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Research Article

Antimicrobial Activity of Two Polysaccharide Edible Films Incorporated with Essential Oils against Three Pathogenic Bacteria

[1]Hamdy A. Shaaban, [2,3]Hatem S. Ali, [2]Gamil F. Bareh, [3]Abdel Rahman S. Al-khalifa and [4]May M. Amer

[1]Department of Chemistry of Flavour and Aroma, National Research Centre, Dokki, Giza, Egypt
[2]Department of Food Science and Technology, National Research Centre, Dokki, Giza, Egypt
[3]Department of Food Science and Nutrition, College of Food Science and Agriculture, King Saud University, Kingdom of Saudi Arabia
[4]Department of Food Toxicology and Contaminants, National Research Centre, Dokki, Giza, Egypt

Abstract

Background: Nowadays, packaging research is receiving considerable attention because of the development of eco-friendly materials made from natural polymers such as chitosan (CH) and hydroxypropyl methylcellulose (HPMC). Essential oils are effective antimicrobials on important some pathogenic bacteria and can be added packaging materials due to absorb various surfaces. **Objective:** The main purposed of this study was to prepared antimicrobial films by incorporating different concentrations of marjoram, clove and cinnamon essential oils, into chitosan and hydroxypropyl methylcellulose films against foodborne pathogens. **Methodology:** Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5% w/w) at 25°C. Following overnight agitation, essential oils were added to the chitosan solution. Hydroxypropyl methylcellulose 1% weight was dispersed in de-ionized water at 80°C. After the dissolution of the polysaccharide, essential oils were added. Essential oils clove (*Syzgium aromaticum*), marjoram (*Origanum majorana*) and cinnamon (*Cinnamomum zeylanicum*) were extracted by hydro-distillation. The antibacterial effects of essential oils were studied against three important food pathogens, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* by application of agar diffusion method. Also, antimicrobial effectiveness of films were studied by tryptone soy agar with 3% NaCl was used as a model solid food system (TSA-NaCl). **Results:** The intensity of antimicrobial efficacy was in the following order: Marjoram>clove>cinnamon. The antibacterial effectiveness of the prepared films against *E. coli*, *S. aureus* and *L. monocytogenes* was studied at 10°C during 12 days. The HPMC-EO and CH-EO composite films present a significant antimicrobial activity against the three pathogens considered. In all film matrices, marjoram exhibited the highest antimicrobial activity. A complete inhibition of microbial growth was observed for CH or HPMC-marjoram films for *E. coli*, HPMC-marjoram for *L. monocytogenes* and HPMC-clove for *S. aureus*. **Conclusion:** The HPMC-EO and CH-EO composite films, containing clove, cinnamon or marjoram, showed a significant antimicrobial activity (bacteriostatic effect) against the three pathogens studied (*E. coli*, *L. monocytogenes* and *S. aureus*). In all film matrices, marjoram exhibited the highest antimicrobial activity.

Key words: Antimicrobial activity, essential oils, chitosan, hydroxypropyl methylcellulose, edible film, pathogens, food packing

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Corresponding Author: Hamdy Shaaban, Division of Food Industries and Nutrition, Department of Chemistry of Flavor and Aroma, National Research Centre, Cairo, Egypt Tel: 2-02-33371010 Fax: 2-02-37601877

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Food quality and safety are the major concerns in the food industry, especially on preventing chemical and microbiological deterioration, as well as the exposure of labile biomolecules (vitamins, essential oils and colorants) to extreme humidity and temperature conditions, which leads them to degradation[1]. The main constraint against the commercialization of essential oils is their strong flavor and high reactivity with food ingredients. This can be minimized by incorporating the oil into a carrier, which can be fabricated into a packaging film. For this, the interest in development of novel ways to prolong the shelf life of food products has increased in the last years. Edible films as a solid sheet can be applied on the surface of the food systems and can be a good alternative to improve food quality by serving as selective barriers to moisture transfer, carbon dioxide permeability, oxygen uptake, lipid oxidation and losses of volatile aromas and flavors[2-4].

These films are thin layers of edible material such as protein, polysaccharide and lipid. Polysaccharides are widely used to produce films[5,6], which can be used to carry active ingredients, for example antioxidant and antimicrobial agents[7]. Studies have focused on the incorporation of natural active compounds in edible film since they may widen the functionality protecting the product from microbial spoilage and thus extend their shelf-life[8-11].

The EOs is liquids from plants material with oily and aromatic characteristics, which have interesting antibacterial activities against food pathogens and food spoiling microorganisms. Being a natural product makes this product attractive for use as a food preservative in the food industry, as consumers have become weary of synthetic additives recently[12,13]. Essential oils also have interesting characteristics in film formation[14].

Essential oils and their components are commonly used as flavoring in the food industry and are categorized as Generally Recognized as Safe (GRAS)[15,12]. Essential oils rich in phenolic compounds have been reported to have a wide spectrum of antimicrobial activity. Among these, cinnamon, clove, marjoram, rosemary, sage and oregano oils have been found to be effective antimicrobial agent[16,17]. Also, they have antioxidant properties. There are some studies explaining compounds are responsible for the major antimicrobial and antioxidant effects of essential oils. For example, thymol, eugenol and carvacrol are the main components responsible for the antimicrobial activity of basil and thyme oils[18,19]. Similarly, Lambert *et al.*[20] reported that essential oils containing a high percentage of phenolic components, such as carvacrol, thymol and eugenol, present stronger antibacterial properties against foodborne pathogens. These compounds are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of cytoplasmic membrane to ATP[20]. According to Smith-Palmer *et al.*[21], Gram-positive bacteria are slightly more sensitive to essential oils than Gram-negative bacteria[21]. Use of antimicrobial agents such as essential oils in edible food packaging can control the microbial population and target specific microorganisms to provide higher safety and quality products[22]. The major advantage of this technology is that the diffusion rate of the antimicrobial agents can be slowed down. Thus, edible films can extend the shelf life of products by keeping high concentrations of active components on the product surface[18].

