International Journal of Food Microbiology 305 (2019) 108246

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Antimicrobial activity of cellulosic pads amended with emulsions of essential oils of oregano, thyme and cinnamon against microorganisms in minced beef meat

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ARTICLE INFO

Keywords:
Cellulosic pads
Emulsions
Essential oils
Oregano
Thyme, meat

ABSTRACT

Cellulosic pads, amended with emulsions containing essential oils of thyme and oregano, exhibited antimicrobial activity against the psychrophilic microbiota of minced beef. In addition, the pads were active against specific meat bacterial species (*Pseudomonas putida*, *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Enterococcus faecalis* and *Lactococcus lactis*) and some common foodborne pathogens (*Salmonella enterica*, *Campylobacter jejuni* and *Staphylococcus aureus*). Three emulsions, IT131017, Mediterranean and Etnic, containing different percentages of carvacrol, thymol, linalool, and α and β-pinene, significantly reduced the growth of *S. enterica* and *P. putida.* Pads derived from emulsions Mediterranean and Etnic induced slight (0.3–0.8 Log_{10} CFUs/g) but reproducible reduction of the psychrophilic microbiota in minced meat and hamburger stored for 12 and 15 days at 4 °C.

1. Introduction

The high water and protein content of meat makes it an optimal medium for microbial growth. Microorganisms that are found in meat belong to two categories: spoilage bacteria such as the genera *Pseudomonas*, *Lactobacillus* and *Enterococcus*, and pathogenic organisms such as *Salmonella enterica* and *typhimurium*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Escherichia coli* and *Listeria monocytogenes* (Giaouris et al., 2014). There are different strategies to preserve the quality and safety of meat products: the addition of chemical preservatives, super-chilling, high pressure and the addition of engineered nanoparticles (Patel, 2015). Each strategy has some drawbacks; for example, some chemical preservatives have been found to be neurotoxic or carcinogenic (Patel, 2015).

Naturally occurring compounds with antimicrobial activity have significant potential as food preservatives because of their low toxicity to non-target species such as mammals and humans. Essential oils (EOs) and other extracts from plants, herbs and spices have shown antimicrobial activity against a number of different food pathogens and spoilage microorganisms (Gyawali and Ibrahim, 2014; Negi, 2012). Extracts from cinnamon, oregano, clove, thyme, tea and citrus fruit are among the most studied natural antimicrobials for food applications (Kaul et al., 2003; Lee et al., 2015; Lin et al., 2004; Negi and Jayaprakasha, 2001; Oussalah et al., 2006) The largest group of natural plant compounds with antimicrobial activity includes: phenolics, phenolic acids, quinines, saponins, flavonoids, tannins, coumarins, terpenoids and alkaloids (Savoia, 2012). More specifically, the activity of these molecules against meat-dwelling microorganisms has been described (Jo, 2013).

Phenolic compounds such as thymol and carvacrol are present in thyme and oregano and have been shown to be the most active EO components of these species (Gutiérrez-Larraínzar et al., 2012; Imelouane, 2009; Oussalah et al., 2007; Teixeira et al., 2013). The antimicrobial activity of these phenolic compounds is related to the presence of –OH group that damages bacterial cell membrane causing leakage of cellular components (Lv et al., 2011; Xue et al., 2013).

There have been recent efforts to incorporate natural extracts (EOs, plant extracts and their constituents) into biopolymeric materials such as cellulose, chitosan or zein films that can be used in food packaging (Arcan and Yemenicioğlu, 2011; Cran et al., 2010; Kanatt et al., 2012; Rodríguez et al., 2007). When antimicrobial compounds are incorporated into a packaging material, the active constituents can be

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https://doi.org/10.1016/j.ijfoodmicro.2019.108246
Received 13 December 2018; Received in revised form 10 May 2019; Accepted 5 June 2019
Available online 11 June 2019
0168-1605/

released in a more controlled fashion from the polymeric matrix onto the surface of the food. Consequently, the minimal inhibitory concentration for bacterial growth inhibition can be maintained for longer periods of time.

Currently the maximum shelf-life for portioned meat products packaged in modified atmosphere and stored in optimal temperature conditions is 11–12 days. An extension of the shelf-life of 2–3 days of the products would allow a significant economic impact generated by the possible expansion of the market.

Cellulosic pads are usually employed to absorb liquid at the bottom of trays of meat packaging and would therefore be a good substrate to incorporate EOs. This manuscript describes the antimicrobial effects of pads amended with emulsions derived from plant EOs and the effect of this substrate on the shelf life of packaged meat.

2. Materials and methods

2.1. Preparation of emulsions and cellulosic pads

Plants materials were purchased from a local garden market. EOs were extracted by steam distillation from seeds of coriander, the cortex of young branches of cinnamon, lemon peel, fruits of pepper, and leaves of thyme and oregano. Three hundred g of ground plant material was placed in a basket inside a container full of water that was boiled for 5 h. The EOs were conveyed through the steam in a container of cold water which allowed separation. Emulsions were prepared in water by mixing EOs (10%), the weighting agent Ester gum (0.6%), the emulsifier Arabic gum (33.6%) and Guar gum (1% w/w in emulsions 1, 2% w/w in emulsions 2) to increase viscosity. Oils and the aqueous phase were homogenized with a high-speed blender (L5M, Silverson Machines, East Longmeadow, MA) for 2 min at room temperature. To minimize droplet size, the premixed emulsion was homogenized with two steps through a high pressure homogenizer (Gea Niro Soavi S.p.A. Parma, Italy) at 180/50 bar. The particle size distribution of resulting sample was measured using dynamic light scattering (Hydro 2000MU, Malvern Instruments, Malvern, UK). The composition of mixed emulsions Etnic, Mediterranean, 81700239 and IT131017 is shown in Table 1. Etnic was also mixed with EOs of cumin to improve odor.

