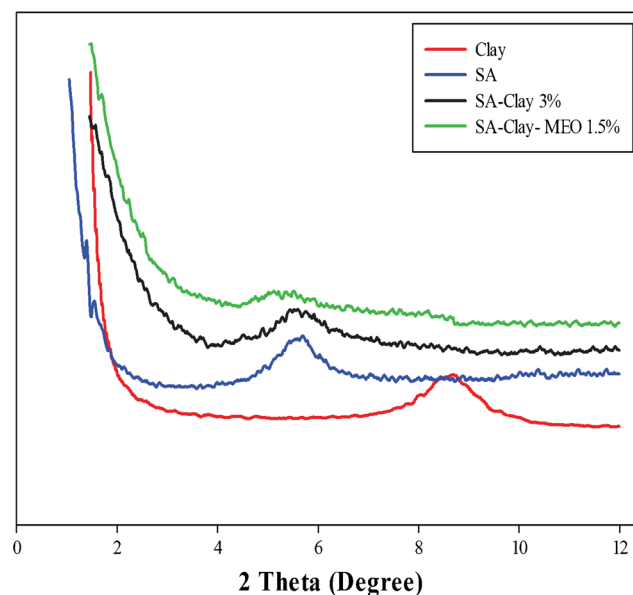


Fig. 1 XRD patterns of MMT powder and alginate-based nanocomposite films

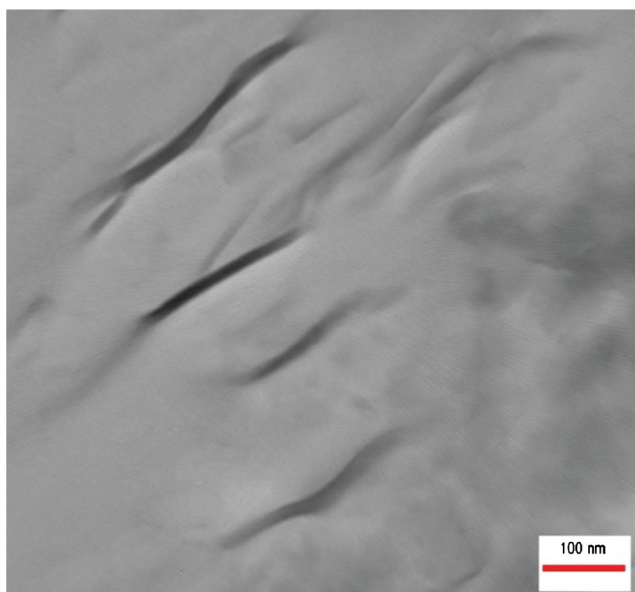


Fig. 2 TEM micrograph of the SA-MMT (3 wt%) nanocomposite film

of MEO, films differed in the surface contour, which had a cracked surface. These results might be due to the microphase separation of sodium alginate and the essential oil's component. Benavides et al. (2011) also showed that the incorporation of oregano essential oil caused a loose texture with sponge-like structure, with pores distributed throughout the alginate film.

Antibacterial activity of the essential oils

Attending to the inhibition zone, the EOs with the highest inhibitory effect was found to be marjoram essential oil and cumin essential oil was the least effective against the *L. monocytogenes*. The intensity of antimicrobial efficacy was in the following order: marjoram > clove > cinnamon > coriander > caraway > cumin (unpublished data). Antimicrobial activity of EOs depends on various factors such as the types of tested microorganism, the type of spice or herb, and the content of extracts and essential oils (Castellano et al. 2008). Generally, EOs are less effective against Gram-negative bacteria compared Gram-positive bacteria. Another factors that affects the antimicrobial activity of EOs is chemical composition that strongly depends on the variety of plant, the part of the plant considered (e.g., seed vs. leaves), origin, time of harvest, the harvesting season and processing, as well as storage conditions (Tajkarimi et al. 2010). EOs can contain about 20–80 constituents at significantly different concentrations. The major group is composed of terpenes and terpenoids, and the other group contains aromatic and aliphatic constituents. The components of EOs are important as their qualitative and quantitative composition determines the characteristics of the oils, which in turn could have an effect on its antimicrobial potential (Burt 2004). In this regard, the major

