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Microbiological and Physicochemical Properties of Meat Coated with Microencapsulated Mexican Oregano (*Lippia graveolens* Kunth) and Basil (*Ocimum basilicum* L.) Essential Oils Mixture

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Abstract: Microencapsulated essential oils (EOs) are increasingly used to protect the safety of foods due to their natural origin. The aim of this work was to determine the chemical composition of Mexican oregano (*Lippia graveolens* Kunth) (MOEO) and basil (*Ocimum basilicum* L.) (BEO) EOs, their combined effect against *E. coli* O157:H7, *Lactobacillus plantarum*, *Brochothrix thermosphacta* and *Pseudomonas fragi*, and their effect on microbiological and physicochemical properties of coated pork meat. EOs chemical composition was determined by GC/MS, their microencapsulated mixture (4 mg MOEO/mL/11 mg BEO/mL) was added to a filmogenic dispersion. Fluorescent probes were used to study the antimicrobial filmogenic active dispersion (FD) effect. Pork meat pieces were coated without microencapsulated EOs (CC), using FD (AC), or uncoated (C), vacuum packed and stored 28 days at 4 °C. Thymol (28.9%) and linalool (23.7%) were the major components of MOEO and BEO, respectively. The cell membrane of all bacteria was damaged by contact with FD. FD-coated samples (AC) exhibited the lowest concentration of 2-thiobarbituric acid reacting substances (TBARS) (0.027 ± 0.001 mg malonaldehyde/kg meat) and natural microbiota growth, while odor and color were the most accepted by untrained judges (range > 6). Coatings added with microencapsulated EOs mixture are a natural food preservation alternative to increase the shelf life of refrigerated meat products.

Keywords: Mexican oregano; basil; active edible coating; microencapsulated essential oil; pork meat; corn starch; physicochemical properties; microbiological properties

1. Introduction

Fresh meat is a good source of nutrients and is also a complex matrix where food-borne pathogens and spoilage microorganisms can easily grow [1–3]. To extend the shelf life and ensure the safety of these products refrigerated storage under vacuum or modified atmosphere packaging are used. However, these conditions allow survival and growth of pathogens and spoilage strains, as well as the

development of oxidation reactions [1,2,4]. To decrease this problem, bioactive edible film-forming suspensions (FD) can be applied on fresh meat as a coating [5] containing EOs obtained from plants such as basil and oregano [2,4,6–8]. EOs are low molecular weight volatile mixtures that are biosynthesized in various organs of plants, the biological activity is determined by their chemical composition that depends on several factors like extraction method [9], climatic and geographical conditions, [7] and genetic diversity [10].

The antimicrobial effect of Mexican oregano essential oil (MOEO) and basil essential oil (BEO), and their components applied individually or in combination has been evaluated against food-borne pathogens (*E. coli*, *Salmonella enterica* ser. Typhimurium, *Staphylococcus aureus*), and spoilage bacteria (*B. thermosphacta*, *Lactobacillus*, *Pseudomonas*), and they showed activity against many microorganisms, mainly causing damage to their cell membrane [6,11–14]. Moreover, EOs may also control lipid oxidation by several mechanisms such as free radical scavenging and reducing Fe^{3+} activities [2,13,15–18]. In some cases, EO combinations increase the number of active compounds and diversity, and extend the mechanisms of action leading to the application of low EOs concentrations to achieve control of microbial growth and lipids oxidation. EOs can also contribute to reduce undesirable impact on food sensory properties [19,20]. BEO combined with EOs from European oregano [6,19] and bergamot [6], has shown effect against some pathogens and spoilage bacteria. However, no reports were found about the combination of MOEO and BEO.

EOs can be used as natural antimicrobials and/or antioxidants in its free or encapsulated form. Microencapsulated EOs show less odor and higher aqueous solubility than free oils [4], which facilitates their incorporation into FD. The microencapsulated EOs powder can be added to the natural polymers comprising the FD with the addition of plasticizers [21]. Free MOEO has been added to FD based on amaranth [22,23], chitosan, starch [22–24] or pectin matrix [25]. On the other hand, chitosan [26] or carboxymethyl cellulose [27] has been used as polymer matrix in FD added with free BEO. Regarding the use of FD-microencapsulated EO in foods, Moreira et al. [28] extended the shelf life and improved microbiological and physicochemical quality of melon cubes coated with a chitosan-pectin-encapsulated *trans*-cinnamaldehyde solution. However, there are no studies related to the application and influence of FD added with microencapsulated EOs on fresh pork meat properties.

The aim of this work was to determine chemical composition of Mexican oregano (*Lippia graveolens* Kunth) (MOEO) and basil (*Ocimum basilicum* L.) (BEO) essential oils, and their combined antimicrobial effect as part of an FD-microencapsulated EOs against *Escherichia coli (E. coli)* O157:H7, *Lactobacillus plantarum (L. plantarum)*, *Brochothrix thermosphacta (B. thermosphacta)*, and *Pseudomonas fragi* (*Ps. fragi*). This work also studied the active coating effect on microbiological and physicochemical properties of fresh pork meat.

2. Materials and Methods

2.1. Materials

Modified (Hi-Cap 100) and waxy corn starches were supplied by CP Ingredients (Querétaro, México); casein peptone and yeast extract were purchased from Bioxon (Estado de México, Mexico); sorbitol, Tween-80, glycerol, streptomycin sulphate, cycloheximide, thallium (I) acetate and 2-thiobarbituric acid (TBA) were obtained from Sigma-Aldrich (St. Louis, MO, USA); all other reagents were purchased from J. T. Baker (Estado de México, Mexico).

2.2. Methods

2.2.1. Plant Material and Extraction of EOs

Mexican oregano was collected and sun-dried in Peñamiller (Querétaro, Mexico). Basil plants were harvested from Huehuetlán El Chico (Puebla, Mexico), and oven dried at 35 °C during 24 h. A

sample from each specimen was authenticated and deposited in the Ethno-botanical Collection of the Querétaro's (Mexico) herbarium "Dr. Jerzy Rzedowski".

Mexican oregano and basil essential oils (EOs) were separately isolated by steam distillation in a stainless steel equipment according to Reyes-Jurado et al. [29]. Each dry material sample (200 g) was placed on a perforated grid above boiling water, and distilled for 2 h. The oils obtained were dried with anhydrous sodium sulfate and stored at 4 °C.