Cellulose derivatives are interesting film forming compounds, as they are odorless, tasteless and biodegradable[23]. In addition, their application cost is low. Hydroxypropyl methylcellulose (HPMC) presents excellent film-forming properties[23,24], with very efficient oxygen, carbon dioxide and lipid barriers. However, HPMC films are highly permeable to water vapor, which is an important drawback that limits their application[25], since an effective control of moisture transfer is a desirable property.

Another biopolymer with excellent film forming ability is chitosan[26]. This non-toxic compound, obtained by the deacetylation of chitin, a structural component present in the shell of some crustaceans. Also, usage of chitosan as a food packaging film has many advantages compared to the other edible films because of its antibacterial activity and metal chelation properties, which partly contribute towards the antimicrobial activity by chelating metal ions that are essential for bacterial growth. The applications of chitosan for the improvement of quality and shelf life of various agricultural, poultry and marine foods have been comprehensively reviewed[27].

The aim of this study is to analyze the effect of essential oil incorporation on the antimicrobial properties of chitosan and HPMC based films. The effectiveness of composite films against three foodborne pathogens, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* were evaluated at 10°C during a storage period of 12 days.

MATERIALS AND METHODS

Materials: Hydroxypropylmethyl cellulose (HPMC) product No. H 7509, CAS No. 9004-65-3, Molecular weight: approximately 86 kDa. Food grade and high molecular weight chitosan (CH) with a deacetylation degree of 82.7% (CAS No. 9012-76-4) was purchased from Sigma Aldrich

were used. The percentages of methoxyl and hydroxypropyl of HPMC ranged between 28-30 and 7-12%, respectively. Apparent viscosity of a 1% CH solution in 0.5% glacial acetic acid 98% and essential oils Clove (*Syzgium aromaticum*), marjoram (*Origanum majorana*), cinnamon (*Cinnamomum zeylanicum*) used to prepare the Film-Forming Dispersions (FFD) were extracted by hydro-distillation from the dried samples by the clevenger type apparatus and the obtained oils stored in a dark container at 4°C until used.

Bacterial strain and maintenance: Stock culture of *Listeria monocytogenes* (ATCC 35152), *Staphylococcus aureus* (ATCC 43300) and *Escherichia coli* (ATCC 27325) were obtained from the culture collections of the Microbiological Department National Research Centre (NRC), Dokki, Giza, Egypt. All strains were kept frozen at -20°C in Tryptone Soy Broth (TSB) supplemented with 30% glycerol. Cultures were then regenerated by transferring a loopful of each bacteria into 10 mL of TSB and incubated at 37°C overnight. A 10 mL aliquot from each overnight culture was again transferred into 10 mL of TSB and grown at 37°C to the end of the exponential phase of growth. Subsequently, these appropriately diluted cultures were then used for the inoculation of the agar plates in order to obtain target inoculums of 10^2 CFU mL^{-1}.

Antimicrobial activity of essential oils: The antibacterial properties of the five mentioned essential oils were studied using the agar diffusion method[28]. Thirty microliters of the essential oils were poured into an agar well (5 mm diameter), previously created with a sterile core bore on the agar, after their plates had been seeded with 0.1 mL of inoculums by swab containing approximately 10^6 CFU mL^{-1} of the indicated bacteria. All the strains were cultured in tryptone soy agar. The plates were incubated at 37°C for 48 h in the suitable incubation chamber. After incubation, the microbial growth was observed and the degree of inhibition was expressed as follows: No inhibition, weak activity (zone of inhibition $\leq$12 mm), moderate activity (12 mm$\leq$zone of inhibition $\leq$20 mm) and strong activity (zone of inhibition $\geq$20 mm[29,30].

Preparation of the film-forming dispersions: Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5% w/w) at 25°C. Following overnight agitation, hydroxypropylmethyl cellulose 1% weight was dispersed in deionised water at 80°C. After the dissolution of the polysaccharide Essential Oils (EO) were added to the chitosan (CH) and hydroxypropylmethyl cellulose solutions in the ratios indicated in Table 1.

Table 1: Concentration of components in aqueous Film-Forming Dispersions (FFD)

Sample	HPMC (%p/p)	CH (%p/p)	Acetic acid (%v/p)	EO (%p/p)
HPMC	1	-	-	-
HPMC-0.5% EO	1	-	-	0.5
HPMC-1% EO	1	-	-	1
HPMC-2% EO	1	-	-	2
HPMC-3% EO	1	-	-	3
CH	-	1	0.5	-
CH-0.5% EO	-	1	0.5	0.5
CH-1% EO	-	1	0.5	1
CH-2% EO	-	1	0.5	2
CH-3% EO	-	1	0.5	3*

*EO concentration was only used for films where no oil phase separation occurred in the film forming dispersion

Both CH-EO and HPMC-EO mixtures were emulsified at room temperature using homogenizer at 13,500 rpm for 4 min. These emulsions were vacuum degasified at room temperature using a vacuum pump.

Preparation of films: Film-Forming Dispersions (FFD) were poured onto a framed and levelled petri dish plate ($\varphi = 8.5$ cm) and subsequently dried under atmospheric conditions. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g m^{-2} in all formulations. Dry films were peeled off from the casting surface and preconditioned in desiccators at 10 °C and at 57.4% Relative Humidity (RH) prior to testing. It is remarkable that the initial concentration of the EO in the FFD was reduced during the film drying step due to the oil evaporation. Studies on this topic[18], revealed that the losses of volatile compounds during the film drying can range between 39 and 99% depending on the ratio essential oil-chitosan in the film forming dispersions. The greater the ratio, the greater the loss. Nevertheless, for sample identification, the initial polymer-EO ratio incorporated in the FFD was used.