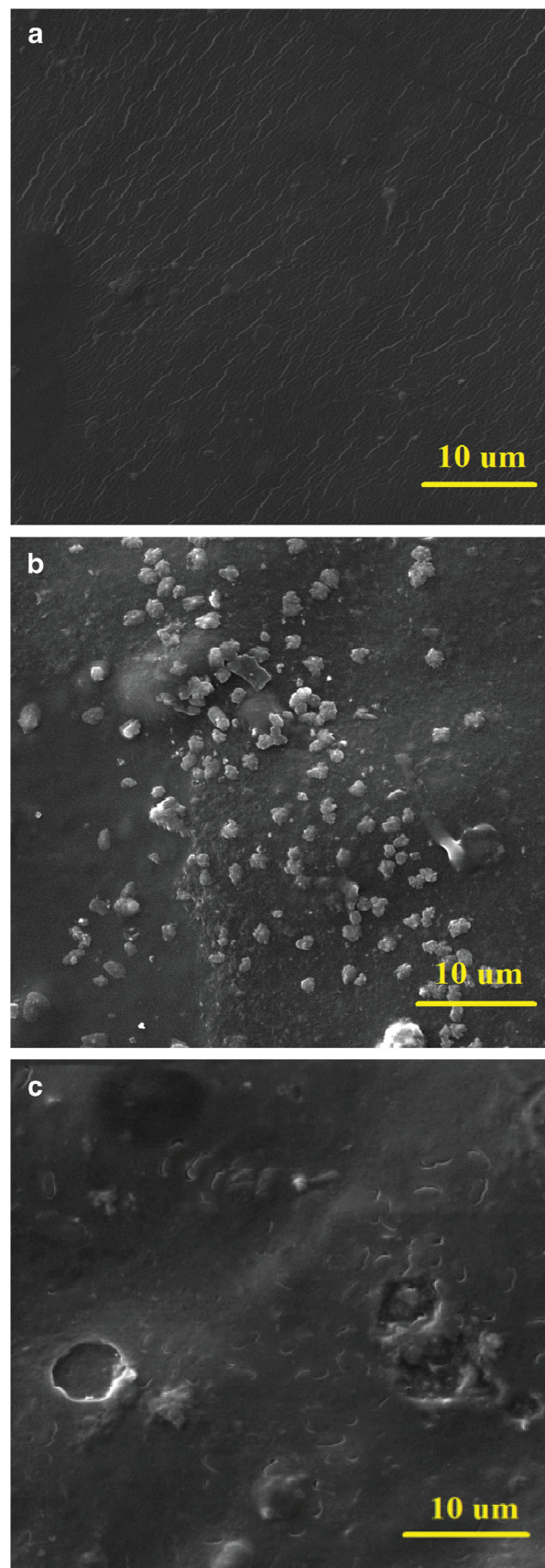


Fig. 3 SEM micrographs of the surface of alginate-based films: **a** SA **b** SA-clay, **c** SA-Clay-MO (1.5 %)

components in marjoram (MEO), cinnamon (CIEO), and clove (CEO) are Terpinen-4-ol, Cinnamaldehyde, and Eugenol, respectively. Previous studies have addressed the strong and consistent inhibitory effect of MEO, CEO, CIEO and their individual terpene and terpenoid components against various pathogens bacteria such *L. monocytogenes*, *E. coli* and *S. aureus* and spoilage microorganisms (Burt 2004; Gómez-Estaca et al. 2010; Lis-Balchin and Deans 2003). Based on these results, MEO, CEO and CIEO were selected for experiment 2.

Inhibition the growth of *L. monocytogenes* in model step

The growth curves of *L. monocytogenes* on control TSA-NaCl plates and TSA-NaCl plates covered with EOs enriched films are presented in Fig. 4. The initial *L. monocytogenes* population on the control TSA-NaCl plates was 2 log CFU/cm²; the microbial count increased substantially during storage, reaching 7.86 log CFU/cm² after 12 days. Our results for the growth of *L. monocytogenes* on TSA-NaCl plates are similar those reported (approximately eight log) for this bacterium by Kristo et al. (2008). No significant differences ($P > 0.05$) were observed between growths of *L. monocytogenes* on control TSA-NaCl plates and plates coated with the SA/clay film without any EOs during the storage period. As can be seen the MMT clay does not have any antimicrobial property and the purpose of addition it to alginate-based films is the improving the physical and mechanical properties of films. The data about the effect of MMT on physical and mechanical properties of films were shown in our previous study (Alboofetileh et al. 2014a). Previous studies have reported the same behavior for both of pure alginate films (Benavides et al. 2011) and MMT clay (Abdollahi et al. 2012). Results also indicate that the alginate-based nanocomposite films containing EOs were effective in reducing the growth of *L. monocytogenes*. In this regards, the films containing MEO showed the lowest *L. monocytogenes* population on inoculated TSA-NaCl plates ($P < 0.05$) in comparison with other films containing CEO and CIEO at the same concentration. A total inhibition of the pathogen growth observed when the highest concentration of MEO (1.5 %) incorporated into the film matrix. However, with 1% of the MEO, nanocomposite films displayed less antimicrobial activity and total inhibition of pathogen growth occurred during the first 7 days of the storage period. Subsequently, the *L. monocytogenes* population increased up to 3.6 log UFC/cm² after 12 days. The *L. monocytogenes* count on TSA-NaCl plates coated with the film containing 1 % MEO decreased 4.3 log after 12-days compared to the control plates (Fig 4a). SA/clay films containing CEO presented less antilisterial activity than SA/clay films containing MEO (Fig. 4b). In the presence of 1.5 % CEO, a total inhibition of the pathogen growth occurred during the first 7 days of the storage period and this film reduced the population of