2.2.2. GC/MS Analysis of Plant EOs

The identification of EOs chemical compounds was conducted following Hernández-Hernández et al. [4], based on comparison of their mass spectra with those listed in the NIST 2010 library, and verified by their Kovats retention indices (KIs) according to Jemma et al. [30]. Quantification was expressed as % peak area of each component relative to total chromatographic area. The analyses were performed using a gas chromatograph model 7890A (Agilent Technologies, Santa Clara, CA, USA) equipped with an MPS2XL autosampler (Gerstel GmbH, Mülheim an der Ruhr, Germany) and coupled to a mass spectrometer detector model 5975 (Agilent Technologies). Two µL of methanol diluted EO (1:10, *v*/*v*) was injected in a split mode (ratio 1:20). The column was DB-5ms (60 m × 0.25 mm i.d. × 0.25 µm film thickness, Agilent Technologies), operated from 40 °C (5 min hold) to 140 °C at a rate of 3 °C/min (20 min hold), then increased to 220 °C at 3 °C/min (5 min hold), and finally raised to 280 °C, at 10 °C/min (5 min hold). Mass spectra were recorded under electron impact at 70 eV, spectral range of 33–600 m/z. The carrier gas was ultrapure He (Infra, Queretaro, Mexico) at a flow rate of 1.0 mL/min.

2.2.3. Bacteria

Bacteria used were from the culture collection of the Food Biotechnology Laboratory of DIPA, Facultad de Química, Universidad Autónoma de Querétaro, Mexico: *L. plantarum*, *B. thermosphacta* and *Ps. fragi*. *E. coli* O157:H7 was a gift from Dr. H. Minor from Tecnológico de Estudios Superiores de Ecatepec, Ecatepec, Mexico. All strains were kept in sterile skim milk and glycerol mixture at −70 °C and activated in nutrient broth (Bioxon, Estado de México, Mexico) at 30 °C, except *E. coli* which was activated at 37 °C.

2.2.4. Minimum Inhibitory Concentration (MIC) of EOs on E. coli O157:H7

Each EO was diluted with 10% (*v*/*v*) Tween 80 to produce concentrations in the range 3–60 mg/mL for BEO, whereas for MOEO ranged 0.4–20 mg/mL. Each concentration was tested in nutrient broth inoculated with 10^5 CFU/ml of *E. coli* O157:H7. The suspension was incubated for 6 h at 37 °C, and then aliquots of 20 µL in triplicate, were incubated in nutrient agar (Bioxon) following the spot method [31], and incubated for 24 h at 37 °C. Minimum inhibitory concentration (MIC) was defined as the lowest EO concentration (mg/mL) that completely inhibited *E. coli* 0157:H7 [8].

2.2.5. Combined Effect of EOs Mixture on E. coli O157:H7

To study the combined antimicrobial effect of MOEO and BEO a checkerboard type arrangement was used [20]. A total of 30 EO combinations were used: 0, 0.4, 2, 5, 10, 15, and 20 mg MOEO/mL and 0, 3, 15, 30, 45, and 60 mg BEO/mL, which included the MIC of each EO. Each test tube contained nutrient broth plus 30 µL of 10^5 CFU/mL of *E. coli* O157:H7, and the appropriate volume of each EO diluted with Tween 80 to reach the tested concentration in a total volume of 3 mL, and then incubated at 37 °C for 24 h. Aliquots of these cultures were spread plated onto nutrient agar followed by incubation as previously mentioned. All tests were carried out in triplicate.

The fractional inhibitory concentration index (FIC_{index}) were calculated as $FIC_{MO} + FIC_B$, where FIC_{MO} = (MIC_{MO} combination/MIC_{MO} alone) and FIC_B = (MIC_B combination/MIC_B alone). The FIC_{MO} and FIC_B values represent the lowest concentrations of MOEO and BEO, respectively, that caused complete inhibition of bacterial growth in the combination tested. The results were interpreted as

synergistic ($FIC_{index} < 1$), additive ($FIC_{index} = 1$) or antagonic ($FIC_{index} > 1$) interactions. Experiments were carried out in triplicate.

2.2.6. Microencapsulation of EOs Mixture

The EO mixture (BEO plus MEO) showing the best combined antimicrobial effect was chosen for nanoencapsulation, according to Hernández-Hernández et al. [2]. A 28.6% (*w*/*w*) suspension of Hi-Cap 100 modified corn starch was utilized as wall material. Then, the modified starch suspension (84%, *w*/*w*) was mixed with the EOs mixture (16% *w*/*w*), homogenized at 13,500 rpm during 3 min in an Ultraturrax (IKA T25, Staufen, Germany), and sonicated for 2.5 min at 20 kHz and 75% amplitude (VCX 500 Vibra-Cell, Newtown, CT, USA), to obtain nanometric particles size (<100 nm). The nanoemulsion was spray dried (mod. B-191, Büchi, Flawil, Switzerland) to produce water soluble microcapsules of 3–8 µm in diameter.

2.2.7. Active Coating Preparation

An aqueous 4% (*w*/*v*) waxy corn starch dispersion containing 3.2% (*w*/*v*) sorbitol was heated at 95 °C during 20 min under stirring, followed by cooling to room temperature using an ice bath. This starch dispersion (20% *v*/*v*), was added with 5% (*v*/*v*) Tween 80 (35% *v*/*v*), and 45% *v*/*v* deionized water. After homogenization, the microencapsulated EOs mixture (5 g/100mL) was slowly incorporated to give a filmogenic active dispersion (FD) containing a final EOs concentration of 2% (*w*/*v*). The suspension was degasified and stored in refrigeration until used.

2.2.8. Cells Population Viability

The viability of cells population was conducted according to Passerini et al. [32], with modifications. The strains *E. coli* O157:H7, *L. plantarum*, *B. thermosphacta*, and *Ps. fragi* were activated twice in nutrient broth and incubated for 24 h, except for *L. plantarum*, which was incubated during 48 h. Incubation temperature was 37 °C for *E. coli* O157:H7, and 30 °C for all other bacteria. After incubation, bacterial suspensions were centrifuged at 10,000× *g* during 20 min at 4 °C, and the pellets were washed twice with saline solution (0.85% *w*/*v*), and then re-suspended in this solution, followed by plating in nutrient agar, using the spot method [31]. Cells population was adjusted to obtain a final concentration of 10^7 CFU/mL.