Cellulosic pads were produced with the Rapid-Kothen Sheet Former (Tendring Pacific, Cambridge, UK). The capacity for liquid adsorption was referenced to that of a commercial pad (LP2) which was determined by the weight equal to 160 g/m^2 and absorption equal to 872 ml/m^2. A volume of 400 ml/m^2 of emulsion was sprayed onto the pads with a Sheen Automatic Film Applicator 1137 (Kingstone, England).

Table 1
Minimal Inhibitory Concentration (MIC) of emulsions from different plant extracts tested on total mesophilic microbial flora from meat suspension (see materials and methods). Percentages reported are those able to completely inhibit the growth of approximately 100 CFUs of bacteria. Percentages tested were 0.2%, 0.5%, 0.75%, 1%, 1.5% and 2%. Emulsion 2 of each category refers to a higher viscosity product that was easier to applied on the pad and it showed the same performance as the original version with lower viscosity.

Essential oil		MIC (%)
Cinnamon	Emulsion 1	0.5
	Emulsion 2	0.5
Oregano	Emulsion 1	0.5
	Emulsion 2	0.5
Thyme	Emulsion 1	0.75
	Emulsion 2	0.75
Black pepper	Emulsion 1	No effect
	Emulsion 2	No effect
Lemon	Emulsion 1	No effect
	Emulsion 2	No effect
Grapefruit	Emulsion 1	No effect
Coriander	Emulsion 1	No effect
60% thyme, 30% oregano, 10% cumin	Etnic	0.75
50% cinnamon, 25% oregano, 25% thymeo	81700239	0.50
60% oregano, 50% thyme	Mediterranean	0.50
55% oregano, 45% thyme	IT131017	0.75

2.2. Gas chromatography and mass spectrometry (GC–MS)

Emulsion samples were diluted 1 to 100 into an internal standard mix of water with 500 µl of 1-propanol. An Agilent 6890 gas chromatograph was equipped with a SPB-20, 30 m, 250 × 0.25 (Supelco #24086) column set to a constant flow of hydrogen at 1.2 ml/min. The GC conditions were as follows: Inlet 250 °C, 1 µl split injection (10:1) into a focus liner (Supelco #2879905-U); initial oven held at 50 °C for 5 min, ramp at 10 °C/min to 280 °C, and hold for 2 min. The Agilent 5973 MSD conditions were as follows: scan range = 25–300 amu, threshold = 100, aux = 250 °C. Analytes were identified based on high quality matches of full scan mass spectra to spectra in the NIST Library.

2.3. Selection of emulsions

The minimal inhibitory concentrations (MIC) of emulsions was determined on suspension obtained from 3 g of beef minced meat mixed in 27 ml of peptoned water (1% w/v peptone, 0.5% w/v NaCl), mixed by vortexing and centrifuged for 3 min at 3000 ×*g* to sediment meat residuals. Aliquots of 100 µl of meat suspension (approximately 100 colony forming units (CFUs) of microorganism) were sprayed onto three plates of PCA agar (Merck KGaA 64271 Darmstadt, Germany) supplemented with 0.2%, 0.5%, 0.75%, 1%, 1.5% and 2% of each emulsion (Table 1). The emulsions were placed in the empty plates and mixed with the warm medium until its solidification. After incubation at 4 °C in a fridge for 48 h, colonies were counted and the lowest percentage of emulsion that completely inhibited the microbial growth was recorded as the MIC.

2.4. DNA extraction from meat and sequencing of 16S gene

Aliquots of 100 µl of meat suspensions, obtained as described in 2.3, were plated on four PCA plates: two were incubated at 4 °C and two at 28 °C. Ten colonies of different morphology were inoculated into 3 ml of liquid medium (5 g/L enzymatic digest of casein, 2.5 g/l yeast extract, 1 g/l glucose) and incubated at 4 and 28 °C for 48 h. An aliquot of 1.5 ml of culture was centrifuged for 5 min at 13.000 ×*g* and re-suspended in 500 µl of TE buffer, mixed with 200 µl of lysozyme (100 mg/ml) and incubated at 37 °C for 30 min. After incubation, 20 µl of proteinase K (20 mg/ml) and 200 µl of AL buffer of the kit Qiagen Stool (Qiagen, Hilden, Germany) were added and the mixture was incubated at 56 °C for 30 min and then at 70 °C for 10 min. Then 200 µl of 100% ethanol were added and the mixture was loaded onto the column of the kit. DNA was eluted out of 50 µl of ultrapure water according to the kit instructions. With the same procedure, DNA was extracted from 30 ml of meat suspension after centrifugation for 5 min at 13.000 x g to recover bacterial pellet and re-suspension in 500 µl of TE.

PCR was conducted on 5 ng of DNA with primers designed for the conserved regions of 16S ribosomal RNA gene (27F-5′-AGAGTTTGAT CCTGGCTCAG-3′/1492R-′5′-ACGGGCTACCTTGTTACGAC-3′) (Lane, 1991). PCR was carried out in presence of 2 µM of primers, 1× GoTaqMasterMix (Promega, Madison, Wisconsin, US) at 95 °C for 5 min, followed by 30 cycles consisting of a denaturation step (95 °C for 1 min), an annealing step (56 °C for 1 min) and an extension step (72 °C for 1.5 min); with a final extension at 72 °C for 8 min.