Antimicrobial effectiveness of films: The methodology was adapted from Kristo *et al.*[31]. Tryptone soy agar (International Diagnostics, UK) with 3% NaCl (Sigma Aldrich, Germany) was used as a model solid food system (TSA-NaCl). Aliquots of TSA-NaCl (20 g) were poured into petri dishes. After the culture medium solidified, properly diluted overnight cultures from each strain were inoculated on the surface and different test films (containing or not antimicrobial substance) of the same diameter as the petri dishes were placed onto the inoculated surface. Inoculated and uncoated TSA-NaCl petri dishes were used as control. Plates were then covered with parafilm to avoid dehydration and stored at 10°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 plastic bag with 100 mL of tryptone phosphate water. The bag was homogenized for 2 min in a Stomacher blender, which allows us to obtain a very homogeneous system where samples taken to the analysis are representative. Serial dilutions were made and then poured onto TSA. Plates were incubated at 37°C for 24 h before colonies were counted. All tests were done in triplicate.

Statistical analysis: The statistical analysis of the data was performed through SPSS version 16.0. Quantitative data were represented in form of Mean±Standard Deviation (SD). Analysis of variance (ANOVA) was used in the analysis of the results. Duncan's multiple range test was used to determine any significant differences in mean log CFU cm^{-2} among treatments at a 95% Least Significant Difference (LSD) intervals.

RESULTS AND DISCUSSION

Antimicrobial activity of the essential oils: The qualitative antimicrobial activity of the essential oils is shown in Table 2. Marjoram essential oil presented the highest inhibitory effect. The intensity of antimicrobial efficacy was in the following order: Marjoram>clove>cinnamon. Among the three tested pathogens, *E. coli* were the most resistant. Based on inhibition zone test results, marjoram, clove and cinnamon EOs were selected for this study.

Results obtained showed that of the 3 essential oils were selected for tested in this study, marjoram, clove and cinnamon exhibited strong antibacterial activities and among the three tested pathogens, *E. coli* were the most resistant. Gomez-Estaca *et al.*[28] showed that clove, lavender, thyme and rosemary had strong and consistent inhibitory effects against various pathogens and spoilage microorganisms. In addition, Winward *et al.*[32] reported that cinnamon essential oil had a good inhibitory effect on pathogenic bacteria such as *E. coli* and *S. aureus*. The lower antimicrobial activity against *E. coli* can be attributed to the fact that Gram-negative are in general more resistant due to the external lipopolysaccharide wall surrounding the peptidoglycan cell wall[33]. The mechanism of antimicrobial activity of essential oils is related with the attack on the phospholipid present in cell membranes, which causes increased permeability and leakage of cytoplasm or in their interaction with enzymes located on the cell wall[33]. Thus, the resistance of Gram-negative bacteria to the essential oils likely lies in the protective role of their cell wall lipopolysaccharides or outer membrane proteins. Moreover, the antimicrobial activity of EOs depends on the type of spice or herb, the chemical composition and the content of extracts and essential oils[34]. Chemical composition of EOs is complex and strongly dependent 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[33,35]. The major components in EOs are phenolic substances, which are thought to be responsible for the antimicrobial properties. In this regard, the major components in marjoram, cinnamon and clove are terpinen-4-ol, cinnamaldehyde and eugenol, respectively. Sublethal concentrations of eugenol and cinnamaldehyde have been found to inhibit production of amylase and proteases by *Bacillus cereus*. Cell wall deterioration and a high degree of cell lysis were also noted[36].

Table 2: Antibacterial activity of the EOs against 3 bacterial strains

	Inhibition zone diameters (mm)		
Microorganisms/essential oil	Marjoram	Clove	Cinnamon
Listeria monocytogens	38	27	22
Staphylococcus aureus	43	30	23
Escherichia coli	21	18	16

Antimicrobial effectiveness of films

***Escherichia coli*:** Growth curves of *E. coli* in control TSA-NaCl plates and in TSA-NaCl plates coated with the different films are shown in Fig. 1. No significant differences were observed between the growth of *E. coli* on control TSA-NaCl plates and plates coated with HPMC film during the storage period. *Escherichia coli* population increased from 2-8.5 logs CFU cm^{-2} after the 12 days. Wu *et al.*[37] also observed the absence of antimicrobial activity of cellulose films against *E. coli* strain. Growth data indicated that HPMC-EO composite films were effective at reducing microbial growth. This reduction increased when the essential oil concentration in the HPMC film rose.

The most effective essential oil is the marjoram oil. In fact, HPMC films with more than 1% of marjoram oil completely inhibited pathogen growth for the first 7 days of storage. At the end of the storage, only HPMC films with the highest concentration of marjoram oil (2%) could maintain a complete inhibition of the microbial growth, whereas a significant, but rather less important, increase (approximately 1 log CFU cm^{-2}) of *E. coli* counts was observed for 1% of essential oil. Even if a complete inhibition of the microbial growth was not observed when 0.5% of marjoram oil was incorporated, *E. coli* counts were significantly reduced after the third day of storage, as compared to the control sample and microbial population did not exceed 4 logs CFU cm^{-2} at the end of the 12 days.