To verify the effect of FD on the aforementioned bacteria, one mL of this suspension was contacted with 1 mL of each washed cells suspension, vortexed for 30 s, and filtered through a 0.45 µm pore size polycarbonate membrane (Nucleopore, Whatman, Cambridge, UK), which was placed on a slide and then stained using the Live/Dead back light bacterial viability kit (Invitrogen, Waltham, MA, USA), using intact cells as control. One hundred µL of the staining solution (1.5 µL of 3.34 mM SYTO 9 plus 1.5 µL of 20 mM propidium iodide, in 1 mL of deionized water) was added to the sample, and was incubated in the dark at room temperature for 1 h. A fluorescence microscope fitted with the filter FICT (Axioskop 40, Carl Zeiss, Oberkochen, Germany), and the image software ZEN 2012 (blue edition, version 1.1.2.0) was used to check cells viability. When exposed to excitation wavelength of 480 nm/500 nm emission fluorescent green color is attributed to intact cell membranes, while fluorescent red color is associated to damaged cell membranes [32].

2.2.9. Active Coating Application on Meat

Meat samples were prepared from the *Longissimus thoracis* muscle of 10 week-old swines, 12 h post-mortem, purchased from a local abattoir. Meat samples were cut into square pieces of 3×3 cm^2 and 1 cm thick. Samples were placed inside a plastic bag, mixed and randomly assigned to 3 coating levels: (1) control without coating (C); (2) coating without microencapsulated mixture of EOs (CC); and (3) coating with microencapsulated mixture of EOs (AC). The coating suspension (0.06 mL/cm^2) was spread on the samples using a glass rod, which is equivalent to 1.2 mg EOs mixture/cm^2. All samples

were vacuum packed (absolute pressure = 50.7 kPa, Food Saver V3800, Antwerp, NY, USA) and stored at 4 °C. Sampling was carried out at 0, 7, 14, 21 and 28 days.

2.2.10. Effect of Coatings on Fresh Meat Properties

Antimicrobial Properties

The microbiota present in the samples was recovered using sterile cotton swabs on both sides of the meat surface moisturized with 10 mL of 0.85% (*w*/*v*) sterile saline solution. The swab was introduced in a saline solution followed by decimal dilutions. Dilutions in triplicate were plated using the following media: Plate count agar (Bioxon) for aerobic mesophilic microorganisms. STAA agar (% *w*/*v*) (casein peptone 2, glycerol 1.5, yeast extract 0.2, dibasic potassium phosphate 0.1, magnesium sulfate 0.1, bacteriological agar 1.5), supplemented with (% *w*/*v*) streptomycin sulfate 0.005, cycloheximide 0.0005, and thallium sulfate 0.0005, for *B. thermosphacta*. De Man, Rogosa, Sharpe (MRS) (BD, Franklin Lakes, NJ, USA) for lactic acid bacteria. Nutrient agar with 2% (*w*/*v*) sucrose for *Pseudomonas*. McConkey-sorbitol agar (Gibco, Ciudad de México, México) for *E. coli* O157:H7. Microbial populations were quantified by spreading 100 µL onto the agar surface, except mesophilic aerobes that were pour-plated (1 mL) [2]. Temperatures and incubation times are described in Section 2.2.8.

Physicochemical Properties

Lipids Oxidation

Meat contains unsaturated fatty acids that can be oxidized producing free radicals that participate in the propagation and termination reactions of autoxidation [16,33]. The peroxides formed decompose to a wide variety of chemicals such as ketones, alcohols, aldehydes, among others such as malondialdehyde (MDA) [15,16]. MDA can react with two molecules of TBA producing a rose pigment with maximum absorption wavelength of 532 nm.

Lipids oxidation determination was carried out according to the American Meat Science Association [34]. The meat (0.50 ± 0.01 g) was homogenized with 2.5 mL of TBA solution (0.375% *w*/*v* TBA, 15% *w*/*v* trichloroacetic acid and 0.25 N HCl), transferred to a boiling water bath for 10 min, and cooled in an ice bath. Then, the sample was centrifuged at 5000× *g* for 10 min at 4 °C, and the supernatant absorbance was measured at 532 nm (A_{532}) in a spectrophotometer (Thermo Scientific, Waltham, MA, USA). The control comprised 0.5 g distilled water instead of the meat sample, and the concentration of TBARS was calculated by Equation (1):

$$\text{TBARS (mg malonaldehyde/kg sample)} = A_{532} \times 2.77 \quad (1)$$

pH Determination

pH was determined in 10 ± 0.2 g of meat previously homogenized with ninety mL distilled water, using a potentiometer (Horiba, Kyoto, Japan).

2.2.11. Sensory Evaluation

Sensory evaluation was conducted following Hernández-Hernández et al. [2], with modifications. Meat samples from the three different treatments (C, AC, and CA) were analyzed by 50 untrained judges comprising equal number of men and women aged between 20 and 58 years. A descriptive hedonic scale of 9 points was used to evaluate color and odor [34], where 1= extremely undesirable; 2 very undesirable; 3 moderately undesirable; 4 slightly undesirable; 5 neither acceptable nor unacceptable; 6 slightly desirable; 7 moderately desirable; 8 very desirable and, 9 extremely desirable. An average score < 6 for any sensory attribute indicated unacceptable meat sample.

2.2.12. Statistical Analysis

To avoid the effect of muscle on independent variables, random blocks statistical design was used, including two factors: coating and storage time. Coating was tested at three levels: control without coating, coating without EO (CC), and active coating (AC); while five levels of storage time were investigated: 0, 7, 14, 21, and 28 days, making up a total of 15 treatments with three independent samples for each one. Therefore, 45 experimental units were used in this study. The independent variables were those described in Sections 2.2.10 and 2.2.11. A two way analysis of variance was conducted on the experimental results, and multiple comparison of the means was carried out by using Tukey's test with significance level $p < 0.05$, employing the SPSS Statistical Software, v. 22.0 (Chicago, IL, USA). All experiments were performed in triplicate, and mean values are reported ± standard error.