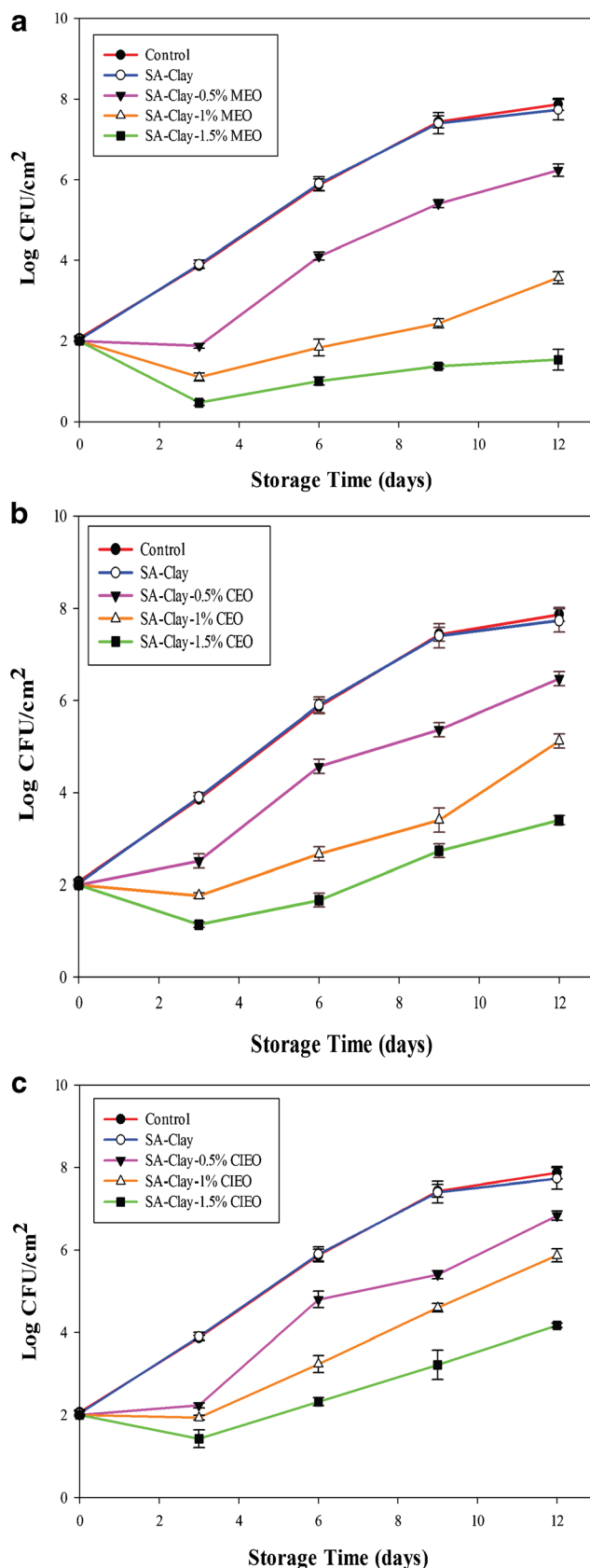


Fig. 4 Effect of different antimicrobial alginate-clay films on the growth of *L. monocytogenes* on TSA-NaCl medium stored at 10 °C for 12 days