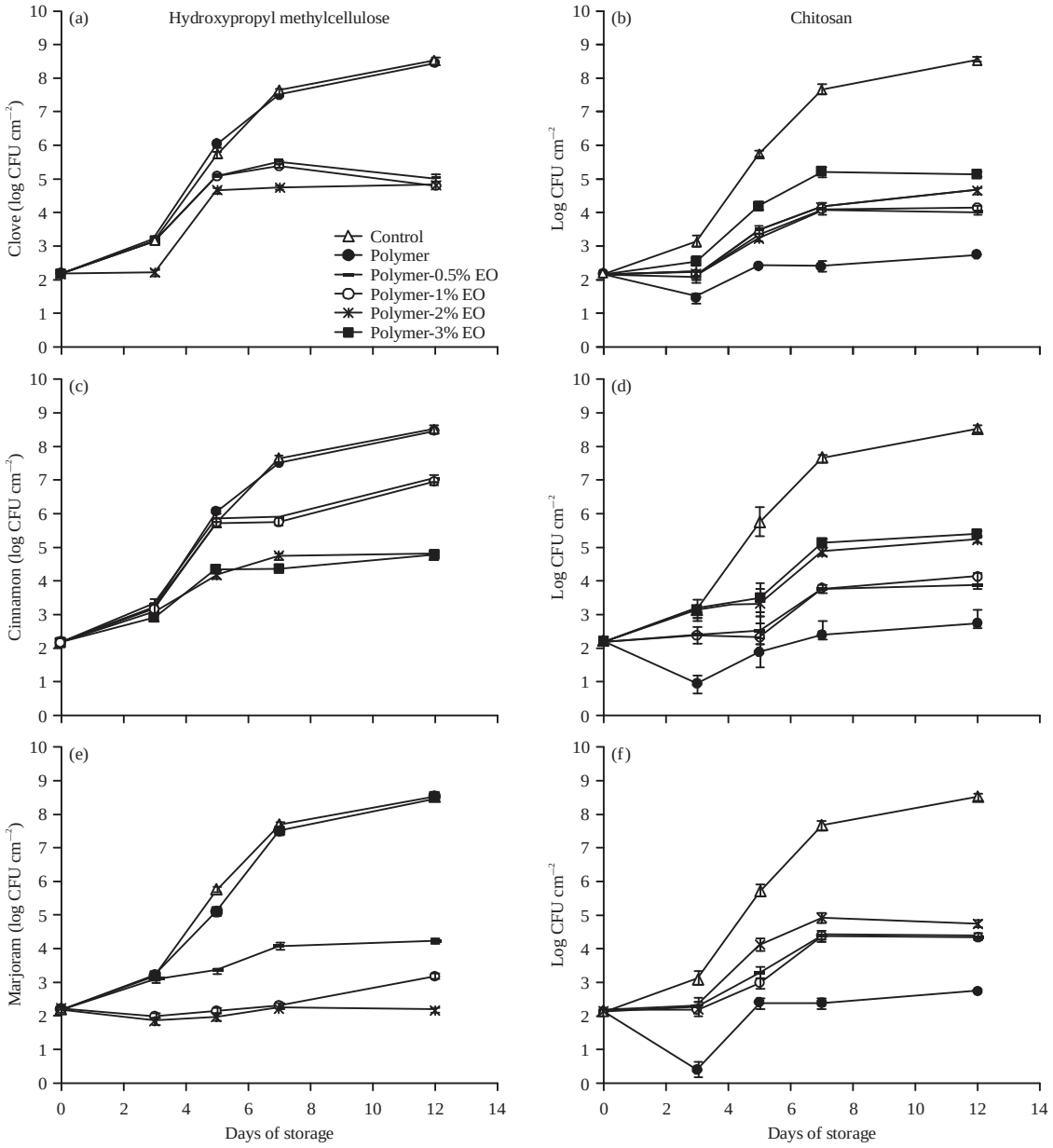

Fig. 1(a-f): Effect of different films on the growth and survival of *Escherichia coli* on TSA-NaCl medium stored at 10°C. Mean values and 95% LSD intervals for each time

Concerning HPMC-clove composite films, differences among films with different clove oil concentration were not significant at the end of the storage period when a microbial reduction of more than 3 logs, as compared with the control plates was observed. With the highest concentration of clove oil (2%), a total inhibition of the pathogen growth occurred during the first 3 days of the storage period.

The HPMC-cinnamon composite films showed a less marked antimicrobial activity for the lowest cinnamon oil concentration levels (0.5 and 1%). A reduction of the *E. coli* counts, with respect to the control was observed after the third day of storage for the highest cinnamon oil (2 and 3%) and after the 5th day for 0.5 and 1%. At the end of the storage period, a pathogen reduction, as compared to the control plates of about 1.5 and 4 logs were reached for the lowest and highest cinnamon oil concentrations, respectively.

The obtained results agree with those reported by Winward *et al.*[32], when analyzing the activities against different *E. coli* strains of plant essential oils. Their results demonstrated that both marjoram and cinnamon essential oils

showed bactericidal activities against *E. coli*, but the Minimum Inhibitory Concentration (MIC) value obtained for cinnamon oil was higher than those found for marjoram oil (2.5 and 0.5 mL/100 mL, respectively). According to our results, when marjoram oil was incorporated into the HPMC matrix, it was also more effective than cinnamon oil at reducing *E. coli* growth.

To understand antimicrobial effectiveness of HPMC-EO and CH-EO composite films, several factors must be considered. Parameters concerning microorganisms such as inoculum sizes, bacteria physiological state and storage conditions (temperature, culture media) are important to explain the antimicrobial activity of chitosan, essential oils and different films. Fernandez-Saiz *et al.*[38] pointed out that the sensitivity of *S. aureus* seems to be higher when bacteria were inoculated in the mid-log phase and microbial reduction was significantly more pronounced compared to the late-log phase or the stationary phase. In the present study, the influence of all these factors was not evaluated, the bacteria were inoculated at stationary phase, the storage conditions were fixed and only one level of infection was considered.