3. Results and Discussion

3.1. GC/MS Analysis of Plant EOs

The extraction yield for MOEO was 1.42% ± 0.17% (*w*/*v*) using water-steam distillation process (WSD). This result is lower than those previously reported using *Lippia palmeri* S. Wats (5%–6%) [7] and *Lippia graveolens* Kunth (4.29% *w*/*v*) [4], using WSD and hydro distillation (HD) respectively. For BEO, the yield was 0.33% ± 0.02% (*w*/*v*), which is higher than that obtained from Neapolitan Basil (0.11%-HD) [35]. Nevertheless, higher BEO yields were reported by Nurzyńska-Wierdak [36] (0.5%–1.5%-WSD), Beatović et al. [37] (0.65%–1.90%-HD) and Cheliku et al. [35] (0.47%–3.4%-HD).

The main components in MOEO were thymol (28.88%), eucalyptol (16.07%), *p*-cymene (6.61%), terpinen-4-ol (3.9%), and linalool (3.64%) (Table 1). Additionally, the major constituents of BEO included linalool (23.74%), eucalyptol (8.12%), estragole (3.77%) and α-bergamotene (3.39%). A previous study on MOEO extracted by HD from Toliman (Querétaro, México) showed higher amount of thymol (66.3%), followed by small amounts of γ-terpinene, α-pinene and β-thujene. Several factors may have influenced these variations including microenvironment, season, and extraction method [9]. Peñamiller is a town located at the GPS coordinates: longitude −99.81500, latitude 21.051944, and 1320 m above sea level, whereas Toliman GPS coordinates are: longitude −99.928889 latitude 20.908611, and at 1569 m above sea level, 15 km away from Peñamiller [38]. Ortega-Nieblas et al. [7] reported variations in yield and composition of EO extracted from *Lippia palmeri* S. Wats leaves coming from two different zones in Sonora, Mexico. Furthermore, Martínez-Natarén et al. [10] found genetic diversity and composition variability in EO extracted from *Lippia graveolens* harvested in Yucatán, Mexico. In relation to BEO, linalool has been reported as the major component [8,35,37,39,40].

Table 1. Mexican oregano (MOEO) and basil (BEO) essential oil composition (% area).

No.	Compound	MOEO	BEO	KI[1]	KI[2]	Ref.
1	Bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro deriv.	0.06	0.03	929	929	[4]
2	α-Pinene	0.6	0.03	935	937	[41]
3	Camphene	0.13	0.04	951	951	[41]
4	3,7,7-Trimethyl-1,3,5-cycloheptatriene	0.01	–	973	972.6	[41]
5	β-Thujene	–	0.31	975	978	[41]
6	β-Pinene	–	0.52	980	980	[41]
7	1-Octen-3-ol	0.06	–	989	986	[41]
8	β-Myrcene	1.06	–	993	991	[41]
9	α-Phellandrene	0.66	0.03	1007	1005	[41]
10	3-Carene	0.62	–	1013	1012	[4]

Table 1. *Cont.*

No.	Compound	MOEO	BEO	KI[1]	KI[2]	Ref.
11	α-Terpinene	–	0.04	1019	1019	[41]
12	2-Carene	0.38	–	1020	1018	[41]
13	*p*-Cymene	6.61	0.34	1030	1030	[41]
14	D-Limonene	–	0.41	1032	1033	[41]
15	*o*-Cymene	2.05	–	1034	1031	[4]
16	Eucalyptol	16.07	8.12	1036	1035	[41]
17	*trans*-β-Ocimene	–	0.04	1040	1039	[41]
18	β-Ocimene	–	0.55	1050	1050	[41]
19	Ocimene	0.02	–	1051	1050	[41]
20	γ-Terpinene	–	0.17	1062	1062	[41]
21	Linalool oxide	0.03	–	1077	1078	[41]
22	α-Terpinolene	0.3	0.31	1092	1090	[41]
23	*p*-Cymenene	0.12	–	1093	1090	[41]
24	Linalool	3.64	23.74	1108	1106	[41]
25	1-Octen-3-yl-acetate	–	0.08	1114	1110	[41]
26	Fenchol	0.05	–	1122	1121	[41]
27	*trans*-2-Menthen-1-ol	0.26	–	1129	1124	[41]
28	4-Acetil-1-methylcyclohexene	0.01	–	1136	1135	[41]
29	Pinocarveol	0.48	–	1148	1142	[41]
30	Camphor	–	0.23	1152	1150	[41]
31	Borneol	1.1	–	1177	1177	[41]
32	Terpinen-4-ol	3.9	1.46	1188	1193	[41]
33	Octyl ester acetic acid	–	0.13	1194	1196	[41]
34	*p*-Cymene-8-ol	0.24	–	1196	1193	[41]
35	Estragole	–	3.77	1198	1196	[41]
36	α-Terpineol	2.62	–	1201	1200	[41]
37	*cis*-Piperitol	0.16	–	1212	1211	[41]
38	*cis*-Carveol	0.14	–	1217	1215	[41]
39	Thymol ether	0.96	–	1218	1217	[41]
40	D-Carvone	0.09	–	1230	1231	[41]
41	*p*-Menth-1(7)-en-2-one	0.08	–	1234	1231	[41]
42	Piperitone	0.13	–	1241	1250	[41]
43	Bornyl acetate	–	1.48	1285	1285	[41]
44	Thymol	28.88	–	1308	1306	[41]
45	Eugenol	–	1.13	1361	1358	[41]
46	β-Elemene	–	1.61	1400	1401	[4]
47	Methyleugenol	0.08	–	1403	1403	[41]
48	Isocaryophyllene	–	0.11	1411	1411	[41]
49	Caryophyllene	2.01	–	1412	1415	[41]
50	β-Cubebene	0.06	0.1	1414	1419	[41]
51	α-Bergamotene	0.29	3.39	1416	1414	[41]
52	α-Guaiene	–	0.49	1417	1413	[41]
53	β-*cis*-Farnesene	0.04	0.1	1423	1428	[41]
54	Humulene	1.3	0.37	1425	1432	[41]
55	Germacrene-D	–	2.09	1437	1442	[41]
56	β-Selinene	0.35	–	1440	1436	[41]
57	Aromandendrene	–	0.06	1441	1439	[41]
58	Amorphene	–	1.66	1454	1452	[41]
59	Guaiol	0.2	–	1602	1602	[41]
60	Humulene epoxide II	0.56	–	1610	1609	[41]
61	Cubenol	–	0.34	1613	1623	[41]
62	γ-Eudesmol	0.1	–	1624	1629	[41]
63	Methyl jasmonate	–	0.1	1632	1629	[41]
64	β-Eudesmol	0.17	0.07	1635	1638	[41]
65	α-Cadinol	0.28	0.09	1637	1635	[41]
66	Hexahydrofarnesylacetone	0.02	–	1826	1828	[41]

KI[1]: Calculated Kovats index; KI[2]: Kovats index reported in literature.