L. monocytogenes more than 4.2 log UFC/cm² compared with the control plates. In the presence of 1 % CEO, a complete inhibition of microbial growth was observed during the first 3 days of the study period. SA/clay films containing CIEO showed a less marked antilisterial activity than other films containing MEO and CEO (Fig. 4c). The highest levels of CEI oil (1 and 1.5 %) led to a complete inhibition of *L. monocytogenes* growth just for the first 3 days; then the *L. monocytogenes* population increased up to 5.86 and 4.16 log UFC/cm², respectively, after 12 days. As expected, in the presence of 0.5 % of all tree essential oils, the antimicrobial effect was less marked and film containing this concentration was not sufficient to control the microbial growth for the entire storage period. Our results are in agreement with the results reported by Sánchez-González et al. (2010) which used chitosan film containing tea tree essential oil to control *L. monocytogenes* in TSA-NaCl plates. Generally, when antimicrobial agents are incorporated into food packaging films, these materials diffuse from film matrix and finally result in an inhibitory effect on bacteria (Hosseini et al. 2009). In this regards, when EOs were incorporated into the SA/clay film, the antimicrobial activity of the EOs was maintained, but the inhibitory effect of the EOs in the film matrix decreased in comparison with the pure essential oil. The possible causes that would explain this result could be the lower amount of EOs in the film matrix in comparison with the well test for pure EOs as well as partial loss of volatile compounds during film preparation (Abdollahi et al 2012; Sánchez-González et al. 2010). Beside the nature of EOs and the type of bacteria that were explained above, other factors such as film structure, method and manufacturing conditions affect the antimicrobial action of films. Like the disk test result, nanocomposite film containing MEO was the most effective in controlling the growth of *L. monocytogenes* on TSA-NaCl plates. This phenomenon can be explained by the high content of phenolic compounds in MEO as well as good interaction between the constituents of the marjoram with the polymer matrix (Burt 2004; Papadokostaki et al. 1998). In the present study, the concentration of the EOs had a significant effect in the inhibition of pathogens growth during the first days of the storage period, but at the end of the period, the nature of the incorporated EOs became more significant factor. Furthermore, it must be highlighted that, as storage time increased, due to the evaporation of volatile compounds responsible for the antimicrobial activity and/or by the migration of EOs components into the agar medium (Sánchez-González et al. 2011) the film effectiveness decreased. In this regards, after the films were placed on the inoculated surface of TSA-NaCl, the SA/clay hydrophilic matrices absorbed water, which induced changes in the film structure. This condition facilitates the evaporation and liberalization of EOs components from the polymer matrix. Consequently, the effectiveness of the films tends to decrease during the storage period.

Inhibition the growth of *L. monocytogenes* on trout slice

The alginate-clay containing MEO essential oil film appeared to be more effective than the other films investigated in the model step. Thus, a moderate-term refrigerated storage study was conducted to evaluate the antimicrobial efficacy of this antimicrobial film in rainbow trout slices to compare the natural condition against the model situation. The effects of alginate-clay films containing 1 % MEO compared to alginate-clay film on *L. monocytogenes* inoculated rainbow trout slices are presented in Fig. 5. This concentration was chosen based on the physical, mechanical and microbial properties of prepared films that the physical, mechanical was not shown. Due to their psychrotrophic nature, populations of *L. monocytogenes* in all samples significantly increased after the 15 days at 4 °C. The population of *L. monocytogenes* on control samples increased during storage reaching a final population density of 7.38 log CFU/g after 15 days of storage. Masniyom et al. (2006) reported that *L. monocytogenes* grew by approximately 6 log CFU/g in modified atmosphere packaged and refrigerated seabass slices after 21 days of storage. The same behavior was also observed in samples treated with alginate-clay films without MEO essential oil. This is an indication that the film components did not inhibit *L. monocytogenes*. This is in agreement with the study by Concha-Meyer et al. (2011) who reported that an alginate film alone cannot inhibit the growth of *L. monocytogenes* on smoked salmon. The use of films containing MEO resulted in a significant reduction of the *L. monocytogenes* population during the entire storage period. The *L. monocytogenes* population of the samples wrapped in the SA-Clay-MEO films at the day 15 was 6.23 log CFU/g. The significant reduction in

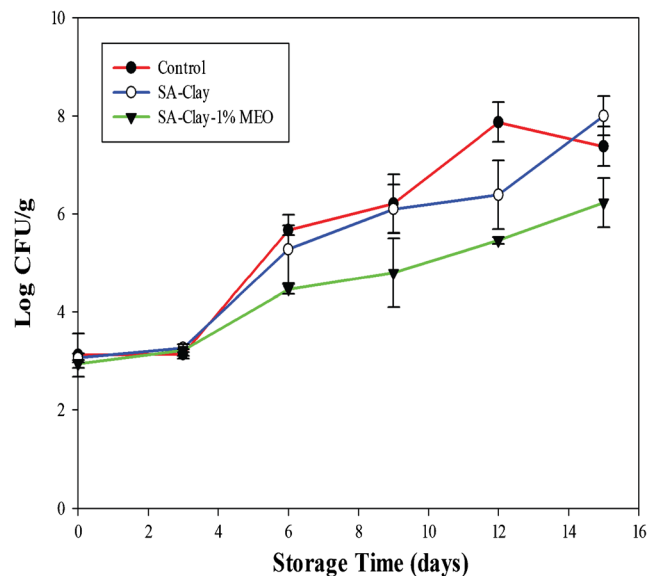


Fig. 5 Effect of antimicrobial alginate-clay films on the growth of *L. monocytogenes* on rainbow trout slices stored at 4 °C for 15 days

L. monocytogenes observed in this treatment can be attributed to the antibacterial activity of MEO incorporated into the alginate-clay films. By comparing Figs. 4a and 5, it can be seen that SA-Clay + 1 % MEO films showed lower antilisterial activity in trout slices compared to model steps. In this regards, Burt (2004) noted that the high levels of fat and/or protein in foodstuffs protect the bacteria from the action of the EOs in several way. For example, if the EOs dissolves in the lipid phase of the food there will be relatively less available to act on bacteria present in the aqueous phase. Furthermore, the greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster (Gill et al. 2002).