The CH films without essential oil presented a significant antimicrobial activity against *E. coli*, a significant decrease in microbial counts after 3 storage days and a complete inhibition of the microbial growth during the rest of the tested storage period were observed, which seems to indicate some bactericide effect of CH. Pathogen population did not exceed 2 logs CFU cm^{-2} throughout the 12 storage days. Previous studies also showed the antimicrobial activity of chitosan films[39]. Other researchers report that the incorporation of the essential oils enhanced the antimicrobial efficiency of chitosan against different strains (*E. coli*, *L. monocytogenes*, *S. aureus*, *Salmonella typhimurium*, *Bacillus cereus*) whereas, it had little effect on the physicochemical properties of films[39,40]. Nevertheless, in this study, when CH films were tested against *E. coli*, the incorporation of essential oils (marjoram oil, clove oil and cinnamon oil) into the films led to a significant decrease of their antimicrobial effectiveness. This can be attributed to the dilution effect of CH when EO is present in Table 3, thus being less available for microorganisms and it reflects the milder antimicrobial effect of the EO compounds as compared to CH, despite the fact that their antimicrobial effect has been proved in the HPMC films as well as in previous studies[41]. The antimicrobial reduction of EO could be due to the bonding of the active compounds in the CH network through the strong interactions with the charged polymer chains which made their access to the microorganisms difficult.

Table 3: Concentration of Essential Oils (EO) and chitosan (CH), expressed as mass percentage (M) of the total solids of the film# and the total concentration (C_F) in the film for each polymer formulation (P-EO)*

	EO-concentration		CH-concentration	
Formulation	M (%)	C_F (mg)	M (%)	C_F (mg)
P	0	0	105	325
P-0.5% EO	34.3	107	68.6	214
P-1% EO	51	163	51	163
P-2% EO	68.6	214	34.3	106
P-3% EO	75	243	30	85

*P-polymer (HPMC or CH), #Film completely covers the surface of TSA-NaCl agar plates

In the case of CH-marjoram oil composite films, a very mild effect of the marjoram oil concentration on the growth of *E. coli* was observed. After 12 days, CH-marjoram oil composite films provoked a microbial reduction of about 4 logs with respect to the control plates, compared to the 6 log reduction observed when pure CH films were used.

A similar loss of activity occurred when cinnamon oil and clove oil were incorporated into the CH matrix and this effect was promoted when the EO concentration increased in the film, especially for cinnamon oil. At the end of the storage period, an inhibition of about 4.5 logs was obtained with the lowest concentrations of cinnamon oil (0.5 and 1%) and clove oil (0.5, 1 and 2%), whereas for the highest levels of clove oil (3%) and cinnamon oil (2 and 3%) the loss of activity was greater (only 3 logs reduction as compared to the control). The dilution effect of CH and its impact on the antimicrobial activity of the CH films were ratified by the EO concentration effect observed in cinnamon oil and clove oil.

The polymer which constitutes the film matrix is also relevant in defining the antimicrobial properties. In the present study, chitosan and HPMC behaved very differently. The first polymer, electrically charged, showed antimicrobial activity itself, Liu *et al.*[42] and Li *et al.*[43] described the mechanisms. According to these researchers, chitosan increased the permeability of the outer and inner membranes and ultimately disrupted bacterial cell membranes, releasing the cellular content. In all likelihood, this damage was caused by the electrostatic interaction between NH_3 groups of chitosan acetate and carbonyl and phosphoryl groups of phospholipid components of cell membranes. Different factors, such as the concentration, degree of acetylation or the molecular weight of chitosan and the incorporation of acetic acid, affect the antimicrobial activity of the polymer.

When the concentration was higher than 2 00 ppm, acetic acid showed a significant antimicrobial activity against *E. coli* strains[44]. In the present study, a part of the antimicrobial activity observed with chitosan films could be attributed to the residual amount of this compound in the film, since some of the acetic acid incorporated in the FFD evaporates during

film drying. According to Brody *et al.*[45], the antimicrobial effect of chitosan occurred without any migration of active agents. As chitosan is in a solid form, only microorganisms in direct contact with the active sites of the polymer are inhibited because chitosan cannot diffuse through the adjacent agar media[46]. In the present study, bacteria are inoculated on the plate surface, so chitosan is in direct contact with microorganisms. Incorporating antimicrobial agents, such as essential oils, into chitosan edible films can improve the antimicrobial efficiency of the film, as the diffusion of the oil compounds would compensate the non-migrated antimicrobial power of CH. Nevertheless, this was only observed for the Gram-positive bacteria tested, *L. monocytogenes* and *S. aureus*, which can be explained because CH is less effective against these pathogens if compared with essential oils. Previous studies show that EO were active against Gram-positive rather than Gram-negative bacteria[35] and generally the latter were more sensitive to CH[47,42]. *Escherichia coli* as opposed to the other microorganisms studied, is classified among Gram-negative bacteria and a more effective antimicrobial activity of pure CH films has been observed.

***Listeria monocytogenes*:** The antimicrobial effect against *L. monocytogenes* at 10°C of CH, HPMC, CH-EO and HPMC-EO composite films was determined on TSA-NaCl medium and shown in Fig. 2. Pure HPMC films were not effective at reducing *L. monocytogenes* growth, since no significant differences were observed in microbial growth with respect to the control TSA-NaCl plates. *Listeria monocytogenes* population increased from 2-8 logs CFU cm^{-2} at the end of the storage period.

Marjoram oil incorporated into the HPMC matrix was more effective than cinnamon oils at the same concentration. Following a storage period of 12 days, films with 2% of Marjoram oil reduced the microbial growth with respect to the control to 6.5 logs whereas only 2 and 4 logs were reduced with the same level of cinnamon oil or clove oil, respectively. At the highest concentration (2%), marjoram oil incorporation into the HPMC matrix led to a reduction of the initial pathogen population throughout the entire storage period and a complete inhibition of growth was observed with 1% marjoram oil until the 5th days of storage, giving rise to a microbial reduction of approximately 4.5 logs after 12 storage days. As expected, the antimicrobial effect is less marked with 0.5% marjoram oil where a microbial reduction of approximately 2-3 logs as compared to the control plates was observed during the first 7 days of storage.