3.2. Minimum Inhibitory Concentration (MIC) of EOs Against Escherichia coli O157:H7

The MOEO concentration (2 mg/mL) that decreased *E. coli* initial population (5 Log CFU/mL) to 2.9 Log CFU/mL after 6 h of incubation, did not maintain its inhibitory effect after 24 h since population

increased to 3.8 Log CFU/mL (Figure 1a). This effect was attributed to bacterial adaptation to a sublethal concentration of MOEO associated to an increase in the saturated fatty acids of the membrane, causing non-specific reduction in cell permeability [12,42,43]. Thus, the MOEO concentration exerting total inhibition after 12 h of incubation was 2 mg/mL, whereas higher concentrations (5, 10, 15 and 20 mg/mL) totally inhibited *E. coli* after 6 h incubation time.

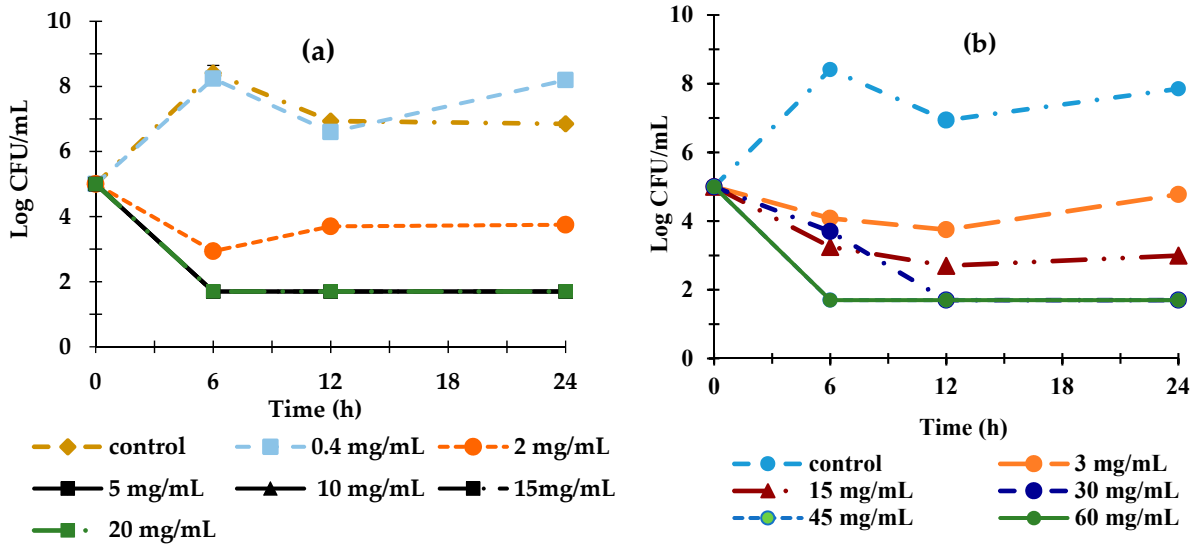

Figure 1. Minimum inhibitory concentration (mg/mL) of MOEO (**a**) and BEO (**b**) against *Escherichia coli* O157:H7.

Using 3 mg/mL BEO the bacterial growth was similar to that observed when 0.4 mg/mL MOEO was used (Figure 1b). Total bacterial inhibition afer 6 h of incubation was reached at 45 and 60 mg/mL of BEO. Therefore, MIC values for *E. coli* O157:H7 were 5 mg MOEO/mL, and 45 mg BEO/mL. It has been reported lower MIC value for BEO (12.5 mg/mL), but obtained by HD [8].

3.3. Combined Effect of EOs Mixture Against E. coli O157:H7

The combination of EOs exhibiting bactericidal effect is shown in Table 2. The mixture of 4 mg MOEO/mL plus 11 mg BEO/mL showed additive effect ($FIC_{index} \approx 1$), indicating that the antimicrobial effect observed was equivalent to the sum of each individual EO acting independently.

Table 2. Combined effect of essential oils against *Escherichia coli* O157:H7.

MOEO [1] (mg/mL)	BEO [2] (mg/mL)	MOEO FIC [3]	BEO FIC	FIC_{Index}
3	23	0.6	0.51	1.11
3	34	0.6	0.76	1.36
3	45	0.6	1	1.6
4	11	0.8	0.24	1.04
4	23	0.8	0.51	1.31
4	34	0.8	0.76	1.56
4	45	0.8	1	1.8
5	11	1	0.24	1.24
5	23	1	0.51	1.51
5	34	1	0.76	1.76
5	45	1	1	2

[1] Mexican oregano essential oil; [2] Basil essential oil; [3] Fractional inhibitory concentration.

The rest of the combinations were antagonic ($FIC_{index} > 1$), suggesting a reduction of the sum of each individual EO effect acting independently [13]. No reports could be found on the combined antimicrobial effect of the EOs in this study against *E. coli*. However, EO from European oregano (*Origanum vulgare*) combined with BEO showed an additive antimicrobial effect [6,19].

Additionally, mixtures of pure compounds present in MOEO and/or BEO like α-terpineol/linalool, eucalyptol/linalool, and α-terpineol/eucalyptol (Table 1), have shown additive effect against *E. coli* O157:H7 [13]. Therefore, the mixture of 4 mg MOEO plus 11 mg BEO per mL was chosen for microencapsulation for the rest of the experiments in this study.

3.4. Determination of Cells Population Viability

The effect of FD on the viability of individual cells was studied using spoilage (*L. plantarum*, *Ps. fragi* and *B. thermosphacta*) and pathogenic (*E. coli* O157:H7) food-related bacteria. A control culture using *Ps. fragi* was tested for cells viability (Figure 2a). After 1 h of CC filmogenic suspension treatment all studied bacteria showed green color, similar to that of the untreated population, and is shown for *B. thermosfacta* (Figure 2b). In contrast, when each bacterial population was contacted with FD, a red color indicated viability loss, which is shown for *B. thermosfacta* (Figure 2c), *L. plantarum* (Figure 2d), and *E. coli* O157:H7 (Figure 2e). However, *Ps. fragi* population was partly damaged because red and green cells were observed (Figure 2f), indicating that it was the most resistant to the EOs mixture. These results confirm that the mixture of 4 mg MOEO/mL plus 11 mg BEO/mL is lethal for most microorganisms tested, which is attributed to the presence of thymol and linalool, that can produce membrane and cell wall damage, causing leakage of macromolecules and cell lysis [29,44].