Amplicons were purified by GFX PCR and a Gel Band Purification Kit (GE Healthcare UK Limited –HP7 9NA, UK) and sequenced. Sequences were aligned to those collected in a non-redundant database (ncbi site https://blast.ncbi.nlm.nih.gov) using the BLAST algorithm. Reference strains *S. enterica*, subsp. *enterica* serovar *typhimurium*, strain SA2380 (ATCC35987), *C. jejuni* subsp. *jejuni*, strain ASD-83-79

(ATCC33291) and *S. aureus* subsp. *aureus* strain MU50 (ATCC700699) were purchased at LGC Promochem (Foster City, CA).

2.5. Halo inhibition test

Single microbial species mentioned above (*C. jejuni*), as well the meat suspension, were grown in liquid medium (Section 2.4) at 37 °C for 24 h until stationary phase was achieved. *C. jejuni* was grown at 42 °C for 48 h in Preston Campylobacter Selective Enrichment Broth (Biolife Italiana, Milano, Italy) under microaereophilic conditions created by CampyGenTM gas pack (Oxoid Ltd., Hampshire, England). One culture derived from the meat suspension was grown at 4 °C to select psychrophilic microorganisms.

Tests for antimicrobial activity were carried out by placing 50 µl of each emulsion into a hole at the center of a PCA plate or on a circle (2.5 cm) of cellulosic pad. Plates were sprayed with 100 µl (approximately 10^8 cells) of a culture of single microbial species or the meat suspension grown for 24 h, and incubated over night at 37 °C to grow mesophilic microorganism or at 4 °C to grow psychrophilic microorganisms. The antimicrobial activity was estimated from the halo extension (mm) of growth inhibition.

2.6. Tests of antimicrobial properties of emulsions and cellulosic pads in small meat samples

Ten g of beef minced meat purchased from a supermarket was amended with 10^3 CFUs/g of *C. jejuni*, *S. enterica* and *S. aureus*. Meat was then mixed with 0.5% of each emulsion or laid over the cellulosic pads (15 × 5 cm). An aliquot of 3 g was immediately used for DNA extraction (T0) and the remaining sample was stored at 4 °C for 7 days (T1). Each amount was subdivided in aliquot of 1 g to conduct three independent DNA extractions for each sample.

Primers specific for each bacterial species that had been identified in meat samples were designed using the software Primer 3 v.0.4.0 available at the site http://bioinfo.ut.ee/primer3-0.4.0/primer3/. Primer sequences and other characteristics are shown in Table 2. Real time PCR (qPCR) was performed in a final volume of 20 µl consisting of 1× SYBR GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen, Karlsruhe, Germany), 2 µM for all primers (except 12S NAD that was used at 1 µM), and 5 ng µl of DNA. The qPCR was conducted in ABI PRISM® 7000 Real-Time PCR System (Applied Biosystem, Foster City, CA) with the following thermal protocol: 50 °C for 2 min, 95 °C for 10 min, 35 cycles of 95 °C for 15 s, and 60 °C for 60 s. Three replicates of each DNA sample were analyzed.

The data from the qPCR were analyzed following the comparative C_T method ($\Delta\Delta C_T$) using the control sample T1 as calibrator (Livak and Schmittgen, 2001) and 16S NAD (Nadkarni et al., 2002) as endogenous control gene (Table 2). Significance of differences between samples was assessed by *t*-test.

2.7. Sensory evaluation

Sensory evaluation was conducted by 16 trained judges between the age of 25–50 years, on samples of raw and cooked minced meat after 4, 12 and 15 days of storage, which corresponds to the expiration date of the commercial products and 3 days after. The raw evaluation was carried out on samples in sealed trays, assessing the color and odor of the meat.

The evaluation of the cooked product was carried out on samples (meatballs) of 15 g each, at the same time intervals after for the raw test. The meat samples had been steamed for seven minutes, without adding salt. The parameters evaluated were color odor and taste. Each judge independently assigned a score between 1 and 10 (1–3: not acceptable; 4–5: not acceptable but improvable; 6–8 acceptable; 9–10 good product). The final score was calculated by averaging all scores. The product was considered acceptable if all the parameters evaluated were acceptable.

2.8. Tests on packaged meat

Minced meat (300 g) was packaged in a modified atmosphere containing 30% CO_2 and 70% O_2 in 20 × 30 cm plastic trays. The meat was placed over the pads, amended with or without the emulsions, with an average thickness of 3 cm. The hamburgers (100 g) were packaged in the same way with a thickness of 2 cm. All trays were sealed securely with a plastic film.

The antimicrobial activity of pads treated with emulsions Mediterranean, Etnic and IT131017 was assessed in three independent productions (test 1, 2, 3) of minced meat and hamburger after 2, 12 and 15 days (T2, T12, T15) of storage at 4 °C. For each storage period, 3 trays of minced meat and 3 trays of hamburger were analyzed. From each tray, 3 g of meat were taken along the entire thickness and suspended in 27 ml of peptoned water by vortex. After centrifugation of 5 min at 3000 ×*g*, 100 µl of supernatant were directly plated on PCA

Table 2
Characteristics of primer pairs employed in this work. Ta is the temperature of annealing.