Although, as commented on above, HPMC-cinnamon oil composite films presented a less marked antimicrobial activity than HPMC- marjoram oil films, the highest levels of cinnamon essential oil (2 and 3%) led to a complete inhibition of *L. monocytogenes* growth for 5 days, reaching a reduction of approximately 2 logs at the end of the storage period. At this final point, the antimicrobial activity was equivalent for all concentrations of cinnamon oil and the pathogen population did not exceed 6 logs CFU cm^{-2}.

The HPMC-clove oil composite films were more effective at controlling *L. monocytogenes* growth than HPMC-cinnamon oil films. During the first 3 days, a complete inhibition of microbial growth was observed with the three clove oil concentrations 0.5, 1 and 2%. At the end of storage, the pathogen population did not exceed 4 and 5 logs CFU cm^{-2} for 2% and both 0.5 and 1% of clove oil, respectively.

The CH films without essential oil showed a significant antimicrobial activity against *L. monocytogenes*, a complete inhibition of the microbial growth was observed during the first five days and the pathogen population did not exceed 6 logs CFU cm^{-2} at the end of the storage period. Previous studies also reported the antimicrobial activity of CH against *L. monocytogenes* strains[48,32]. The incorporation of essential oils into the CH matrix slightly improved the antimicrobial effect of CH as was previously reported by Zivanovic *et al.*[40] for oregano essential oil. In the cases of marjoram oil and clove oil, this was more intense when the EO concentration increased, but for cinnamon oil no effect of the EO concentration was observed.

In this study, cinnamon oil, clove oil and marjoram oil incorporated into CH or HPMC films were effective at inhibiting or reducing pathogen growth. Nevertheless, antimicrobial activity of EO varied as a function of the type of bacteria, the nature of the essential oil and the characteristics of the film matrix where they were included. Although, EO were effective against the three pathogens considered, they were more effective, at the same concentration, against *L. monocytogenes* than *E. coli* when incorporated into HPMC films. Some previous studies Smith-Palmer *et al.*[21] have also found that Gram-positive bacteria were more sensitive to EO than Gram-negative bacteria, due to the relatively impermeable outer membrane that surrounds Gram-negative bacteria. In some cases, antimicrobial effectiveness of EO decreased throughout storage, which could be explained by the evaporation of volatile compounds responsible for the antimicrobial activity and/or by the migration of EO components into the agar medium. Essential oils contain around 85-99% volatile and 1-15% non-volatile components. The antimicrobial activity of these natural compounds is essentially due to a complex mixture of terpens which constitute the volatile fraction[49].

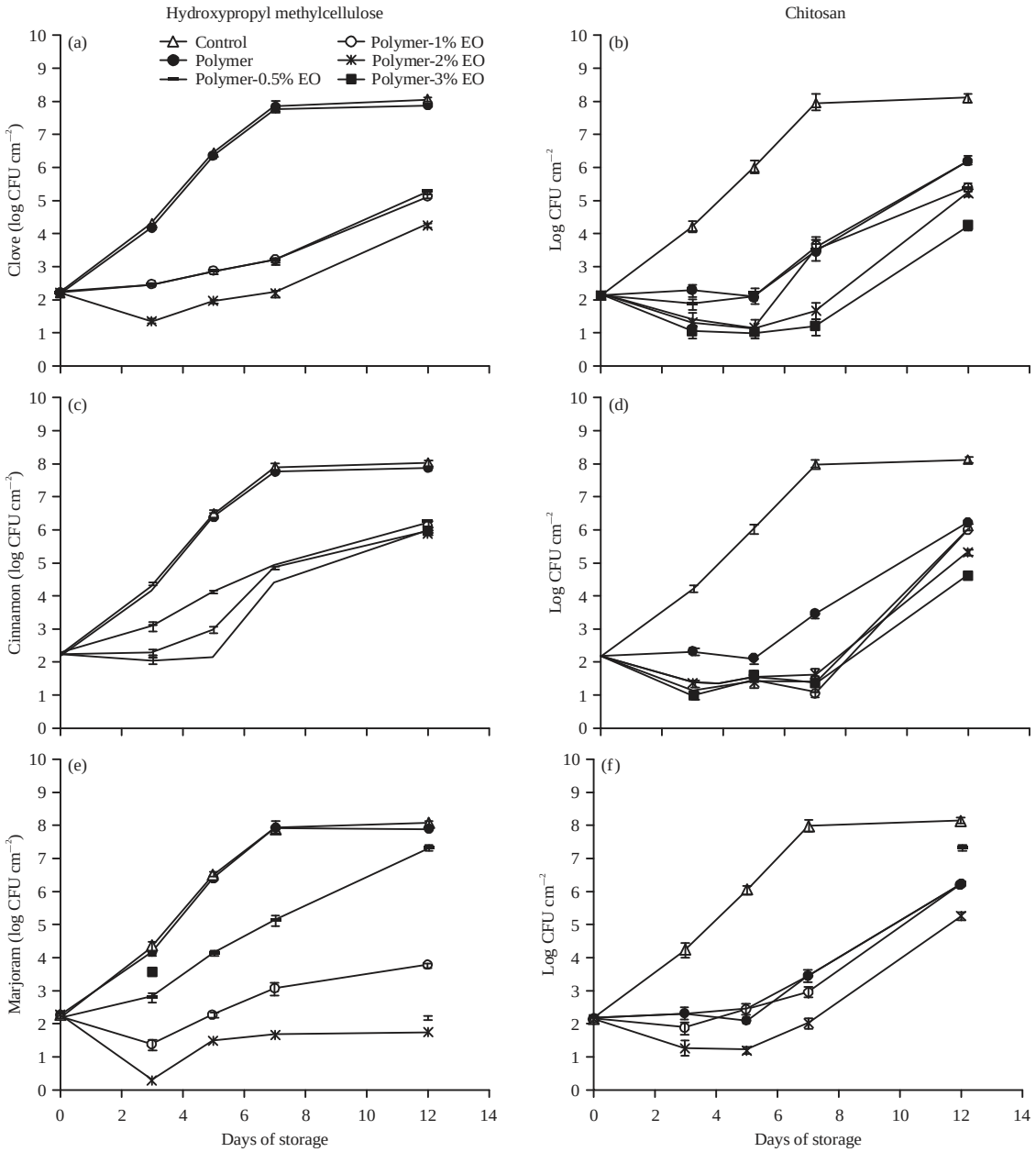

Fig. 2(a-f): Effect of different films on the growth and survival of *Listeria monocytogenes* on TSA-NaCl medium stored at 10°C. Mean values and 95% LSD intervals for each time