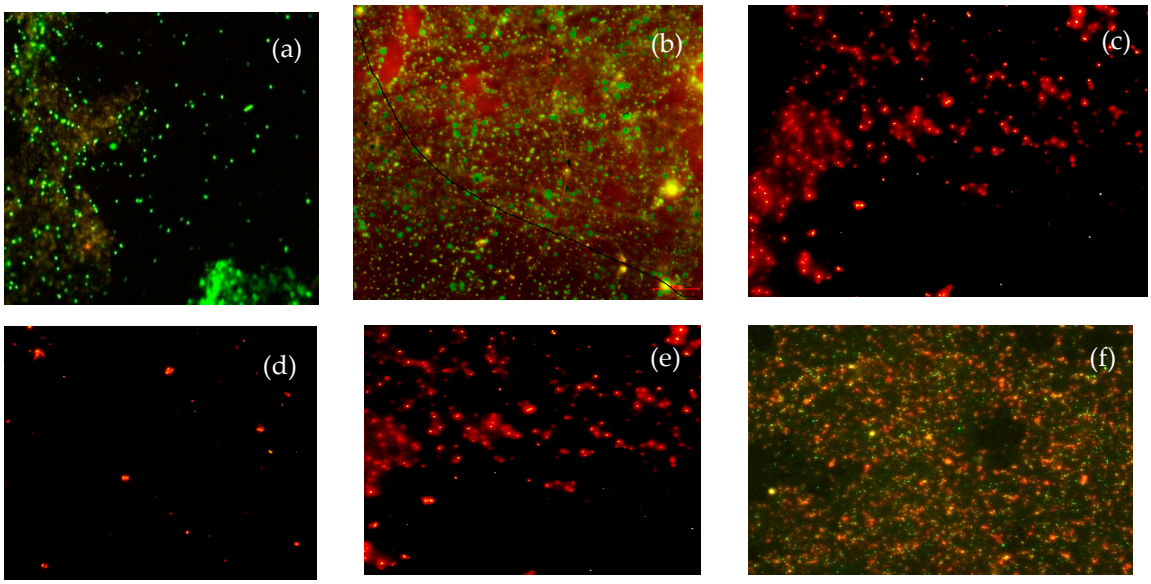

Figure 2. Viability of pathogenic and spoilage microorganisms contacted with coating without microencapsulated mixture of EOs (CC), or coating with microencapsulated mixture of EOs (AC). (**a**) control of intact viable cells (*Ps. fragi*); (**b**) *B. thermosfacta* contacted with CC. Bacteria after contact with AC: (**c**) *B. thermosfacta*; (**d**) *L. plantarum*; (**e**) *E. coli* O157:H7; (**f**) *Ps. fragi*.

When BEO was contacted with *E. coli* the external membrane was broken leading to increased permeability [13]. Similar results were found by combining BEO with European oregano EO (*Origanum vulgare*), or bergamot EO (*Citrus bergamia*) [6]. Eugenol, another compound present in MOEO (Table 1) has been involved in cell membrane disruption of *E. coli* O157:H7, *B. thermosphacta* [12] and *Lactobacillus sakei* [45]. In addition, *p*-cymene acted against *E. coli* cell wall [11], whereas limonene altered the membrane structure of *Pseudomonas* spp [12]. On the other hand, thymol can reduce the elasticity and

produce morphological changes to dipalmitoyl-phosphatidylcholine, one component of microbial cell membranes [14].

Active compounds in the mixture, such as eucalyptol/linalool produced pore formation in *E. coli* O157:H7 external membrane, and to other Gram-positive and Gram-negative bacteria resulting in efflux of small molecules and ions leading to cell collapse [13].

3.5. Effect of Coatings on Fresh Meat Properties

3.5.1. Antimicrobial Properties

The coating applied on meat samples was allowed to get dry for 2 h in a laminar flow cabinet, and then sampling of the native microbiota was conducted. Serial dilutions in the appropriate media were used to quantify the different mesophilic bacteria tested in this study, which showed similar population ($p < 0.05$), regardless of the treatment. This suggests that the meat matrix compounds protected the native microbiota, delaying the EOs antimicrobial activity [46,47].

Storage time affected mesophilic microorganisms growth ($p < 0.05$); bacterial population of control and CC samples increased from 5.28 ± 0.02 Log CFU/cm^2 at the beginning (Table 3) to >8 Log CFU/cm^2 at day 28. Conversely, the population after AC was kept close to the initial value (5.28 ± 0.02 Log CFU/cm^2), with a reduction of 2.99 Log CFU/cm^2 at the end of the experiment, which prevented meat deterioration considering that 7 log CFU/cm^2 of mesophilic microorganisms is a decay sign [48].

Table 3. Effect of active coating containing microencapsulated MOEO and BEO on total population, *B. thermosphacta*, LAB, *Pseudomonas*, and *E. coli* O157:H7 growth (Log CFU/cm^2) of fresh pork meat stored at 4 °C.