Microrganism	Primer name	Primer sequence	Accession number of target sequence or reference	Ta (°C)	Amplicon length (bp)
Campylobacter jejuni	CAMP1	F 5′-TGGTAATGTTTTAGGTTCTAGTGGTGTG-3′	Y11648	59	201
		R 5′-TTCACAGGCTTACCCATATCCA-3′		59	
Enterococcus faecalis	Entero 3	F 5′-CACAAGTCGCTGCTGTTTCA-3′	AF245684	59	84
		R5′-CGCCGTCGTTACCAACTTTT-3′		59	
Lactococcus lactis subsp. *cremoris*	Lacto 3	F 5′-ACCGCTGATCATTTGGGACT-3′	U81621	59	100
		R 5′-GGTCAACTGCCAAGCGATTT-3′		59	
Pseudomonas fluorescens	16SPSEflu	F 5′-TGCATTCAAAACTGACTG-3′	Scarpellini et al., 2004	60	850
		R 5′- AATCACACCGTGGTAACCG-3′		60	
Pseudomonas fragi	Fragi 3	F 5′-TTGTTTCATCGACAGGCACG-3′	U89151	59	92
		R 5′-TCGCCGATCAAGTCAAACAC-3′		59	
Pseudomonas putida	Pu1	F 5′-TGGCACATCCCGCAGTT-3′	NC 002947	58	83
		R 5′-TCGATGACGAAGCGTGAGTACT-3′		59	
Salmonella enterica	SA4	F 5′-CCAATGGCGGCGAATTAC-3′	Agrimonti et al., 2013	59	71
		R 5′-GGATCCCTTTGCGAATAACATC-3′		58	
Staphylococcus aureus	STAF1	F 5′-GGCATGGAGATGAAAGAAGCA-3′	AF105976.2	59	151
		R 5′-CTGTAGCAGCTAATCCTTTCGAAA-3′		60	
Microbial population (end point PCR)	16S rRNA	F 5′-AGAGTTTGATCCTGGCTCAG-3′	Lane, 1991	56	1450
		R 5′-ACGGGCTACCTTGTTACGAC-3′		56	
Microbial population (real time PCR)	16S NAD	F 5′-TCCTACGGGAGGCAGCAGT-3′	Nadkarni et al., 2002	59	466
		R 5′-GGACTACCAGGGTATCTAATCCTGTT-3′		58	

Fig. 1. Chemical profiles. Chemical profiles of emulsions IT131017, Mediterranean, 81700239 and Etnic as determined by GC–MS.

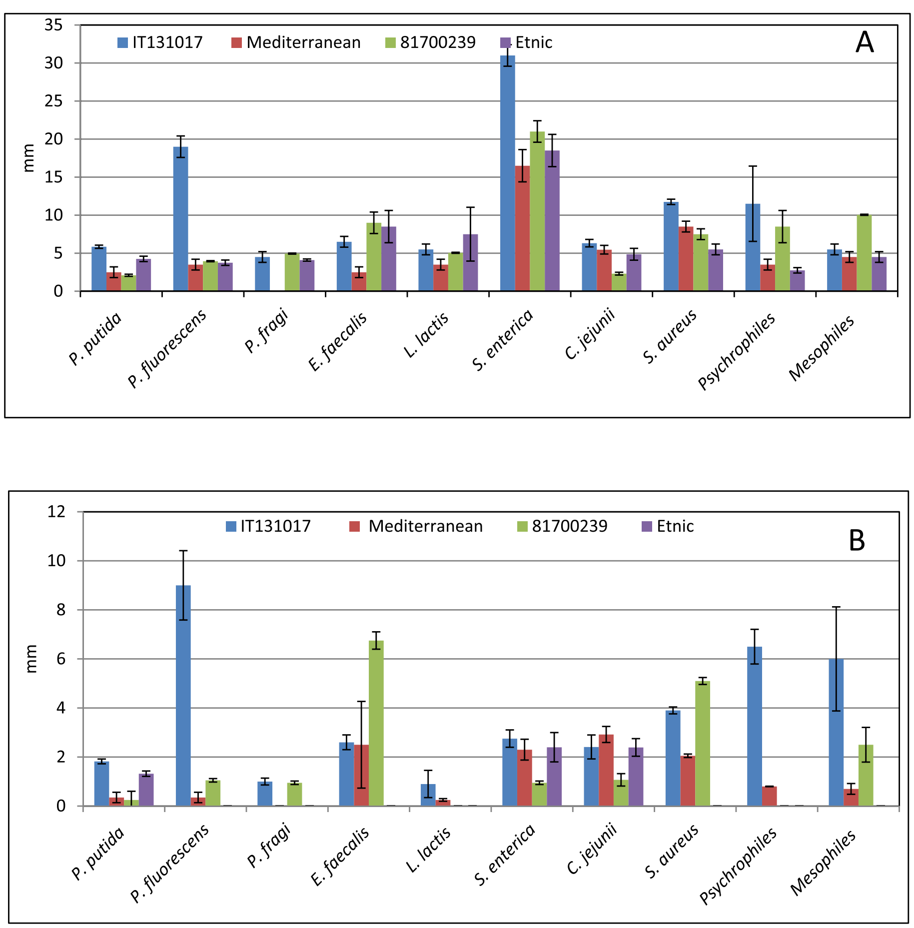


Fig. 2. Halo inhibition test. Dimensions (mm) of the halo of growth inhibition of emulsions IT131017, Etnic, Mediterranean and 81700239 (A) and respective pads (B). The values are the averages of three replicates; the bars are standard errors.

Table 3
Sensory tests conducted on samples of minced meat packaged in the presence of pads amended with different emulsions. Rating scale: 0–3: not acceptable; 4–5: not acceptable but improvable; 6–8: acceptable; 9–10: good product.