The selective activity of the EO compounds is reflected in the obtained results since the response of each microorganism was different for each EO. Likewise, interactions of these compounds with the film matrix also have a great impact on the antimicrobial activity. In fact, both for HPMC and CH, the influence of the nature and the amount of the essential oil differ with the pathogen considered. Concerning *E. coli*, the nature of the essential oil incorporated into CH or HPMC matrix was more relevant than the concentration of EO in the film to determine the antimicrobial effect. During the first days of the storage period, the concentration of the EO, rather than the type of EO, was the most relevant parameter in the inhibition of *S. aureus* growth. Nevertheless, after 12 days, this tendency changed and the nature of the incorporated EO became more significant. Regarding *L. monocytogenes*, results differed depending on the nature of the polysaccharide. The amount of EO incorporated into the HPMC matrix was more relevant than the nature of the EO, whereas for CH films this only occurred during the last days of storage.

Cellulose and these derivatives as opposed to chitosan, do not show antimicrobial activity and so, only the action of

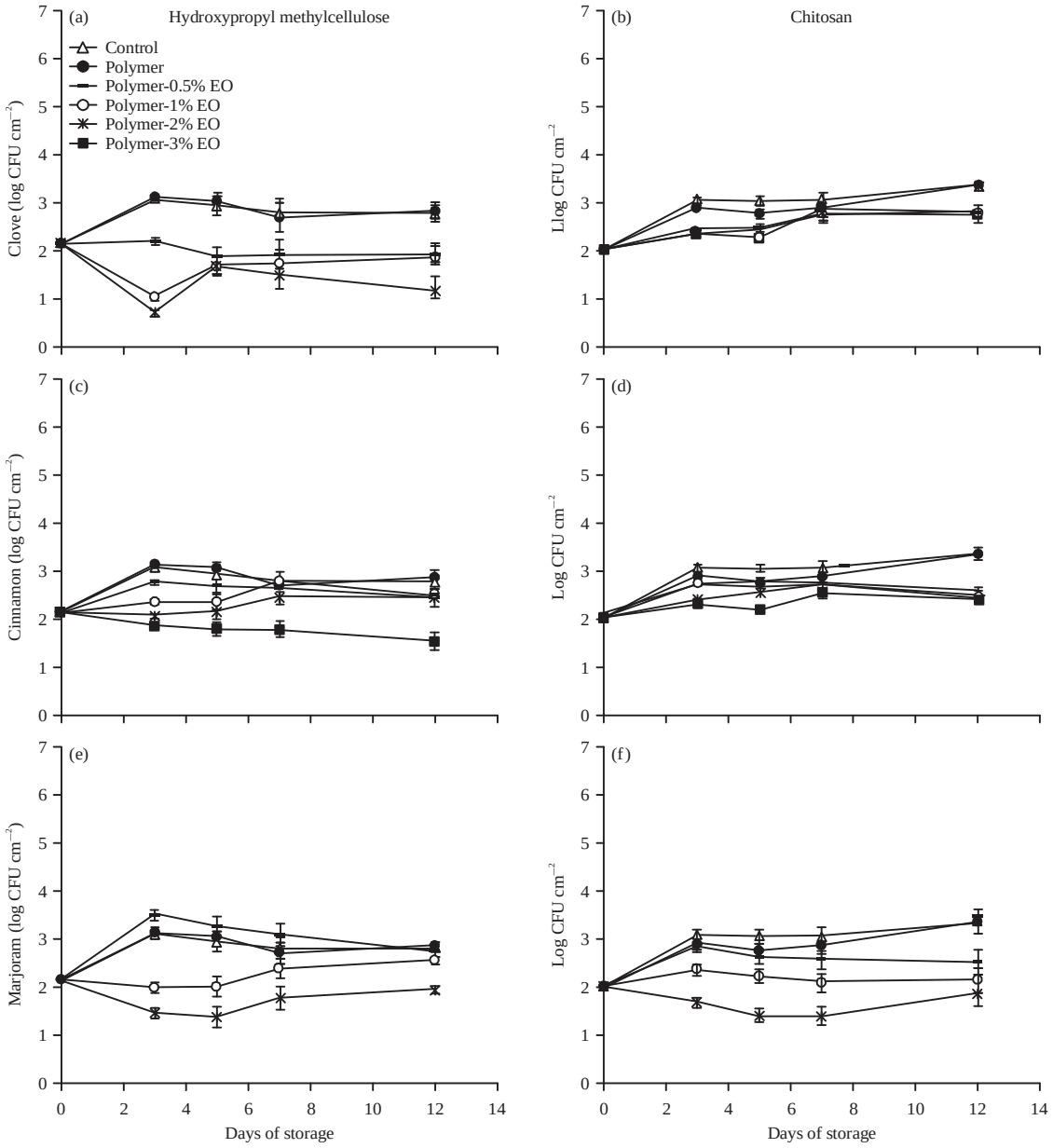

Fig. 3(a-f): Effect of different films on the growth and survival of *Staphylococcus aureus* on TSA-NaCl medium stored at 10°C. Mean values and 95% LSD intervals for each time

the EO compounds can be observed, since the polymer acts only as a neutral support releasing the active compounds to the surface where the pathogens were plated. In this sense, the particular interactions of EO compounds and the polymer matrix can also play an important role. For the same concentration of a particular EO incorporated into HPMC or CH films, significant differences were observed in the behavior of each pathogen under consideration. The reduction growth of the studied pathogens was more heavily influenced by the type of matrix than by the nature of the essential oil. Chitosan could bind with terpens which are the major components of essential oils[50]. These interactions could limit the release of antimicrobial compounds. The rate of microbial growth rose in line with a progression in the release of the antimicrobial compounds which leads to a scarce availability of the active substances on the surface, where the contamination is prevalent[32,51].