Microorganism	Time (days)	Treatment		
		Control	CC	AC
Mesophilic	0	5.28^{cA} ± 0.02	5.28^{eA} ± 0.02	5.28^{aA} ± 0.02
	7	5.50^{cA} ± 0.09	5.81^{dA} ± 0.03	4.78^{aB} ± 0.15
	14	7.29^{bB} ± 0.01	7.63^{cA} ± 0.03	5.25^{aC} ± 0.08
	21	8.58^{aA} ± 0.04	8.00^{bB} ± 0.03	2.92^{bC} ±0.05
	28	8.42^{aA} ± 0.03	8.65^{aA} ± 0.02	2.29^{cB} ± 0.06
B. thermosphacta	0	3.75^{eA} ± 0.01	3.75^{eA} ± 0.01	3.75^{aA} ± 0.01
	7	4.24^{dB} ± 0.01	4.73^{dA} ± 0.04	3.35^{bC} ± 0.08
	14	5.64^{cA} ± 0.02	5.21^{cB} ± 0.02	3.32^{bC} ± 0.13
	21	6.12^{bA} ± 0.03	6.14^{bA} ± 0.01	1.73^{cB} ± 0.04
	28	6.51^{aA} ± 0.03	6.32^{aB} ± 0.01	1.69^{cC} ± 0.03
LAB	0	2.33^{eA} ± 0.07	2.33^{cA} ± 0.07	2.33^{bA} ± 0.07
	7	2.62^{dA} ± 0.02	2.60^{cA} ± 0.02	2.62^{aA} ± 0.02
	14	3.12^{cB} ± 0.09	3.45^{bA} ± 0.10	1.69^{dC} ± 0.02
	21	4.23^{bA} ± 0.07	3.55^{bB} ± 0.07	1.92^{cC} ± 0.08
	28	5.11^{aA} ± 0.01	4.45^{aB} ± 0.04	1.87^{cC} ± 0.08
Pseudomonas	0	3.26^{dA} ± 0.16	3.26^{cA} ± 0.16	3.26^{eA} ± 0.16
	7	4.84^{cA} ± 0.10	4.37^{bA} ± 0.28	3.71^{dB} ± 0.05
	14	6.12^{bA} ± 0.09	5.47^{aB} ± 0.08	5.37^{aB} ± 0.08
	21	5.00^{cB} ± 0.08	5.58^{aA} ± 0.06	4.15^{cC} ± 0.09
	28	6.68^{aA} ± 0.04	6.12^{aB} ± 0.09	4.64^{bC} ± 0.03
E. coli 0157:H7	0	4.03^{dA} ± 0.05	4.03^{dA} ± 0.05	4.03^{aA} ± 0.05
	7	4.95^{cA} ± 0.03	4.59^{cB} ± 0.08	3.84^{abC} ± 0.13
	14	4.85^{cA} ± 0.06	5.00^{cA} ± 0.05	3.29^{cB} ± 0.03
	21	6.37^{bA} ± 0.01	5.63^{bB} ± 0.06	3.63^{bC} ± 0.06
	28	7.67^{aA} ± 0.02	6.58^{aB} ± 0.02	1.97^{dC} ± 0.02

CC= coating without microencapsulated mixture of EOs; AC= coating with microencapsulated mixture of EOs (AC). Within each column and for each microorganism, mean values having the same lowercase letter are not significantly different ($p < 0.05$). Means in the same row having the same capital letter are not significantly different ($p < 0.05$).

B. thermosphacta population of control and CC samples increased throughout storage, from 3.75 ± 0.01 Log CFU/cm^2 to more than 6.0 Log CFU/cm^2 (Table 3), whereas AC reduced bacterial count from 3.75 ± 0.01 Log CFU/cm^2 (day 0) to 1.69 ± 0.03 Log CFU/cm^2 (day 28), due to EOs antibacterial effect.

LAB population was significantly lower ($p < 0.05$) on days 14, 21 and 28 for samples coated with AC (Log CFU/cm^2: 1.69 ± 0.02, 1.92 ± 0.08, and 1.87 ± 0.08, respectively, Table 3), which indicates the high sensitivity of these bacteria to the EOs' active compounds. At day 28 the population of control sample was highest among the three treatments (5.11 ± 0.01 Log CFU/cm^2). It is known that LAB are involved in meat spoilage, and may cause off odor and color loss in vacuum sealed meat [49].

Regarding *Pseudomonas* group, CC and AC samples presented similar population ($p < 0.05$) on day 14 (Log CFU/cm^2: 5.47 ± 0.08 and 5.37 ± 0.08, respectively), which were lower than those of the control (6.12 ± 0.09 Log CFU/cm^2) (Table 3). AC meat samples showed the lowest population compared to those of the control and CC at 21 and 28 days of storage. However, in contrast to all other microorganisms, final population increased 1.38 Log CFU/cm^2 compared to initial values, indicating that *Pseudomonas* spp. was the most resistant to the MOEO/BEO effect. This result is in agreement with the bacterial viability outcome (Figure 2f). Semeniuc et al. [50] identified high resistance of *Ps. aeruginosa* to BEO, whereas Gutiérrez et al. [46] found similar effect when using European oregano, thyme and marjoram EOs against *Ps. fluorescens* and *Ps. putida*.

The EO combination was found highly effective against the pathogen *E. coli* O157:H7 [3], as 2.06 Log CFU/cm^2 reduction was achieved after 28 days, indicating its sensitivity to the EO mixture. On the other hand, this pathogen population increased in the control and CC samples at least 2.5 Log CFU/cm^2 after 28 days (Table 3).

Except for *Pseudomonas*, all other bacteria studied showed high sensitivity to the microencapsulated EO mixture incorporated in the coating (AC). This coating showed pH < 5.4 (Table 4), which could have enhanced its antimicrobial activity. It has been shown that low pH (about 5.32) increased European oregano and thyme EOs hydrophobicity facilitating their solubilization in the cell membrane, and thus enhancing their antimicrobial effect [46]. It should be noted that most microorganisms studied here revealed extensive damage to their cell wall after being contacted with the microencapsulated MOEO/BEO within the coating.

Table 4. Effect of active coating containing microencapsulated MOEO and BEO on TBARS (mg malonaldehyde/kg meat), and pH of fresh pork meat stored at 4 °C.

Physicochemical Properties	Time (days)	Treatment		
		Control	CC	AC
TBARS	0	0.04^{bA} ± 0.02	0.04^{aA} ± 0.02	0.04^{aA} ± 0.02
	7	0.08^{bA} ± 0.004	0.04^{aB} ± 0.00	0.04^{aB} ± 0.00
	14	0.08^{bA} ± 0.00	0.03^{aB} ± 0.00	0.01^{aC} ± 0.00
	21	0.09^{bA} ± 0.00	0.04^{aB} ± 0.00	0.02^{aC} ± 0.00
	28	0.28^{aA} ± 0.01	0.08^{aB} ± 0.00	0.01^{aC} ± 0.00
pH	0	5.23^{bA} ± 0.09	5.27^{bcA} ± 0.08	5.25^{abA} ± 0.06
	7	5.45^{bA} ± 0.12	5.40^{abA} ± 0.15	5.06^{bcA} ± 0.04
	14	4.80^{cB} ± 0.02	4.91^{cA} ± 0.01	4.74^{dB} ± 0.02
	21	6.00^{aA} ± 0.15	5.74^{aAB} ± 0.10	5.35^{aB} ± 0.06
	28	5.14^{bcA} ± 0.06	5.05^{bcA} ± 0.06	4.93^{cdA} ± 0.06

Within each column and for each physicohemical property, mean values having the same lowercase letter are not significantly different ($p < 0.05$). Means in the same row having the same capital letter are not significantly different ($p < 0.05$).