Raw	Time	Score		Judgement
		Color	Odor	
Mediterranean	T4	7.12 ± 1.09	7.19 ± 1.82	Acceptable
	T12	6.12 ± 0.72	6.25 ± 0.93	Acceptable
	T15	6.12 ± 0.50	5.44 ± 0.82	Not acceptable but improvable
Etnic	T4	9.06 ± 0.44	8.06 ± 0.77	Acceptable
	T12	8.06 ± 0.77	8.00 ± 0.81	Acceptable
	T15	6.12 ± 0.50	6.19 ± 0.98	Acceptable
81700239	T4	8.00 ± 0.63	7.81 ± 0.65	Acceptable
	T12	6.00 ± 0.63	6.25 ± 0.68	Acceptable
	T15	5.31 ± 0.70	5.50 ± 0.63	Not acceptable but improvable
IT137110	T4	8.06 ± 0.77	7.75 ± 0.68	Acceptable
	T12	6.69 ± 0.79	7.00 ± 0.63	Acceptable
	T15	6.94 ± 0.68	7.25 ± 0.88	Acceptable

Cooked	Time	Score			Judgement
		Color	Odor	Taste	
Mediterranean	T4	6.50 ± 0.82	7.12 ± 0.88	6.25 ± 0.58	Acceptable
	T12	6.12 ± 0.72	6.25 ± 0.93	6.06 ± 0.77	Acceptable
	T15	6.12 ± 0.50	6.25 ± 0.93	6.06 ± 0.68	Acceptable
Etnic	T4	6.31 ± 0.60	6.69 ± 0.60	6.45 ± 0.74	Acceptable
	T12	7.06 ± 0.77	6.94 ± 0.77	7.06 ± 0.44	Acceptable
	T15	6.12 ± 0.50	6.19 ± 0.98	6.19 ± 0.75	Acceptable
81700239	T4	6.06 ± 0.77	4.06 ± 0.68	4.12 ± 0.81	Not acceptable
	T12	5.62 ± 0.62	3.81 ± 1.17	3.12 ± 0.88	Not acceptable
	T15	5.19 ± 0.65	3.75 ± 0.68	2.75 ± 0.93	Not acceptable
IT137110	T4	8.06 ± 0.57	7.8 ± 0.62	8.06 ± 0.77	Acceptable/Good
	T12	6.44 ± 0.96	6.44 ± 0.73	6.12 ± 0.81	Acceptable
	T15	6.25 ± 0.68	6.31 ± 0.70	6.06 ± 0.77	Acceptable

agar (T2) or after dilution 10^{-1} (T12) and 10^{-2} (T15). Plates were incubated at 4 °C for 2 days and colonies were counted. Statistical analysis (ANOVA) was aided by Daniel's XL Toolbox for Excel, version 7.3.2, by Daniel Kraus, Würzburg, Germany (www.xltoolbox.net).

3. Results

3.1. Selection of emulsions and chemical analysis

The emulsions of cinnamon and oregano inhibited the growth of microorganisms in the PCA medium at a concentration of 0.5%, whereas the emulsion of thyme inhibited growth at 0.75% (Table 1). The inhibition tests on cultures of meat microorganisms showed that emulsions IT131017, Mediterranean and 81700238 were active at concentration of 0.5%, while Etnic was active at 0.75% (Table 1).

The chemical profile of these four emulsions was characterised by GC–MS (Fig. 1): IT131017 and Mediterranean contain the highest percentages of thymol (69.2% and 55.4%, respectively) and carvacrol (22.9 and 22.1%, respectively); Mediterranean also contains α- and β-pinene (5.02 and 5.11% respectively). Etnic contains less carvacrol and thymol, but is characterised by the presence of linalool (11.1%) and 2-methyl-3-phenylpropanal (53.2%). As expected, emulsion 81700238, derived from EOs of cinnamon, contains high percentage of (E)-Cinnamaldehyde (77.6%).

3.2. Effectiveness of emulsions against specific microorganisms

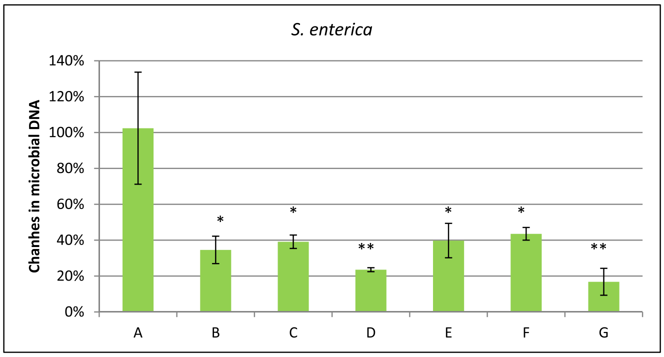
The 16S gene sequence of colonies isolated from meat showed high homology (> 99.99%) with the same genes of *P. putida*, *P. fluorescens*, *P. fragi*, *L. lactis* (subsp. cremorii) and. *E. faecalis*. The GenBank accession number of these sequences was respectively MK691960, MK691699, MK691688, MK680825 and MK680825. These species, as well the total cultivable mesophilic and psicrophylic microorganisms and some common foodborne pathogens, (*S. enterica*, *C. jejuni*, *S. aureus*) were the targets to assess the antimicrobial properties of the emulsions.

