Antilisterial effect of citrus essential oils and their performance in edible film formulations

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ABSTRACT

The antimicrobial activity of eight essential oils (EOs) extracted from the fruit peel of Citrus genotypes (orange, mandarin and lemon) was evaluated against 76 strains of Listeria monocytogenes, previously isolated from different food matrices. EOs showing the most (EO L2 and EO L8) and least (EO O3 and EO M7) effective inhibition activities were chemically characterized by gas chromatography coupled with mass spectrometry (GC/MS) to compare their composition. EO L2 and EO L8 were chosen to determine the MIC and to evaluate the cell viability of the most sensitive strains (L. monocytogenes LM35 and LM69) after 1, 2, 4 and 6 h of exposure. The effectiveness of chitosan (CH) and methylcellulose (MC) edible films, alone and in combination with EO L2 and EO L8, was determined against LM35 and LM69 at 37 °C for 0, 8 and 24 h and at 8 °C for 0, 1, 3 and 7 days. In addition, the analysis of the microstructure of the films were performed by scanning electron microscope (SEM) to evidence the interactions between the polymers and EOs. Thirty-five and twenty-nine strains were clearly inhibited by EO L2 and EO L8, respectively, while the other Citrus EOs showed poor (EO M1, O4, O5, O6) or minimal (EO O3 and M7) antimicrobial activity. A total of 36 chemical volatile substances was identified by GC/MS to detect the compounds that might play an important role in the characterization of the EOs. The chemical characterization points to oxygenated monoterpenes as relevant compounds in inhibiting Listeria strains, since they have been detected in lemon EOs in concentrations four/five folds higher than orange EOs. Generally, CH- and MC-based films containing EO L2 and EO L8 showed antilisterial activities, even though, the best performances were observed in case of CH-films at 8 °C, with a major reduction up to 3 log (CFU/cm^2) in case of EO L2 incorporation. The microstructures observed by SEM suggested a better incorporation of the EOs in CH matrix, where a higher amount of oil droplets was distinguished. Therefore, lemon EOs incorporated into chitosan films could be an efficient tool to control Listeria monocytogenes, especially in refrigerated applied conditions.

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1. Introduction

Listeria monocytogenes is the causative agent of several outbreaks of food-borne listeriosis in America and in Europe (CDC, 2014; ECDC, 2013). This disease primarily affects people with weakened immune systems, such as older adults, pregnant women and newborns. Even though listeriosis is relatively rare and sporadic, it is a disease with high fatality rate (up to 30%) (FAO/WHO, 2004). In the United States, Listeria spp. annually induces, on average, 1600 cases of illnesses and 260 deaths, and is the third leading cause of death from food poisoning (Scallan et al., 2011). In 2012, 1642 cases of listeriosis have been reported in Europe with an increasing trend in comparison with previous years (ECDC, 2013). The highest proportions of food samples that exceeded the safety

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Table 1
Inhibitory activity[a] of citrus EOs against *Listeria monocytogenes* isolated from food tested by disc diffusion assay.