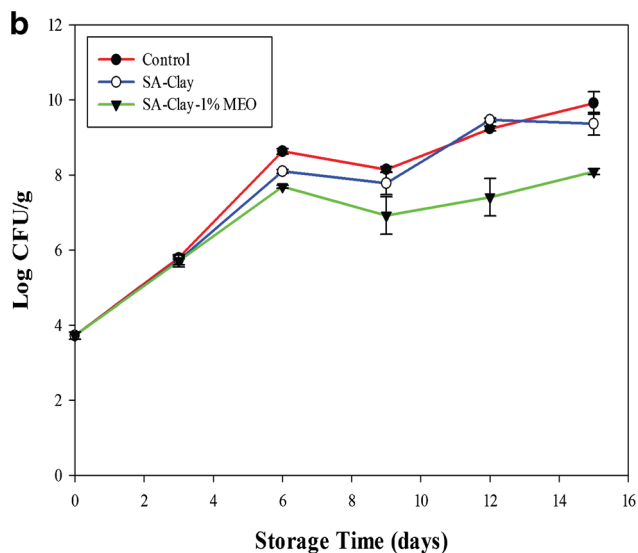
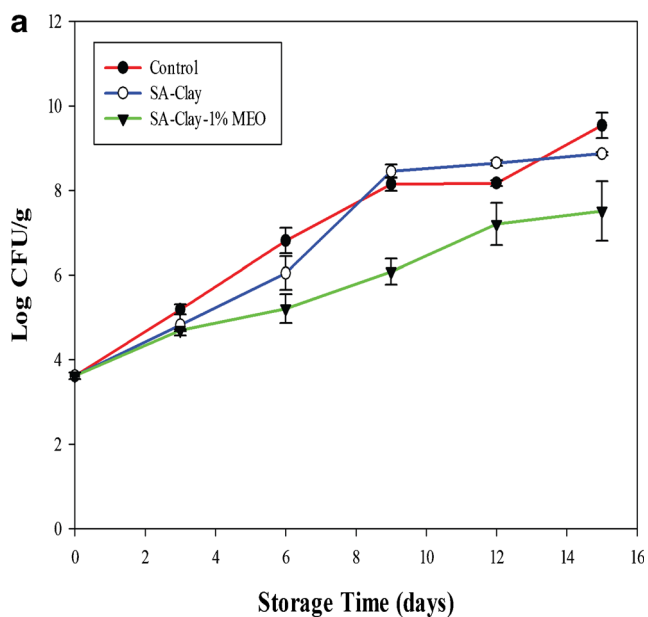


Fig. 6 Effect of antimicrobial alginate-clay films on the growth of total viable counts (a) and psychrotrophic bacterial counts (b) on rainbow trout slices stored at 4 °C for 15 days

Inhibition growth of spoilage bacteria on trout slices

The changes in the microflora of rainbow trout slice during storage under aerobic conditions and film packaging with or without addition of MEO are presented in Fig. 6. The initial number of total viable counts (TVC) in rainbow trout slices was 3.61 log CFU/g which indicated the good quality of fish used in this study. Generally, the initial microbial load of freshwater fish is different because of water condition and temperature. According to available literature reports, the bacterial counts of different freshwater fish species are between 2 and 6 log CFU/g (Chytiri et al. 2004). Figure 6a shows that the TVC of all samples increased with storage time. The increase of TVC in fish flesh during storage has been demonstrated by previous related researches (Ojagh et al. 2010; Pezeshk et al. 2011). As can be seen, bacterial growth on the slices wrapped with antimicrobial-free films was slightly lower than control meat samples, indicating that the presence of SA-Clay film alone did not affect the growth of any of the bacteria studied. Zinoviadou et al. (2009) noted that under the high a_w conditions of the meat surface the contacting films are highly hydrated and probably do not exhibit high O_2 barrier properties and for this reason no differences in the microflora profile were observed using the antimicrobial-free film. The use of films containing MEO resulted in a significant reduction of the TVC population during the entire storage period. The TVC population of the samples wrapped in the films with MEO at the day 15 was 7.51 log CFU/g, while for the control it was 9.54 log CFU/g. The antibacterial effect found with nanocomposite films containing MEO was to be expected, since preliminary studies showed that MEO possessed