Measuring the halo inhibition of bacterial growth showed that emulsions IT131017 and Mediterranean were the most active against psychrophilic microorganisms, while 81700238 was the most active against mesophilic species (Fig. 2A). All four emulsions were active against *S. enterica*: in particular, IT131017 produced a halo of 31 mm, while the others yielded halos > 15 mm. IT131017 was also the most active against *C. jejuni*, *P. fluorescens*, *P. putida* and *S. aureus*. The other emulsions were active against all bacterial species tested, although with different levels of efficacy (Fig. 2A).

The tests conducted with the pads yielded remarkably consistent results with those obtained from the emulsions; some minor discrepancies may be due to a different migration of some components from cellulose. IT131017 and Mediterranean were the most active against the psychrophilic bacteria. Pad IT131017 was the most active against *P. fluorescens*, *S. enterica*, *P. putida* and mesophiles, while pad Mediterranean showed activity against all bacterial species except *P. fragi*. Pad Etnic was active against the psychrophilic bacteria, *S. enterica*, *P. putida* and *C. jejuni* (Fig. 2B). Pad 81700239 was particularly active against *E. faecalis* and *S. aureus*.

3.3. Sensory tests on meat packaged with emulsified pads

Sensory evaluation established that all the samples of meat examined had a perceptible taste and odor of the EOs; however, all were



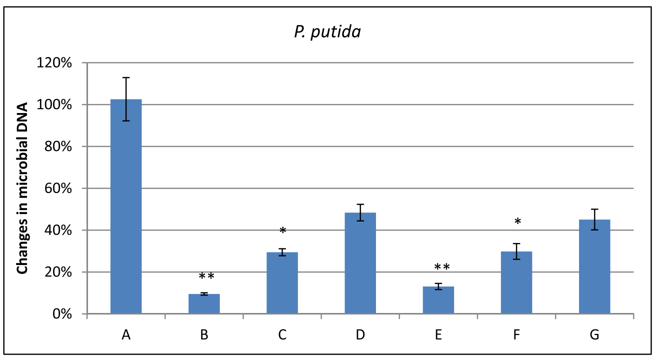


Fig. 3. Microbial quantification in meat by relative qPCR. Results of qPCR performed on DNA of *S. enterica* and *P. putida* extracted from samples of meat. Untreated control (A), meat mixed with emulsion IT131017 (B); Mediterranean (C), Etnic (D) meat laid on pad IT131017 (E), Mediterranean (F), Etnic (G). Data are expressed as percentages of variation with respect to the control A. Bars represent standard deviations of nine replicates (3 DNA extraction x 3 qPCR). Asterisks mark significant difference with respect to the control A: $^{*}p < 0.05$; $^{**}p < 0.01$.

considered acceptable according to the parameters evaluated. The exception was 81700239, which was then excluded from the subsequent tests (Table 3).

3.4. Effects of emulsions on specific microorganisms in meat

The relative quantification of specific microbial genes through qPCR was used to assess the growth of specific microbial species in samples of meat. The untreated control at time 1 (sample A1) was used as calibrator. Relative qPCR indicated a reduction of *S. enterica* growth in all treated samples as compared with the control (Fig. 3). This reduction was particularly evident in samples treated with the emulsion and the Etnic pad (to 23% and to 16% of the control, respectively) but all samples differed significantly from the control ($p < 0.05$). The growth of *P. putida* was significantly reduced in samples treated with the emulsions IT131017 and Mediterranean (to 9 and 2% of the control respectively) and their cellulosic pad (13 and 29%) (Fig. 3). No significant inhibition of the growth of other microorganisms was observed.

3.5. Tests on packaged meat

In samples of minced meat and hamburgers packaged in the presence of Mediterranean pad, there was a reduction of the bacterial load in all three independent tests conducted at the times T12 and T15 with respect to the control (Fig. 4). Although the decrease in microbial load was rather modest (0.3–0.8 Log CFUs/g), it is observable in all samples and in some cases, highly significant, especially at time T12 (Figs. 4, 1A, B). Also, packaging in presence of the Etnic caused a decrease in the microbial count, although the decrease was more modest than that caused by the Mediterranean pad and significant only at T12 (Figs. 4, 2A, B). The pad IT131017 had no significant effect on microbial growth in either in minced meat or in hamburgers (Fig. S1).

4. Discussion

This work explored the possibility of an active packaging which exploits emulsions derived from EOs of oregano, thyme and cinnamon. As expected, GC–MS analysis has shown that all four selected emulsions contained thymol and carvacrol. Emulsion 8170023881, deriving from EOs of cinnamon, also contains a high percentage of cinnamaldheyde, which is known to be highly effective against food borne pathogens (El-Baky et al., 2013).

Although the basic composition of IT131017, Mediterranean and Etnic is quite similar, there are some differences that may be responsible for the different activities observed against the microorganisms tested. Mediterranean contains α and β-pinene, two monoterpenes initially isolated from EOs of pine, but found also in thyme (Imelouane, 2009) and oregano (Ozkan et al., 2010) active against food borne pathogens, as *E. faecalis* and *S. aureus* (Ozogul et al., 2015). Etnic is characterised by a significant percentage of 2-methyl-3-phenylpropanal derived from the addition of cumin (Jalali-Heravi et al., 2007) and linalool, a monoterpene identified primarily in EOs of basil, (Suppakul et al., 2003) but also present in some species of cumin (Mohammadpour et al., 2012), and in varying amounts in thyme EOs (Bagamboula et al., 2004). Recent *in vitro* and *in vivo* studies have demonstrated that linalool has a comprehensive range of bioactive properties that can be exploited for pharmaceutical and medicinal applications, including some activity against Gram positive bacteria (Aprotosoaie et al., 2014; Pereira et al., 2018).