Strain code	EO M1	EO L2	EO O3	EO O4	EO O5	EO O6	EO M7	EO L8	Statistical significance[b]	Source of isolation[c]
LM01	0.6	0.8	0.6	0.6	0.6	0.6	0.7	1	***	M
LM02	0.7	0.8	0	0.6	1	0.8	0.8	1	***	M
LM03	0.8	0.8	0	0	0.8	1	0.6	0.8	**	M
LM04	1	1	0.6	0.6	0.6	0.6	0.6	0.6	***	D
LM05	0	0.8	0	0.8	0.6	1	0.6	1.4	**	D
LM06	0.7	0.8	0.6	0.6	0.6	0.8	0.7	0.8	***	M
LM07	0.6	0.8	0.7	0.6	0.6	0.6	0.7	0.8	***	D
LM08	0.6	0.6	0.8	0.6	0.7	0.8	0.6	1.1	***	F
LM09	0.6	0.8	0	0.8	0	0.8	0	0.8	*	F
LM10	0.8	0.8	1	0.8	1.4	1	1	1	***	D
LM11	0.6	1	0.6	0	0.8	0.8	0.8	0.8	***	F
LM12	0.8	0.9	0.6	0.6	0.6	0.8	0.6	0.8	***	M
LM13	0.8	1	0.6	0.6	0.6	0.8	0.8	1.2	***	D
LM14	0.6	0.8	0	0.6	0.6	0.8	0.6	0.8	***	F
LM15	0.6	1	0	0.8	0.6	0.8	0	0.8	**	D
LM16	0	1	0	1	0.6	1	0.8	1	**	F
LM17	0.8	1.2	0.6	0.8	0.7	0.7	0.6	0.8	***	D
LM18	0.7	1	0.6	0.8	0.6	0.6	0.8	0.8	***	F
LM19	0.6	0.9	0.6	0.6	0.8	0.8	0.7	0.8	***	F
LM20	0.7	1.1	0.6	0.8	0.6	0.8	0.6	1	***	F
LM21	0.7	0.8	0.7	0.7	1	1	0.6	0.8	***	M
LM22	0.6	0.8	0.6	0.6	0.8	1	0.8	1	***	F
LM23	0.7	1	0.6	0.8	0.6	0.6	0	0.7	***	D
LM24	0.6	0.9	0.8	0.8	0.6	0.6	0.6	0.8	***	F
LM25	0.6	0.8	0.6	1	0.8	0.7	0	1	***	F
LM26	0.7	0.8	0.6	0.7	0.8	0.6	0.7	1	***	M
LM27	0.8	1.3	0.8	0.8	0.6	0.8	0.6	1	***	D
LM28	0.7	0.8	0.6	0.6	0.6	0.7	0.6	0.8	***	M
LM29	0	0.8	0	0.6	0.6	0.8	0	1	*	M
LM30	0.6	1	0.6	0.8	0.6	0.6	0.6	1	***	PF
LM31	0.6	0.8	0.6	0.7	0.6	0.6	0.6	0.8	***	M
LM32	0.6	1	0	0.6	0.6	0.6	0.6	0.8	***	F
LM33	0	1	0.6	0.8	0.6	0.8	0.6	1	***	F
LM34	0.6	0.6	0.6	0.6	0.6	0.8	0.6	0.8	***	F
LM35	1	1	0	0.8	0.8	1.2	1	1.4	***	V
LM36	0.8	1	0.8	0.8	0	0.8	0.8	1	***	F
LM37	0.6	1.2	0.6	0.6	0.6	0.6	0.6	0.8	***	F
LM38	1.2	1	0.7	0.7	0.6	0	0	0.8	**	D
LM39	0.8	0.8	0.7	0.7	0.8	0.8	0.6	1	***	D
LM40	0.6	1	0	0.6	0.6	0.6	0.6	1	***	D
LM41	0.7	1.2	0.8	0.6	0.6	0	0.7	1	***	F
LM42	0	0.6	0.6	0.6	0.6	0.8	0	0.8	**	M
LM43	0.6	1.2	0.6	0.8	0.6	0.6	0	0.8	***	D
LM44	0.6	0.8	0.6	0.6	0.6	0.8	0.7	1	***	M
LM45	0.8	0.6	0.6	0.6	0.6	0.6	0.6	0.8	***	PF
LM46	0.7	1	0.6	0.7	0.6	0.8	0.6	1	***	F
LM47	0.6	1.2	0.6	0.8	0.6	0.8	0.6	1	***	M
LM48	0.6	1.2	0.6	0.6	0.8	0.8	0.8	1	***	D
LM49	0.6	1	0.6	0.6	0.7	0.8	0.6	0.8	***	M
LM50	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	F
LM51	1.2	1.3	0.8	0.8	0.6	0.6	0.6	0.8	***	M
LM52	0.6	1	0.6	0.6	0	0.6	0.6	0.7	***	F
LM53	0.6	0.9	0.6	0.6	0.6	0.6	0.6	0.8	***	D
LM54	0.9	1	0.6	0.6	0.6	0.8	0	0.8	***	M
LM55	0.6	0.8	0.7	0.6	0.6	0.6	0.6	0.8	***	M
LM56	0.6	0.8	0.6	0.6	0	0.6	0	0.8	**	D
LM57	1	1	0.7	0.6	0.6	0.8	0	1.1	***	D
LM58	0.6	0.8	0.6	0.7	0.7	0.6	0.6	0.8	***	M
LM59	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	D
LM60	0.6	1	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM61	0.9	1	0.9	0.6	0.6	0.6	0.6	0.9	***	PF
LM62	0.6	0.6	0.8	1	0.6	0.6	0.6	1.2	***	D
LM63	0.6	1	0.6	0.6	0	0	0	1	*	F
LM64	0.6	1	0.8	08	0.8	0	0	0.9	**	F
LM65	0.6	1	0.6	0	0.6	0.6	0	0.8	**	PF
LM66	0.6	0.8	0.6	0.6	0	0	0	0.8	*	F
LM67	0.6	0.6	0.6	0.6	0.6	0.7	0.6	0.9	***	F
LM68	0.6	0.6	0	0.6	0	0	0	0	ns	F
LM69	1	1.2	0.8	1	1	1.1	0.8	1.4	***	D
LM70	0.8	0.6	0.8	0.6	0.6	0.8	0.6	1	***	V
LM71	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM72	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM73	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.8	***	F
LM74	0.9	1.1	0.8	0.8	0.8	0.8	0.6	0.8	***	D

(continued on next page)

Table 1 (*continued*)

Strain code	EO M1	EO L2	EO O3	EO O4	EO O5	EO O6	EO M7	EO L8	Statistical significance[b]	Source of isolation[c]
LM75	0.8	0.9	0.7	0.7	0.6	0.6	0.6	0.8	***	M
LM76	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	D
CECT 936	0.8	1	0.6	0.6	0.7	0.6	0.6	0.8	***	CECT

[a] Results indicate mean value of four determinations (carried out in duplicate and repeated twice). The values are expressed in cm.
[b] P value: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
[c] M, Meat; D, Dairy; F, Fish; V, Vegetable; PF, Packaged food; CECT, Colección Española de Cultivos Tipo (http://www.cect.org/).

threshold (zero tolerance in all ready-to-eat products) for *L. monocytogenes* in EU, in 2012, were ready-to-eat (RTE) fishery and meat products (ECDC, 2013).

L. monocytogenes is widely present in soil, water, food (McCarthy, 1990; Kaclíková, Kuchta, Kay, & Gray, 2001) and food processing environments (Donnelly, 2001). Its capacity to adhere and colonize inert food contact surfaces such as polypropylenes, rubbers, stainless steel and glass, is well established (Beresford, Andrew, & Shama, 2001; Rieu et al., 2008). Moreover, its ability to grow at a wide range of temperatures (−0.4 °C up to 50 °C), at a relative low pH (5.0−5.7 at 4 °C and 4.3−5.2 at 30 °C) and its capacity to form biofilms makes the control of this pathogen very difficult (Luber et al., 2011).