Other authors have reported MOEO activity against *E. coli* O157:H7 [7], *B. thermosphacta*, *L. plantarum*, and *Ps. fragi* [2], and BEO against *E. coli* [6,8,40,50–52]; *Ps. aeruginosa* [40,50,51], and *L. plantarum* [40]. It should be noted from the cell population viability study (Figure 2), that most microorganisms studied here revealed extensive damage to their cell wall after being contacted

with the microencapsulated MOEO/BEO within the coating. Other authors have reported MOEO activity against *E. coli* O157:H7 [7], *B. thermosphacta*, *L. plantarum*, and *Ps. fragi* [2], and BEO against *E. coli* [6,8,40,50–52]; *Ps. aeruginosa* [40,50,51], and *L. plantarum* [40].

3.5.2. Physicochemical Properties

The initial TBARS content of the meat was 0.04 ± 0.02 mg malonaldehyde/kg meat (Table 4). The control and CC samples increased their MDA content with storage time reaching after 28 days 0.28 ± 0.01 and 0.08 ± 0.001 mg MDA/kg meat, respectively. Lipid oxidation of AC samples was small and decreased significantly ($p < 0.05$) among treatments from day 14 onwards (Table 4). All TBARS values reported in Table 4 are <0.3 mg MDA/kg meat, and thus after 28 days of storage the meat was below the off odor threshold of 0.5 mg MDA/kg meat, reported for meat patties [18]. Oxidation of unsaturated fatty acids of meat depends on several factors, such as the presence of oxygen, metals like heme iron, and free iron, among others [16,18,33]. The coating without EOs (CC) provided an oxygen transfer barrier resulting in samples showing lower TBARS than the control. The antioxidant effect showed by the AC samples is in agreement with a report using 4%–6% BEO that did not find TBARS in raw minced meat packed in polyethylene bags at 4 °C [17]. In addition, BEO contains eugenol (Table 1) that is able to donate electrons to inactivate free radicals involved in the propagation step of autoxidation, besides its protective effect against prooxidants like iron [15,16,18]. Moreover, MOEO extracted from Tolimán (Querétaro, México), thymol, and BEO showed capacity to inactivate free radicals as evaluated by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method [2,27]. On the other hand, a report showed that α-terpineol (MOEO) and linalool (MOEO and BEO) (Table 1), exert strong antioxidant capacity and iron reducing properties, confirming the advantage of using mixtures of EOs [13]. However, properties of EO mixtures should be verified to avoid antagonic effect among the active ingredients.

The increase in pH of control and CC meat samples on day 21 relative to the initial value can be attributed to the accumulation of microbial metabolites during storage (Table 4) [18,33,53]. This pH increase may show negative effect on sensory properties like meat color, flavor, tenderness, and water holding capacity, and off flavor due to microbial growth [53]. At the end of the experiments pH values of the control and CC treatments were not significantly different ($p < 0.05$) from the initial value, whereas the pH from AC samples was significantly lower than the initial value (Table 4). The similar pH values between final and initial experiments is in agreement with Gómez and Lorenzo [54] using vacuum packed foal meat stored at 2 °C, and with Stojanovic-Radic et al. [55] working with chicken meat coated with basil, rosemary and rosemary/basil mixture of EOs stored at 4 °C. Similar pH values are attributed to the presence of ammonia, aldehydes and ketones produced by protein hydrolysis [56,57] that tend to neutralize the lactic acid resulting from LAB metabolism [49].

3.6. Sensory Evaluation

Color and odor from control meat samples was acceptable (range > 6) only on the first day. On the other hand, CC samples color was accepted after 7 and 21 days, whereas odor was undesirable when samples were kept for 28 days (acceptance < 6) (Figure 3a,b). AC sample color and odor were accepted by judges throughout the study, and from day 7 onwards showed the highest values from all treatments, which coincided with "slightly desirable" in the hedonic scale used. Therefore, the microencapsulated mixture MOEO/BEO at the concentration added to the coating showed favorable effect on sensory properties of treated samples, reflecting a greater acceptance than the control.

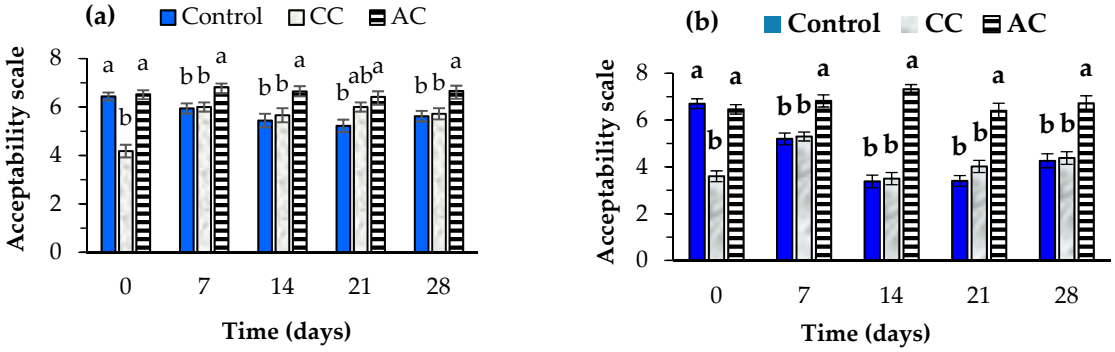

Figure 3. Effect of active coating containing microencapsulated MOEO and BEO on color (**a**) and odor (**b**) aceptability of fresh pork meat stored at 4 °C.

4. Conclusions

Thymol and linalool were the major components of MOEO and BEO, respectively. Using fluorescent probes it was found that coating added with microencapsulated mixture of MOEO (4 mg/mL) and BEO (11 mg/mL) caused damage to *B. thermosphacta*, *E. coli* O157:H7, *L. plantarum*, and partly to *Ps. fragi* cell membranes. This effect was responsible for the low bacterial populations in fresh pork meat stored for 28 days at 4 °C.

The active coating showed an antioxidant effect by decreasing lipid oxidation, and also demonstrated favorable effecta on the sensory properties of treated samples. Therefore, coatings added with microencapsulated EOs mixture represent a natural preservation alternative to increase the shelf life of refrigerated meat products.

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