***Staphylococcus aureus*:** Growth curves of *S. aureus* on control TSA-NaCl plates and TSA-NaCl plates coated with the different films are shown in Fig. 3. *Staphylococcus aureus* grow slowly, the population only increased approximately

from 2-3 logs CFU cm^{-2} at the end of the storage period. This can be explained by the culture conditions and, more specifically, the incubation temperature. The Food Safety and Inspection Service (FSIS) reported that *S. aureus* can survive and grow slowly at various refrigeration temperatures (4.4, 10 and 15.5°C)[52]. This observation agrees with our results and with that reported in previous studies[52-54]. The HPMC films were not effective at reducing *Staphylococcus aureus* growth, since no significant differences appeared with respect to the microbial growth on control TSA-NaCl plates. Wu *et al.*[37] also observed the absence of antimicrobial activity of cellulose films against *S. aureus* strain.

The EO incorporation into HPMC films reduced the pathogen growth. Clove essential oil seems to be the most effective antimicrobial compound tested. During the first 3 days the highest concentrations of clove oil (1 and 2%) led to a microbial reduction of the initial population in the plates and although the antimicrobial effectiveness slightly decreased afterwards, the pathogen population remained lower than the initial level of infection after 12 days. In the presence of 0.5% clove oil, a complete inhibition of microbial growth was observed during the entire storage period.

The incorporation of cinnamon oil into HPMC films was less effective, but with the highest concentration of cinnamon oil (3%) a reduction of 1 log with respect to the control plate was observed throughout the whole storage period.

The HPMC-marjoram oil composite films showed a similar antimicrobial activity to those of HPMC-cinnamon essential oil composite films. Up to 1% of marjoram oil, films did not show antimicrobial effectiveness, as compared to control plates, but with the highest marjoram oil concentration (2%), a microbial reduction of approximately 1 log was exhibited during the entire storage period.

Pure CH films did not show antimicrobial activity against *S. aureus*, since no significant differences were observed during the complete storage period with respect to the control plates, but the EO incorporation into CH films reduced the pathogen growth. The antimicrobial effectiveness of CH-cinnamon essential oils was more limited than in HPMC composite films and very small differences appeared as a function of the essential oil concentration used. Throughout the whole storage period, the incorporation of cinnamon or clove supposed a microbial reduction of approximately 1 log. However, CH-marjoram oil composite films were more effective at controlling *S. aureus* growth than CH-cinnamon EO films. During the storage period, a complete inhibition of microbial growth was observed with the two highest marjoram oil concentrations (1 and 2%). The maximum amount of marjoram oil exhibited a pathogen reduction of almost 2 logs with respect to the control plates.

Mayachiew *et al.*[55] studied the effect of the drying method, particularly the drying temperature, on the antimicrobial activities of chitosan films. The antimicrobial activity, swelling and functional group interaction of the CH films with galangal extract were found to be affected by the drying methods and conditions. Ambient drying and low-temperature hot air drying led to films with the highest antimicrobial activity.

Following 12 days at 10°C, the effectiveness of the films tends to decrease significantly which can be attributed to the film's restructuration. After the films were placed on the inoculated surface of TSA-NaCl, the CH and HPMC hydrophilic matrices absorbed water, which induced changes in the film structure. At the end of storage, films were completely dissolved and, consequently, the EO compounds were liberated and they were able to evaporate or diffuse into the agar medium.

CONCLUSION

The HPMC-EO and CH-EO composite films, containing clove oil, cinnamon oil or marjoram oil showed a significant antimicrobial activity (bacteriostatic effect) against the three pathogens studied (*E. coli*, *L. monocytogenes* and *S. aureus*). In all film matrices, marjoram oil exhibited the highest antimicrobial activity. A complete inhibition of microbial growth was observed for CH films or HPMC with 2% marjoram oil for *E. coli*, HPMC with 2% marjoram oil for *L. monocytogenes* and HPMC with 2% clove oil for *S. aureus*. The nature and amount of the EO, the EO-polymer ratio in the film and the possible interactions between the polymer and the active compounds of EO play an important role in the film's antimicrobial activity. When the polymer has intense antimicrobial activity (such as chitosan against Gram-negative bacteria) the incorporation of EO reduced this activity due to the effective reduction of the available polymer concentration. Nevertheless, the antimicrobial activity is enhanced when the EO is more active than the polymer, such as in the case of CH films against Gram-positive bacteria (case of *L. monocytogenes*). When the polymer did not show antimicrobial activity, the antimicrobial effect of the EO generally increased as the ratio of EO-polymer rose in the matrix. Nevertheless, the effect of the EO ratio is affected by the interactions of the active compounds in the film matrix, which determine their diffusion rate to the infected surface.

SIGNIFICANCE STATEMENTS

- The intensity of antimicrobial efficacy was in the following order: Marjoram (clove), cinnamon
- The antibacterial effectiveness of the prepared films against *E. coli*, *S. aureus* and *L. monocytogenes* was studied at 10°C during 12 days
- HPMC-EO and CH-EO composite films present a significant antimicrobial activity against the three pathogens considered. In all film matrices, marjoram exhibited the highest antimicrobial activity
- A complete inhibition of microbial growth was observed for CH or HPMC-marjoram films for *E. coli*, HPMC-marjoram for *L. monocytogenes* and HPMC-Clove for *S. aureus*

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