The recent resurgence of listeriosis has prompted the food industry, the public and the government to question the adequacy of the current methods of food safety and preservation. All the recommendations of the Codex Alimentarius to providing guidance on the controls and associated tools that can be adopted by regulators and industry to minimize the likelihood of illnesses arising from the consumption of RTE foods containing *L. monocytogenes* (CAC/GL61, 2007), converge on the reduction of the risk through safe food preparation, consumption and storage practices. Moreover, consumer concern created a demand for more "natural" and "minimally processed" food. As a result, the application of naturally produced antimicrobial compounds, such as essential oils (EOs) extracted from plants, has received great attention. EOs are complex mixtures of lipophilic substances which exert different biological properties (Bakkali, Averbeck, Averbeck, & Idaomar, 2008) enjoying a "generally recognized as safe" (GRAS) status by the Foods and Drugs Administration (FDA).

The antimicrobial properties of EOs depend on their chemical composition (Espina et al., 2011; Lanciotti et al., 2004; Moreira, Ponce, Del Valle, & Roura, 2005) which is influenced by raw plant material (genotype and, part of the plant), harvest time, geographical and ecological conditions (Settanni et al., 2014) and extraction method (Burt, 2004).

Citrus spp. have been extensively investigated for EOs (Fisher & Phillips, 2008; Tirado, Stashenko, Combariza, & Martinez, 1995), but the biological activities of the EOs are still under study. Some authors reported EOs to be highly effective, while other stated that the effects are variable (Burt, 2004). Recent reports demonstrated that some EOs extracted from *Citrus* in Sicily (south Italy) showed good potential as antimicrobial compounds effective against food spoilage and/or pathogen microorganisms *in vitro* (Settanni et al., 2012, 2014).

Since the intense aroma, the potential toxicity and the extraction costs limit the direct use of EOs in food preservation, the reduction of the doses to be applied to food matrixes is the clue to be pursued to extensively apply EOs. The use of edible coatings as carriers of antimicrobial compounds could be an alternative tool to contrast food spoilage and/or pathogen agents (Aider, 2010; Bakkali et al., 2008; Burt, 2004; Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011) and, at the same time, to reduce the amount of EOs to be applied in the food.

In this way, the chemico-physical properties of the polymer constituting the film and acting as a selective barrier to gas transport (Vargas, Pastor, Chiralt, McClements, & González-Martínez, 2008), together with the antimicrobial properties of EOs included, can be the goal of an hurdle technology applied to food to extend its commercial shelf-life (Park, 1999; Perdones, Sánchez-González, Chiralt, & Vargas, 2012). To this end, the use of biopolymers, such as chitosan (CH) and methylcellulose (MC), piques the interest of food industries and research groups thanks to their excellent film forming properties, non-toxicity, odorless, tasteless, biodegradability and edibility (Krochta & De Mulder-Johnston, 1997; Villalobos, Hernández-Muñoz, & Chiralt, 2006; Vargas et al., 2008). Chitosan is a cationic polysaccharide obtained from chitin by deacetylation in the presence of alkali (Sánchez-González, González-Martínez, Chiralt, & Cháfer, 2010) that shows antimicrobial activity itself (Vargas & González-Martínez, 2010; Zheng & Zhu, 2003) and can also acts in synergy with EOs.

The aim of this work was (i) to evaluate the effect of citrus EOs against several *Listeria monocytogenes* strains and (ii) to assess the antimicrobial properties of *Citrus* EOs incorporated into chitosan and methylcellulose coatings.

2. Materials and methods

2.1. Listeria monocytogenes strains

Seventy-six strains of *L. monocytogenes* were used in this study. All strains, belonging to the Department of Biotechnology − Microbiology Area, ETSIAMN (Universitat Politècnica de València, Spain), were previously isolated from food matrices including dairy products, fish, meat and vegetables, following the ISO method 11290−1:1996 (ISO 11290−1:1996). Bacterial strains were stored in cryovials (Microbank™ Prolab Diagnostics, Austin, USA) at −80 °C. The strains were reactivated and sub-cultured onto Tryptic Soy Agar (TSA, Merck Millipore, Darmstadt, Germany) incubated overnight at 37 °C.

2.2. Citrus samples and extraction of EOs

The EOs analyzed in this study were obtained from the peels of eight different citrus fruits cultivated in Sicily (Table 2) and collected during March 2014. Samples EO M1 and EO L2 derived from mature trees cultivated in the collection orchard "Parco d'Orleans" of the Agricultural Faculty of Palermo, while samples EO O3, EO O4, EO O5, EO O6, EO M7 and EO L8 from the "Azienda Sperimentale Palazzelli C.R.A. − Centro di ricerca per l'agrumicoltura e le colture mediterranee Contrada Palazzelli Scordia" (CT, Italy).

After peeling, the peels were immediately subjected to hydro-distillation for 3 h using a Clevenger-type apparatus (Comandè, Palermo, Italy) collecting the oil in hexane. EOs were dried over anhydrous sodium sulfate and stored at 4 °C in air-tight sealed glass vials covered with aluminum foil.

Table 2
Sicilian EOs used in the antilisterial screening.