The emulsion IT131017 contained the largest amount of thymol and carvacrol and was active against most of the bacteria analyzed; in particular, its activity against *P. putida* and *S. enterica* was also confirmed by qPCR in small samples of meat. As reported in the introduction, the activity of thymol and carvacrol against a large spectrum of microorganisms has been demonstrated (Nabavi et al., 2015). The antimicrobial activity of EOs of thyme and oregano against *Pseudomonas* spp. has also been shown by other authors. Oral et al., observed a 2 log CFUs/g reduction of *Pseudomonas aeruginosa* in chicken packaged with absorbent pads (Oral et al., 2009); others observed more modest activity, with about a 0.9–0.5 log CFUs/g reduction (Oussalah et al., 2004; Božik et al., 2017).

Pad 81700239 showed the highest activity against the cultures of *E. faecalis*, a Gram negative enterococcus frequently isolated in beef meat; this presence, like that of all enterococci, must be minimized because it causes serious infections and is a reservoir for antibiotic resistance (Jahan et al., 2013). Pad 81700239 also showed the highest activity against cultures of *S. aureus* in accordance with other authors (Zhang et al., 2015; Condo et al., 2018) that found good activity of cinnamon EOs against this species. Alternatively, See and Jenwitheesuk did not report any significant activity (See and Jenwitheesuk, 2018). The antibacterial activity of cinnamon EOs also depends on the food product: Van Haute et al. found that these EOs are active against yeast and molds in salmon but not in scampi (Van Haute et al., 2016). However, pad 81700239 was not tested in meat because the strong taste of cinnamon, and resulting negative evaluation in the sensory tests.

The antimicrobial efficiency of emulsions and treated pads was not the same once they were added to meat. This discrepancy, also observed by other authors, has been attributed to the greater availability of nutrients in meat that could allow a more rapid repair of bacterial damaged structures (Burt, 2004) or to a binding between meat proteins or fatty acids with EO components, which hinders access of the bioactive molecules to the microbial targets (Burt, 2004; Gutierrez et al., 2008). de Oliveira et al. observed that the EOs of oregano are particularly active against *S. enterica* during the first 1–2 days of minced meat storage (de Oliveira et al., 2013). Similarly, Amariei et al.