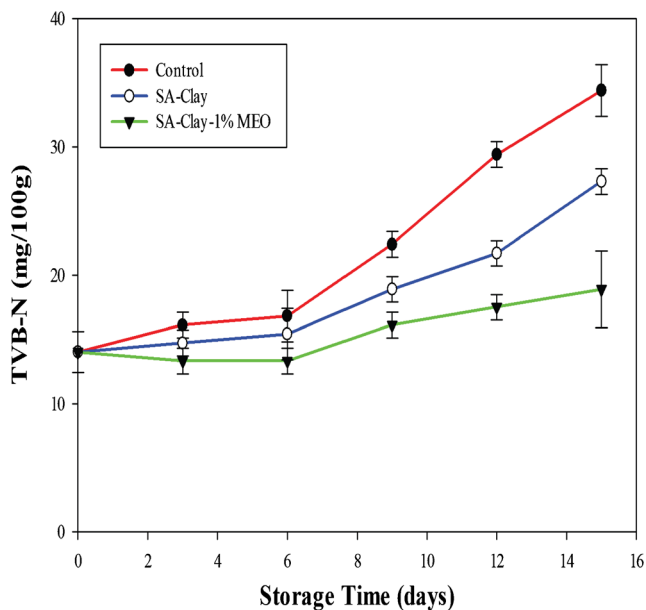


Fig. 7 Effect of antimicrobial alginate-clay films on the value of TVB-N on rainbow trout slices stored at 4 °C for 15 days

significant antilisterial effects. Furthermore, TVC exceeded the value of 7 log CFU/g, which is considered as the upper acceptability limit for fresh water and marine species (ICMSF 1986) on day 6 for the control samples, on day 7 for the films without MEO and on day 12 for the films containing MEO.

The gram-negative psychrotrophic bacteria (PTC) are the major group of microorganisms responsible for spoilage of aerobically stored fresh fish at chilled temperatures (Ibrahim Sallam 2007). In our study, the initial PTC (day 0) of raw rainbow trout slice was 3.3 log₁₀ CFU/g (Fig. 6b). Additionally, the growth pattern of PTC showed the same behavior as that of TVC, with control also being the highest at day 15 (9.91 log₁₀ CFU/g), followed by samples wrapped with SA-Clay film (9.36 log₁₀ CFU/g), and samples wrapped with SA-Clay + MEO film showed a lowest PTC population (8.09 log₁₀ CFU/g). PTC counts of fish slices was about 0.5 log CFU/g higher than TVC counts, indicating that fish bacteria flora is composed mainly of psychrotrophic bacteria. Furthermore, the SA-Clay films enriched with MEO showed a lower antibacterial activity for PTC in comparison with TVC in trout slice during the storage period. This can be attributed to the fact that PTC classified as Gram-negative bacteria and these bacteria due to the external lipopolysaccharide wall surrounding the peptidoglycan cell wall are more resistant to antimicrobial agents. Nevertheless, the hydrophobic constituents of the EOs are capable of gaining access to the periplasm of Gram (–) bacteria through the proteins of the outer membrane (Zinoviadou et al. 2009). 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 (Tajkarimi et al. 2010). Previous studies have also reported similar results for rainbow trout fillets (Ojagh et al. 2010; Pezeshk et al. 2011). TPC in aerobically stored rainbow trout slice samples exceeded the microbiological limit of 7 log CFU/g after 5 days for all of samples.

Total viable counts (TVB-N)

Changes in the value of TVB-N during the refrigerated storage are presented in Fig. 7. The initial value of TVB-N content (mg N per 100 g of fish) in samples was 12.60 which was similar to the other reports, in which the initial TVB-N value of rainbow trout was 10.73 mg per 100 g (Pezeshk et al. 2011). As can be seen, TVB-N value of all samples increased with storage time and the value of the control and the samples wrapped with SA + Clay increased faster than samples covered with SA + Clay containing MEO. Compared with the control samples, the significant reduction in TVB-N observed in the samples wrapped with SA-Clay films. This increase is related to the activity of spoilage bacteria and endogenous enzymes (Kyrana et al. 1997; Vareltzis et al. 1997). The TVB-N value of the all samples showed a slow increase in

the early storage, but then it significantly increased. This trend agrees with previous related research concerning other fish species (Song et al. 2011; Lu et al. 2010). Gimenez et al. (2002) proposed a value of 25 mg N/100 g flesh as the highest acceptable level. In present study, at day 8, the TVB-N value of the control samples exceeded the acceptable level, while the limit of acceptability was reached after 11 days for the samples wrapped with SA + Clay. However, the values for the samples wrapped with SA-Clay-MEO were much lower than the acceptable level at the entire of the storage period (18.90 mg N per 100 g). Therefore, this treatment markedly reduced the TVB-N content. This phenomenon can be attributed to either a more rapidly reduced bacterial population or decreased the capacity of bacteria for oxidative deamination of non-protein nitrogen compounds or both (Fan et al. 2008), which was due to the effect of MEO on fish fillets. Ojagh et al. (2010) found that chitosan coating enriched with cinnamon oil could also significantly lower the TVB-N content of rainbow trout fish during refrigerated storage.