EO	Species	Variety	Experimental orchard
M1	Mandarin (*Citrus reticulata* Blanco)	Mandarino Tardivo di Ciaculli	Campo dei Tigli (Palermo)
L2	Lemon (*Citrus limon* L. Burm.)	Femminello Santa Teresa	Campo dei Tigli (Palermo)
O3	Sweet Orange (*Citrus sinensis* L. Osbeck)	Moro Nucellare	Campo Palazzelli (Acireale)
O4	Sweet Orange (*Citrus sinensis* L. Osbeck)	Lane Late	Campo Palazzelli (Acireale)
O5	Sweet Orange (*Citrus sinensis* L. Osbeck)	Tarocco Tardivo	Campo Palazzelli (Acireale)
O6	Sweet Orange (*Citrus sinensis* L. Osbeck)	Sanguinello Nucellare	Campo Palazzelli (Acireale)
M7	Hybrid of Oroval clementine × Tarocco orange	Alkantara mandarin®	Campo Palazzelli (Acireale)
L8	Lemon (*Citrus limon* L. Burm.)	Femminello siracusano 2Kr	Campo Palazzelli (Acireale)

2.3. Chemical characterization

GC/MS analysis of the EOs was performed by gas chromatography couple with mass spectrometry (GC/MS) (EI) on a GCMS-QP2010 (Shimadzu, Milan, Italy). NIST 21,107,147 library was used for data acquisition. The analysis was carried out through a fused silica capillary column SLB-5MS (5% diphenyl:95% methylsiloxane) 30 m × 0.25 i.d. ×0.25 mm film thickness (Supelco, Milan, Italy); helium gas was used as the carrier gas at a constant linear rate 30 cm s^{-1} (30.6 kPa); split/splitless injector port; injector temperature 250 °C; injection mode split (split ratio 100:1). The oven temperature was programmed as follows: 50 °C, hold 3 min; 3 °C/min to 240 °C; 15 °C/min to 280, hold 1 min. MS scan conditions were: source temperature 200 °C, interface temperature 250 °C, EI energy 70 eV; mass scan range 40–400 amu. GC/MS analysis was carried out in duplicate.

2.4. Screening of antilisterial activity

The antibacterial activity of the eight EOs against *L. monocytogenes* strains was tested by the paper disc diffusion method applied by Kelmanson, Jager, and Van Staden (2000) and with the modifications of Militello et al. (2011). Bacterial cells were grown at 37 °C overnight before tests on tryptone soy broth (TSB). A concentration of about 10^7 CFU/mL of each strain was inoculated into 7 mL of TSA soft agar (0.7%, w/v) and poured onto TSA. Sterile filter paper discs (Filter-Lab Anoia, Spain) of 6 mm diameter were placed onto the surface of the double agar layer and soaked with 10 μL of each undiluted EO. Sterile water was used as negative control. Antibacterial activity was positive when a definite halo of inhibition (in cm) was detected around the paper disc. Each test was performed in duplicate and the experiments were repeated twice. Resulting data were subjected to statistical analysis using the ANOVA procedure with Statistica 10 (Statsoft, USA) software. Differences between means were determined by Tukey's multiple-range test.

2.5. Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was used to measure the antibacterial activity, since it represents a common method to express the EO antibacterial performances (Burt, 2004). MIC is defined as the lowest concentration of an active compound inhibiting visible growth of the tested organisms (Karapinar & Aktuğ, 1987). The strength of the antibacterial activity is determined using dilutions of EO in order to determine the end-point by means of the disc diffusion assay. Each *L. monocytogenes* strain was inoculated into TSA at 10^6 CFU/mL, the paper discs were disposed onto the agar surface, soaked with 10 μL of the serial dilutions of EOs and incubated O/N at 37 °C. Serial dilutions (dilution factor = 2) were obtained with dimethyl sulfoxide (DMSO, Sigma–Aldrich, Milan, Italy). DMSO alone was used as negative control. Each test

was performed in duplicate and the experiments were repeated twice.

2.6. Viability of L. monocytogenes strains by fluorescence microscopy

The viability of the most sensitive *L. monocytogenes* strains after treatment with EOs was evaluated by Viability Kit LIVE/DEAD® *Bac*Light™ (Molecular Probes Inc. Eugene Oregon) and plate counts onto TSA. The viability test was carried out with the strains inoculated at a final density of 10^4 CFU/mL in broth containing 0.125% (v/v) EO. Cells were counted as follows: 500 μl of each broth collected at 0, 1, 2, 4 and 6 h of treatment with EO was added with 0.8 μl of the fluorochromes mix (1:1 v/v, EO/mix) and incubated in darkness at room temperature for 15 min. Five microliters of the resulting mixture were placed onto a poly-L-lisina slide (Poly-Prep® slides, Sigma Diagnostics, U.S.A.). After 10 min of incubation at room temperature, the counts were carried out by the epifluorescence microscope Olympus BX 50 (with a mercury bulb of 100 W) equipped with a double filter (XF 53, Omega) (Olympus Optial Co., Hamburg, Germany). Digital colored photos were taken with Olympus DP10 digital camera (results not shown).

2.7. Antilisterial effect of edible EOs-based films

Chitosan-based (CH) and methylcellulose-based (MC) films were used to perform the antilisterial assay. High molecular weight chitosan (1.2 Pa s viscosity at 1% w/w in 1% w/w glacial acetic acid, acetylation degree: 4.2%, Sigma–Aldrich, USA) was dispersed at 1% w/w in an aqueous solution of acetic acid (1% v/v) and stirred overnight at room temperature. Methylcellulose (0.3–5.6 Pa s viscosity at 1% w/w in water solution, VWR BDH ProLabo, Spain) was dispersed in distilled water (1% w/w) and heated up to 80 °C to promote solubilization.

Once the polymer solutions were obtained, each EO was added at a concentration of 0.5% (polymer: EO ratio 2:1) and stirred for 10 min. The mixtures were then sonicated by the Vibra Cell VCX750 sonicator (Sonics & Materials, Inc., USA) at 20 kHz and 40% power for 480 s (1 s on and 1 s off) in order to obtain the film forming dispersions (FFD). FFDs were casted in plates (diameter 53 mm), weighted up to 6.7 g, to keep polymer amount constant in dry films (30 g polymer/m^2). The films were dried at room temperature and 60% relative humidity (RH).