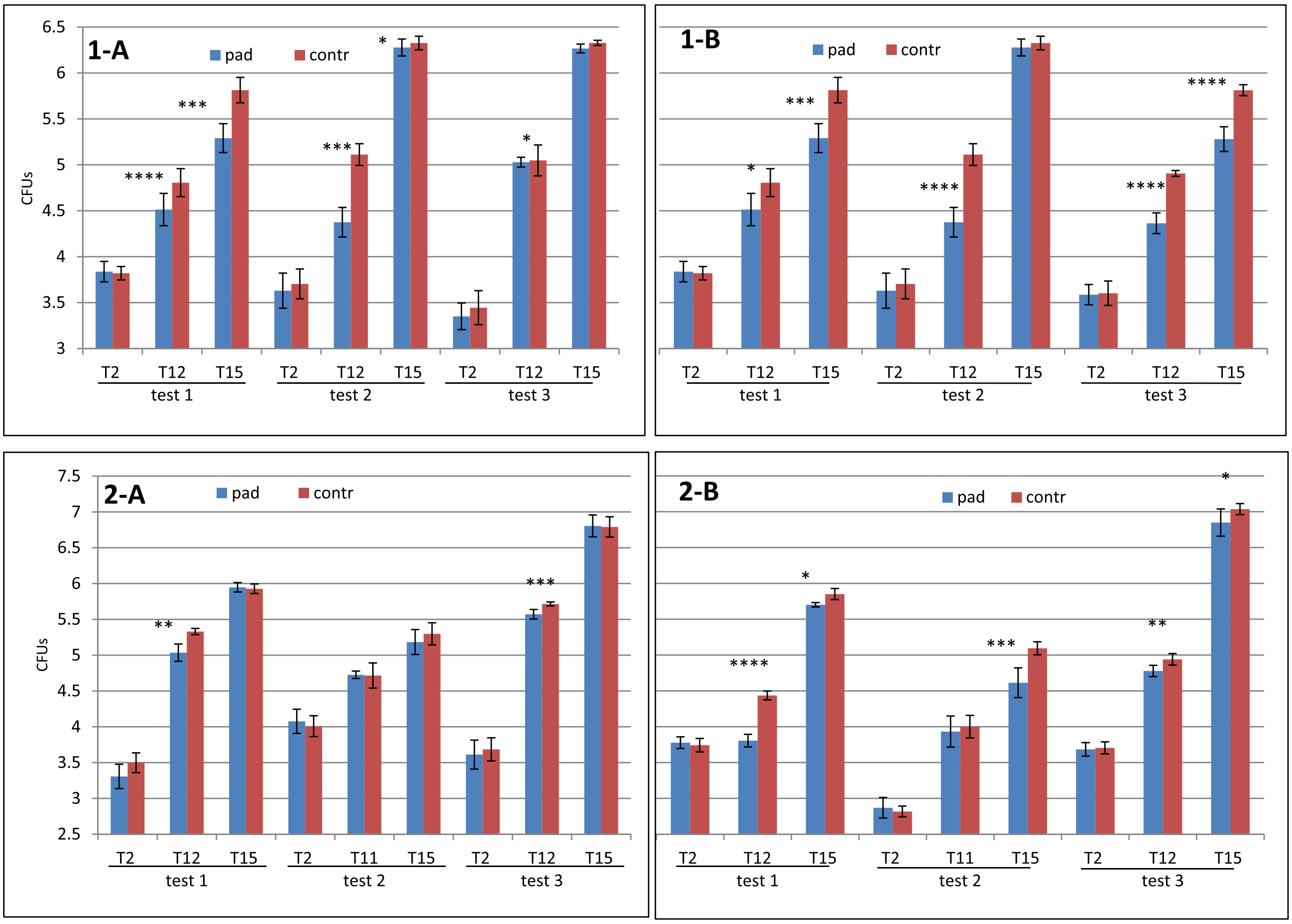


Fig. 4. Tests on packaged meat. Total count of psychrophilic microorganisms in samples of meat packaged in presence of pads Mediterranean (1) and Etnic (2) and relative controls. A: minced meat; B: Hamburger. The asterisks indicate the significance level determined by ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

observed a reduced count of lactic bacteria, yeast and molds after one day of storage, but after four days CFUs/g were similar to those in the control (Amariei et al., 2016). It is possible that during the early storage phase, these active molecules are more available for the bacterial targets; then, upon dissolution in the lipid phase of the meat or when reacting with proteins, the compounds become less available for bactericidal action (de Oliveira et al., 2013). Differently, Zengin and Baysal observed a slight decrease in the aerobic mesophilic microorganisms in minced meat samples treated with EOs of thyme and clove in the later days of storage (Zengin and Baysal, 2015). In this work we did not analyze early storage as our goal was to establish whether the emulsions were able to prolong the self-life of the minced meat, however, the fact that the major differences between the control and the treated samples were observed at time T12 but not at T15 suggests there was a progressive reduction of the antimicrobial activity of the EOs.

The Mediterranean pad proved to be the most effective at slowing the growth of psychrophilic microorganisms in commercial trays of minced meat and hamburger, whereas IT131017 had no significant effect. This result is somewhat unexpected as the tests conducted on pure microbial cultures revealed that the IT131017 pad had equivalent efficacy to the Mediterranean against the psychrophilic microorganisms and was highly effective against most microbial species tested. In addition to the different antimicrobial capacity of EOs in culture media and food matrices discussed in the previous paragraph, the reasons for this difference can be attributed to microbial species that were not necessarily the same in culture and in meat which contains a complex microbiome; here, some species may not be cultivable in synthetic media or their relative abundance may be different from that used in microbiological cultures.

IT131017 contains the highest percentages of carvacrol and thymol, but Mediterranean contains other components such as α and β-pinene and linalool, which also possess antimicrobial activity. Linalool is contained at a slightly higher percentage in the Etnic emulsion, which also causes a reduction of the microbial load in the meat samples, albeit to a more modest extent compared to the Mediterranean. It is possible that these different components exert synergistic activity that strengthens the overall antimicrobial activity. This is in agreement with Burt, who demonstrated that mixtures of minor components of EOs are more effective than mixtures of major components (Burt, 2004). Synergistic effects of EO mixtures have also been observed by other authors (Lopez et al., 2007; Gutierrez et al., 2008; Zengin and Baysal, 2014).

The composition of the EOs is highly dependent on the variety of the species and the environmental conditions in which the plants grow; this makes difficult to standardize the technology (Suppakul et al., 2003; Boskovic et al., 2015; Ozogul et al., 2015). Another problem is the strong aroma of EOs, perceptible even at low concentrations below which antimicrobial activity is lost (Van Haute et al., 2016). From our tests, we have excluded pad 81700239, which from the *in vitro* tests was quite efficient against *E. faecalis* and *S. aureus*, as the cinnamon aroma interfered negatively with that of the meat. EOs of oregano and thyme gave a more pleasant taste when added to meat; in fact, samples treated with them were positively evaluated in the sensory test. This was also observed by Yemiş and Candoğan (2017), who reported good scores for beef packaged in edible soy incorporated with up to 3% of thyme and oregano. However the aroma of oregano and thyme changed the taste of the meat in a way that it could be considered a new product, and thus needs to be submitted to consumer evaluation before it can be placed on the market. Therefore, the application of lower concentrations of EOs is advisable. Some authors observed that combination with other compounds such as nisin (Solomakos et al., 2008), caprylic acid (Hulankova et al., 2013) or modified atmosphere packaging (Karabagias et al., 2011) can significantly improve the antimicrobial activity of EOs of oregano and thyme. Indeed, extraction with supercritical CO_2 reduces the aromatizing effect of EOs, but this can affect their antimicrobial properties and increase the cost of the technology (Bagamboula et al., 2004).

5. Conclusions

In conclusion, the modest but reproducible reduction of the microbial burden in meat packaged in presence of Mediterranean and Etnic pads shows that the incorporation of EOs in cellulose can be a strategy to extend product shelf-life. Notably, this reduction after 12 and 15 days of storage is rather modest and not exploitable at this stage. However, these results represent a starting point for further investigation to improve the antimicrobial activity of cellulosic pads, looking more closely to single chemical components or to more complex mixtures of these of natural origin.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108246.

Declaration of Competing Interest

There is no conflict of interest to declare.

Acknowledgment

Funding: This work was supported by the European Commission [projects INTENSE: grant numbers 652515 and FOOD INTEGRITY: grant number 613688]. The authors are also grateful to Technopole SITEIA-Parma University of Parma for technical infrastructures, to Dr. Elena Masocco for providing meat packs, to Ms. Terri Arsenault for scientific and technical assistance in GC–MS analysis and to Dr. Giorgio Capretti for providing cellulosic pads.

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