Conclusion

According to the results of agar diffusion test, the intensity of antilisterial efficacy was in the following order: marjoram (MEO) > clove > cinnamon > coriander > caraway > cumin. Obtained results from TSA-NaCl plates (model step) indicate that the nanocomposite films containing marjoram essential oil more efficiently inhibited the growth of *L. monocytogenes* than films containing clove or cinnamon. Finally, when the SA-Clay + MEO (1 %) film was applied to trout slices, *L. monocytogenes*, aerobic mesophilic and psychrophilic cell counts were reduced and TVB-N maintained lower than acceptable values throughout the storage period. Therefore, this study demonstrates the effectiveness of alginate-clay films containing MEO to control the growth of *L. monocytogenes* and enhance the microbiological safety of rainbow trout slice under refrigerated storage. However, these films are still hydrophile and need a supportive packaging for commercial application.

References

- Abdollahi M, Rezaei M, Farzi G (2012) A novel active bionanocomposite film incorporating rosemary essential oil and nanoclay into chitosan. *J Food Eng* 111:343–350
- Alboofetileh M, Rezaei M, Hosseini H, Abdollahi M (2014a) Effect of nanoclay and cross-linking degree on the properties of alginate-based nanocomposite film. *J Food Process Preserv* 38:1622–1631
- Alboofetileh M, Rezaei M, Hosseini H, Abdollahi M (2014b) Antimicrobial activity of alginate/clay nanocomposite films enriched with essential oils against three common foodborne pathogens. *Food Control* 36:1–7