The surface of TSA plates (10 g) was seeded with 0.35 mL of cell suspensions (10^4 CFU/mL) and covered with CH and MC films. Inoculated coated TSA and inoculated non-coated TSA dishes were used as controls. Plates were then sealed with parafilm to avoid dehydration and incubated at 37 °C for 0, 8 and 24 h and at 8 °C for 0, 1, 3 and 7 d. The two temperatures were chosen to investigate the effect of the EOs at the optimal growth temperature for the test strains (37 °C) and simulating the conditions of a domestic refrigerator (8 °C). The agar layer was then aseptically removed from

each Petri dish and placed into a sterile stomacher bag with 90 mL of Peptone Water (Merck Millipore, Darmstadt, Germany) and homogenized for 60 s in the stomacher Bag Mixer 400 (Interscience, Saint Nom, France).

Serial dilutions were set up with Ringer's solution (Sigma–Aldrich, Milan, Italy) and 0.1 mL of cell suspensions were spread plated onto TSA plates. Colonies were enumerated after 24 h at 37 °C. The experiment was carried in duplicate.

2.8. Microstructure

Film microstructure was observed by Scanning Electron Microscopy in cross-sectioned cryofractured specimens, using a JEOL JSM-5410 (Tokyo, Japan) electron microscope in order to qualitatively assess the EOs incorporation into the polymeric matrix. The films (3 samples per formulation) were equilibrated in P_2O_5 to eliminate water prior cryofracturing them by immersion in liquid nitrogen. Afterwards, cryo-fractured samples were mounted on copper stubs. After gold coating, the images were captured using an accelerating voltage of 10 kV.

3. Results and discussion

3.1. Screening of the antilisterial activity

The results of the disc diffusion assay are shown in Table 1. All EOs resulted statistically different ($P \leq 0.001$) in inhibiting the strains tested, confirming previous statements that the sensitivity to natural antimicrobial compounds is strain-dependent (Settanni et al., 2014). EO L2 and EO L8 showed the widest spectra of inhibitory activity. In particular, EO L2 inhibited all tested strains and for thirty-five of them the clear halos were larger than 10 mm. Except *L. monocytogenes* LM68, all other strains were sensitive to EO L8 and the halos were registered at diameters larger than 10 mm for twenty-nine indicator strains.

Regarding the inhibition by the other EOs, only *L. monocytogenes* LM10, LM16, LM35 and LM69 were particularly sensitive. On the contrary, strains LM09, LM29, LM63, LM66, LM68 were not inhibited by at least three EOs. EOs O3 and M7 did not show interesting antilisterial activities. In general, the antibacterial effects of citrus EOs depend on the compounds and the species/isolate under study (Fisher & Phillips, 2008) and similar results, in terms of number of strains inhibited and inhibition areas, were previously registered for EOs extracted from *Citrus* in Sicily (south Italy) (Settanni et al., 2012, 2014). It is worth noting that in those previous studies, *L. monocytogenes* resulted the species most sensitive among the bacteria tested which included Gram-positive (*Staphylococcus aureus*), as well as Gram-negative (*Salmonella* spp. and *Enterobacter* spp.) strains.

MICs were calculated only for the most effective EOs (EO L2 and EO L8) against *L. monocytogenes* LM35 and LM69, which were registered as the most sensitive strains. Both strains were equally inhibited and the values registered were 0.625 μL/mL for EO L2 and 1.25 μL/mL for EO L8. The two strains LM35 and LM69 were chosen to be better characterized and then used to register their behavior in edible film formulations. In our opinion, the best strategy to evaluate the efficacy of the incorporation of a given EO in films should be based on the use of the most sensitive strains. In fact, the *in situ* activity can be strongly reduced by the interaction of the EOs with the films and the inhibitory effect on the test strains masked.

3.2. Characterization of EOs by GC/MS

Analysis of volatile compounds was carried out after extraction of EOs. Based on the antilisterial activity, EO L2 and EO L8, as most

effective, and EO O3 and EO M7, as less effective oils, were chemically analyzed by GC–MS. The identified volatile compounds and their relative amounts are given in Table 3. A total of 36 compounds were characterized among the four EOs. The phytochemical groups included monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons. Monoterpene hydrocarbons were quantitatively relevant, ranging from 88.35% (EO L2) to 98.07% (EO O3). Limonene accounted for the major proportion by quantity in all samples. The oxygenated monoterpenes of lemon EOs were four/five folds those of EO O3 and EO M7, indicating a direct role in the mechanisms of inhibition. Sesquiterpene hydrocarbons were detected in minimal percentages in lemon EOs, only traces were found in EO M7 while they were absent in EO O3.

Monoterpene hydrocarbons such as α-Thujene, p-Cymene and cis-2,6-Dimethyl-2,6-octadiene were found only in lemon EOs. Among the oxygenated monoterpenes, 1-Octanol, Fenchol, Citronellal, cis-Geraniol, α-Citronellol, β-Citral, cis-p-Mentha-2,8-dien-1-ol, Geranyl acetate and Neryl acetate were identified only in EO L2 and EO L8. On the contrary, β-Terpinol was only found in EO O3 and EO M7. Almost all compounds showed statistical differences in quantitative terms among EOs. The higher presence of oxygenated monoterpenes in volatile composition profile of EO L2 and EO L8 could explain the greater inhibitory activity than the EO O3 and EO M7.