- Appendini P, Hotchkiss JH (1997) Immobilization of lysozyme on food contact polymers as potential antimicrobial films. *Packag Technol Sci* 10(5):271–279
- Azeredo H (2009) Nanocomposites for food packaging applications. *Food Res Int* 42(9):1240–1253
- Benavides S, Villalobos-Carvajal R, Reyes J (2011) Physical, mechanical and antibacterial properties of alginate film: effect of the crosslinking degree and oregano essential oil concentration. *J Food Eng* 110(2):232–239
- Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods—a review. *Int J Food Microbiol* 94(3):223–253
- Castellano P, Belfiore C, Fadda S, Vignolo G (2008) A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Sci* 79(3):483–499
- Chytiri S, Chouliara I, Savvaidis IN (2004) Microbiological, chemical and sensory assessment of iced whole and filleted aquacultured rainbow trout. *Food Microbiol* 21:157–165
- Concha-Meyer A, Schöbitz R, Brito C, Fuentes R (2011) Lactic acid bacteria in an alginate film inhibit *Listeria monocytogenes* growth on smoked salmon. *Food Control* 22:485–489
- Fan W, Chi Y, Zhang S (2008) The use of a tea polyphenol dip to extend the shelf life of silver carp (*Hypophthalmichthys molitrix*) during storage in ice. *Food Chem* 108:148–53
- Gill AO, Delaquis P, Russo P, Holley RA (2002) Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *Int J Food Microbiol* 73:83–92
- Jimenez B, Roncales P, Beltran JA (2002) Modified atmosphere packaging of filleted rainbow trout. *J Sci Food Agric* 84:1154–1159
- Gómez-Estaca J, López de Lacey A, López-Caballero M, Gómez-Guillén M, Montero P (2010) Biodegradable gelatin–chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. *Food Microbiol* 27(7):889–896
- Hosseini M, Razavi S, Mousavi M (2009) Antimicrobial, physical and mechanical properties of chitosan based films incorporated with thyme, clove and cinnamon essential oils. *J Food Process Preserv* 33:727–743
- Ibrahim Sallam K (2007) Antimicrobial and antioxidant effects of sodium acetate, sodium lactate, and sodium citrate in refrigerated sliced salmon. *Food Control* 18:566–575
- International Commission on Microbiological Specifications for Foods (ICMSF) (1986) *Microorganisms in Foods. In: Sampling for microbiological analysis: principles and specific applications, vol. 2.* University of Toronto Press, Toronto, Canada
- Kristo E, Koutsoumanis KP, Biliaderis CG (2008) Thermal, mechanical and water vapor barrier properties of sodium caseinate films containing antimicrobials and their inhibitory action on *Listeria monocytogenes*. *Food Hydrocoll* 22(3):373–386
- Kyranas VR, Lougovois VP, Valsamis DS (1997) Assessment of shelf-life of maricultured gilthead sea bream (*Sparus aurata*) stored in ice. *Int J Food Sci Technol* 32:339–347
- Lavorgna M, Piscitelli F, Mangiacapra P, Buonocore GG (2010) Study of the combined effect of both clay and glycerol plasticizer on the properties of chitosan films. *Carbohydr Polym* 82:291–298
- Lis-Balchin M, Deans S (2003) Bioactivity of selected plant essential oils against *Listeria monocytogenes*. *J Appl Microbiol* 82(6):759–762
- Lu F, Ding Y, Ye X, Liu D (2010) Cinnamon and nisin in alginate-calcium coating maintain quality of fresh northern snakehead fish fillets. *LWT Food Sci Technol* 43:1331–1335
- Lv F, Liang H, Yuan Q, Li C (2011) In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Res Int* 9:3057–3064
- Marcos B, Aymerich T, Monfort JM, Garriga M (2007) Use of antimicrobial biodegradable packaging to control *Listeria monocytogenes* during storage of cooked ham. *Int J Food Microbiol* 120:152–158
- Masniyom P, Benjakul S, Visessanguan W (2006) Synergistic antimicrobial effect of pyrophosphate on *Listeria monocytogenes* and *Escherichia coli* O157 in modified atmosphere packaged and refrigerated seabass slices. *LWT Food Sci Technol* 39:302–307
- Mayachiew P, Devahastin S, Mackey BM, Niranjana K (2010) Effects of drying methods and conditions on antimicrobial activity of edible chitosan films enriched with galangal extract. *Food Res Int* 43:125–132
- Neetoo H, Ye M, Chen H (2010) Bioactive alginate coatings to control *Listeria monocytogenes* on cold-smoked salmon slices and fillets. *Int J Food Microbiol* 136:326–331
- Ojagh SM, Rezaei M, Razavi SH, Hosseini SMH (2010) Development and evaluation of a novel biodegradable film made from chitosan and cinnamon essential oil with low affinity toward water. *Food Chem* 122:161–166
- Papadokostaki K, Amarantos S, Petropoulos J (1998) Kinetics of release of particulate solutes incorporated in cellulosic polymer matrices as a function of solute solubility and polymer swellability. II. Highly soluble solute. *J Appl Polym Sci* 69(7):1275–1290
- Pezeshk S, Rezaei M, Hosseini H (2011) Effects of turmeric, shallot extracts, and their combination on quality characteristics of vacuum-packaged rainbow trout stored at 4 ± 1 °C. *J Food Sci* 76(6):M387–M391
- Rhim JW, Ng PK (2007) Natural biopolymer-based nanocomposite films for packaging applications. *Crit Rev Food Sci Nutr* 47(4):411–433
- Sánchez-González L, Cháfer M, Hernández M, Chiralt A, González-Martínez C (2011) Antimicrobial activity of polysaccharide films containing essential oils. *Food Control* 22(8):1302–1310
- Sánchez-González L, González-Martínez C, Chiralt A, Cháfer M (2010) Physical and antimicrobial properties of chitosan–tea tree essential oil composite films. *J Food Eng* 98(4):443–452
- Solomakos N, Govaris A, Koidis P, Botsoglou N (2008) The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. *Food Microbiol* 25:120–127
- Song Y, Liu L, Shen H, You J, Luo Y (2011) Effect of sodium alginate-based edible coating containing different anti-oxidants on quality and shelf life of refrigerated bream (*Megalobrama amblycephala*). *Food Control* 22:608–615
- Sorrentino A, Gorrasi G, Vittoria V (2007) Potential perspectives of bio-nanocomposites for food packaging applications. *Trends Food Sci Technol* 18(2):84–95
- Tajkarimi M, Ibrahim S, Cliver D (2010) Antimicrobial herb and spice compounds in food. *Food Control* 21(9):1199–1218
- Tunc S, Duman O (2011) Preparation of active antimicrobial methyl cellulose/carvacrol/montmorillonite nanocomposite films and investigation of carvacrol release. *LWT Food Sci Technol* 44:465–472
- Varelzidis K, Koufidis D, Gavriilidou E (1997) Effectiveness of a natural Rosemary (*Rosmarinus officinalis*) extract on the stability of filleted and minced fish during frozen storage. *Z Lebensm Unters Forsch* 205:93–96
- Zinoviadou KG, Koutsoumanis KP, Biliaderis CG (2009) Physicochemical properties of whey protein isolate films containing oregano oil and their antimicrobial action against spoilage flora of fresh beef. *Meat Sci* 82:338–345