3.3. Viability assay

Dead and viable cells were detected and counted using epifluorescence microscopy. Plate counts of the untreated samples showed an increase of 10^3 CFU/ml for both strains within the six hours of treatment. Divergent results were obtained comparing the counts assessed by epifluorescence microscopy and plate counts.

Based on epifluorescence microscopy, viable cells amounted to 10^{3-4} CFU/ml for LM35 and 10^{4-5} CFU/ml for LM69, while dead cells reached up to 3 and 4 log CFU/ml in case of LM35 and LM69, respectively. These results are in contrast with those of direct plate counts, where no cultivable cells were detected after 1 h (or 2 h in case of LM35 added with EO L8) of incubation. This could be explained by an active but non-culturable (ABNC) state of cells stressed by EOs (Boulos, Prevost, Barbeau, Coallier, & Desjardins, 1999). This was confirmed by Nexmann Jacobsen, Rasmussen, and Jakobsen (1997) who registered significantly fewer viable *L. monocytogenes* cells counted by culture-based techniques compared to the active bacteria detected using fluorescent direct counts. Similar results were achieved with lactic acid bacteria (Moreno et al., 2006) using fluorescent flow cytometric measurements (Boulos, Prevost, Barbeau, Coallier, J., & Desjardins, 1999). According to Joux and Lebaron (2000), bacterial cells cannot be necessarily considered active if they show intact membranes, but it would seem to be more accurate to assume that membrane-compromised cells are dead (Berney, Weilenmann, & Egli, 2006). The EOs antimicrobial activity is due to their hydrophobic nature affecting the lipid bilayer of microbial cells, as confirmed by the evidences of this assays, since the kit used enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, differentiating between active and dead cells (Sachidanandham, Yew-Hoong Gin, & Laa Poh, 2005).

3.4. Antilisterial effect of edible EOs-based films and film microstructure

Antilisterial performances of CH- and MC-based edible films determined on TSA, alone and in combination with EO L2 and EO L8, are shown in Fig. 1. The overall effect of CH- and MC-based films, in terms of trend, was similar for both strains tested. The addiction

Table 3
Chemical composition[a] of citrus EOs.

Compound	RT	EO L2		EO O3		EO M7		EO L8		Statistical significance[b]
Monoterpene hydrocarbons		88.35		98.07		97.81		90.93		
α-Thujene	9.801	0.215	B	n.d.	A	n.d.	A	0.305	C	***
α-Pinene	10.129	1.290	B	0.340	A	0.410	A	1.325	B	***
Sabinene	11.900	1.105	B	0.210	A	0.220	A	1.135	B	***
β-Pinene	12.155	9.890	C	0.025	A	0.025	A	9.125	B	***
β-Myrcene	12.666	1.105	A	1.695	C	1.890	D	1.425	B	***
α-Phellandrene	13.467	0.185	C	0.105	B	0.055	A	0.065	A	***
3-Carene	13.560	n.d.	ns	0.090	ns	0.040	ns	n.d.	ns	ns
α-Terpinene	13.944	n.d.	ns	0.040	ns	0.040	ns	0.340	ns	ns
p-Cymene	14.275	11.515	C	n.d.	A	n.d.	A	0.440	B	***
ᴅ-Limonene	14.854	62.780	A	95.445	C	94.910	C	64.505	B	***
ϒ-Terpinene	16.080	0.025	A	0.075	A	0.180	B	9.525	C	***
(+)-2-Caren	17.315	n.d.	A	0.045	B	0.035	B	0.510	C	***
cis-2,6-Dimethyl-2,6-octadiene	29.716	0.240	B	n.d.	A	n.d.	A	2.225	C	***
Oxygenated monoterpenes		10.770		1.930		2.175		8.275		
1-Octanol	16.736	0.065	C	n.d.	A	n.d.	A	0.050	B	***
Linalol	18.024	0.425	A	1.005	B	1.555	C	0.410	A	***
Nonanal	18.252	0.190	B	0.040	A	0.020	A	0.135	B	**
Fenchol	18.972	0.030	B	n.d.	A	n.d.	A	0.015	B	**
Limonene epoxide	19.608	0.815	B	n.d.	A	n.d.	A	n.d.	A	***
Limonene oxide, trans	19.820	1.000	ns	n.d.	ns	n.d.	ns	n.d.	ns	ns
β-Terpinol	20.507	n.d.	A	0.035	C	0.020	B	n.d.	A	**
Citronellal	20.556	0.065	B	n.d.	A	n.d.	A	0.095	C	***
4-Terpineol	21.971	0.630	B	0.235	A	0.225	A	1.010	C	***
α-Terpineol	22.705	1.445	D	0.415	B	0.265	A	1.100	C	***
Decanal	23.157	0.085	A	0.200	A.C	0.090	A	0.040	A,B	*
trans-Carveol	23.801	0.180	B	n.d.	A	n.d.	A	n.d.	A	***
cis-Geraniol	24.087	0.175	B	n.d.	A	n.d.	A	1.245	C	***
α-Citronellol	24.200	0.070	A	n.d.	A	n.d.	A	0.325	B	**
β-Citral	24.704	1.550	C	n.d.	A	n.d.	A	1.355	B	***
(−)-Carvone	24.947	0.165	B	n.d.	A	n.d.	A	n.d.	A	***
cis-p-Mentha-2,8-dien-1-ol	26.058	0.220	B	n.d.	A	n.d.	A	1.790	C	***
α-Citral	26.090	1.980	B	n.d.	A	n.d.	A	n.d.	A	***
Geranyl acetate	30.116	0.980	C	n.d.	A	n.d.	A	0.325	B	***
Neryl acetate	30.979	0.700	C	n.d.	A	n.d.	A	0.380	B	***
Sesquiterpene hydrocarbons		0.880		n.d.		0.020		0.800		
α-Bergamotene	33.375	0.315	C	n.d.	A	n.d.	A	0.275	B	***
β-Bisabolene	36.474	0.480	C	n.d.	A	n.d.	A	0.385	B	***
Caryophyllene oxide	39.463	0.085	C	n.d.	A	0.020	B	0.140	D	***

Abbreviations: RT, retention time on SLB-5MS column; ns, not significant; n.d., not detectable.
[a] Data are means of two replicates expressed as percent area.
[b] P value: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

of the EOs into the films enhanced their bactericidal activity. The highest antimicrobial effect was obtained for CH films at 8 °C (Fig. 1E and G). When sample EO L2 was added to the films, a reduction in the range of 2–3 Log CFU/cm² was obtained as compared to control plates (Fig. 1A and E). This oil sample determined the lowest listeria counts in both film matrices (CH or MC). In general, the EO L2-based films showed the best inhibition activity compared with the CH or MC control films, and also, compared to EO L8-based films.

After a storage period of 24 h at 37 °C and 7 days at 8 °C, pure MC films showed no significant effect on the growth of both strains. MC films incorporating EO L2 promoted a slight reduction in *Listeria* counts at 37 °C after 8 h of incubation (≤ 1–2 log CFU/cm²) (Fig. 1B).

A stronger antilisterial effect was evidenced for the CH-based films, alone and in combination with EOs. Specifically, CH-films were more effective in reducing the microbial growth at 8 °C rather than 37 °C. In fact, CH-films added with EOs led to a reduction up to 3 and 6 log CFU/cm², in the case of LM35 and LM69, respectively, when incubated at 8 °C for 7 days (Fig. 1E and G).

The highest significant antibacterial effect evidenced in case of the incubation at 8 °C may be related to the influence of the temperature in promoting the permeability of cell membranes and, thus, dissolving more easily EOs in the lipid bilayer when low temperatures occur (Sánchez-González et al., 2011).

Fig. 2 shows the SEM microstructures of the cross-sections of CH and MC films. Pure MC and CH films (Fig. 2A, D) exhibited a homogeneous and continued microstructure in line to that observed in previous studies (Vargas, Albors, Chiralt, & González-Martínez, 2011). The addition of the lemon EOs to the film matrix promoted discontinuities (Fig. 2B, C, E, F), in agreement with the results reported by Perdones et al. (2012) in CH-based films containing essential oil. The presence of EO droplets is more noticeable in CH-based films (Fig. 2B, C), and especially in films containing EO L2 (droplets size 1–8 μm). The observations pointed to a better incorporation of the EOs in CH matrix, where a higher amount of oil droplets was distinguished.

Furthermore, the higher inhibition activity recorded for EO L2 included into CH matrix can be due not only to the better incorporation, but also to the subsequent release of the active compounds. A good incorporation of EO into the films slows down the diffusion rate of the antimicrobial compounds, keeping high concentrations of EOs for extended period of time and reducing the levels of microorganisms on the surface.

The two strains LM35 and LM69 chosen to evaluate the efficacy of the inclusion of EOs in films had different food origin, specifically vegetable and dairy products, respectively. Thus, this study demonstrated the potential application of the EOs to inhibit *L. monocytogenes* from different sources. Although the resistant
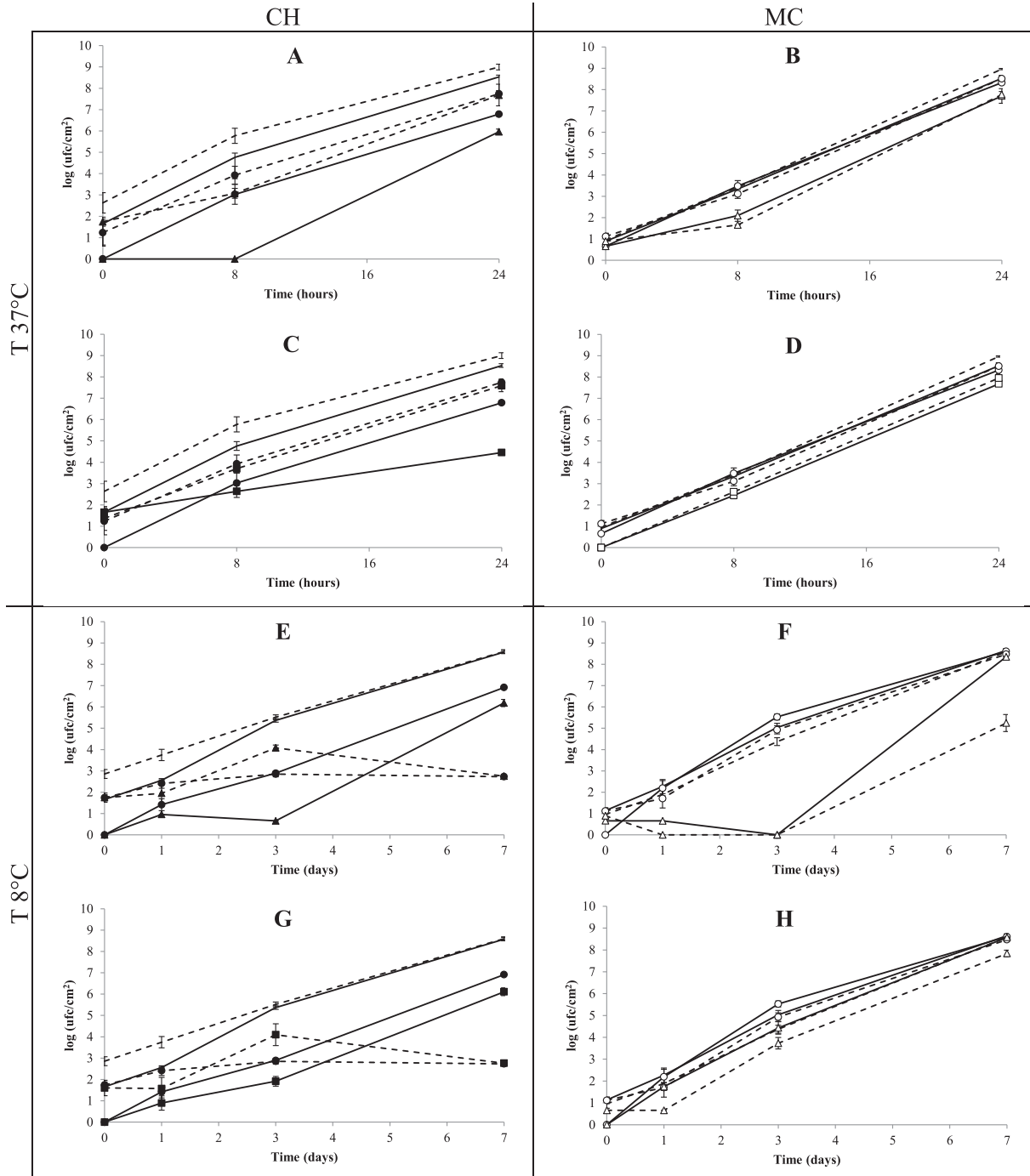
CH | MC

T 37°C

T 8°C

Fig. 1. Effect of incorporation of EOs in chitosan and methylcellulose films on the growth of *L. monocytogenes* at 37 °C for 24 h (A, B, C and D) and 8 °C for 7 d (E, F, G and H). Symbols:—, strain LM35; – –, strain LM69; black marks indicate chitosan films; empty marks indicate methylcellulose films; unmarked lines indicate control strains; ●,○, indicate control films; ▲, △, indicate films with EO L2; ■, □, indicate films with EO L8. A and E, chitosan films with EO L2; B and F, methylcellulose films with EO L2; C and G, chitosan films with EO L8; D and H, methylcellulose films with EO L8.

strains will not be inhibited by this strategy, a strong reduction of this pathogen can be obtained in terms of sensitive strains.

4. Conclusion

Citrus EOs showed bioactive properties against *L. monocytogenes*. The efficacy of the inclusion of EOs in films was tested against the most sensitive strains, in order to better evaluate their suitability. A masking effect of the film matrices on the inhibitory properties of the active substances cannot be excluded and could be relevant determining negative results in presence of low sensitive strains. The antibacterial effect of the EOs showing the highest inhibitory power was maintained when they were incorporated into biodegradable films based on chitosan or methylcellulose. Chitosan films containing EO L2 were the most effective in reducing *L. monocytogenes* counts. Chitosan edible films enriched with lemon oils represent an alternative tool to control surface contaminations of *L. monocytogenes*, especially in refrigerated
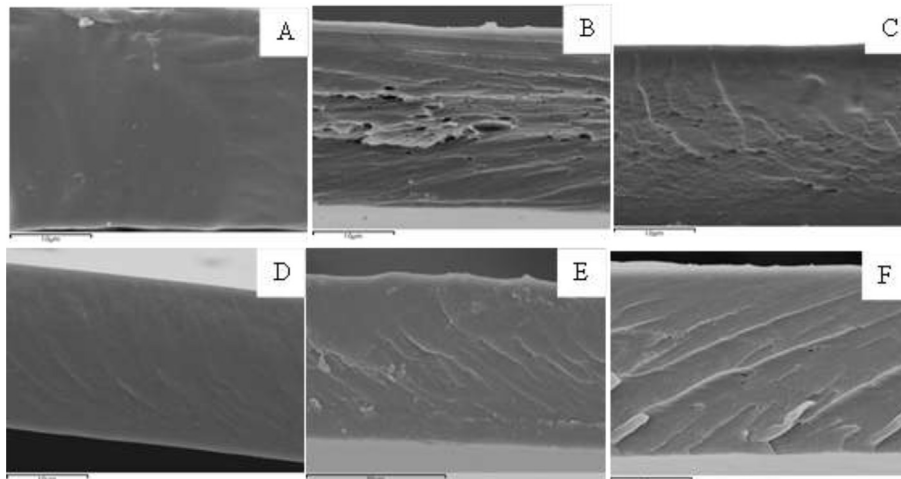
Fig. 2. SEM microstructure of cross sections of chitosan and methylcellulose films with essential oils. Magnification is ×3500. A, chitosan films; B, chitosan film with EO L2; C, chitosan film with EO L8; D, methylcellulose film; E, methylcellulose film with EO L2; F, methylcellulose film with EO L8.

conditions. The reduction in EO concentration needed for film applications, as compared to direct contact treatments, can reduce the possible sensory impact on food. Works are being prepared to refine the technology for the production of EO-based films, to evaluate the suitability of the films tested in this study on food matrices, as well as the impact of the EO released on the sensory quality. Hence, the foreseeable potential practical application of this study is to reduce the presence of *L. monocytogenes* in foods, but also to valorise citrus fruit peel that basically constitutes a waste of the fruit juice industry in Sicily.

Acknowledgments

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