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Characterization of oregano (*Origanum vulgare*) essential oil and definition of its antimicrobial activity against *Listeria monocytogenes* and *Escherichia coli in vitro* system and on foodstuff surfaces

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First aim of this research was to characterize oregano (*Origanum vulgare*) essential oil and the characterization of its minimum inhibitory concentration against the pathogenic species, *Listeria monocytogenes* and *Escherichia coli*. Moreover, the oregano essential oil antimicrobial activity was tested against these pathogenic species, inoculated onto wood and stainless steel surface. The GC/MS profile of oregano essential oil revealed the presence of 34 compounds, principally terpinolene, carvacrol and p-cymene accounting for about 70% of the total area of the identified molecules. Oregano essential oil showed higher antimicrobial activity against *L. monocytogenes* in comparison with *E. coli*. In fact, the *L. monocytogenes* minimum inhibitory concentration ranged between 125 and 200 mg/L while those for *E. coli* ranged between 250 and 350 mg/L. Regarding the decontamination efficacy, the washing of the two surfaces with oregano fastened the viability decrease of both the inoculated microorganisms over time. This phenomenon was more pronounced for wood as compared to steel. The data obtained suggests the great potential of this essential oil to be employed, as alternative to traditional chemicals, and as sanitizing strategy for surfaces.

Key words: Oregano essential oil, GC/MS, surface decontamination, minimum inhibitory concentration.

INTRODUCTION

The adhesion and persistence of microorganisms in equipment surfaces have the potential to spread pathogens and spoilage microorganisms to foods, influencing their shelf-life and safety (Bae et al., 2012). This is particularly significant in the food processing industry (Giaouris and Nychas, 2006) as well as in the domestic environment (Humphrey et al., 2001; Choi et al., 2012). The surfaces of equipment used for food handling, processing and storage are considered as major sources of microbial contamination (Bae et al.,

2012). Several studies have shown the ability of microorganisms to attach to surfaces commonly found in the food processing environment, such as stainless steel, polystyrene, hydroxyapatite, rubber, glass and wood (Soares et al., 1992; Barnes et al., 1999). Additionally, if certain microorganisms remain on a given surface for a relatively long time, they can continue to replicate and eventually form biofilms (Uhlich et al., 2006). The microbial attachment and the eventual biofilm formation, acting as reservoir of spoilage and pathogenic species, increase significantly the risk for food contamination (Valeriano et al., 2012). In fact, microorganisms can be easily detached from surfaces and/or biofilms and contaminate foods, causing reduced product shelf-life and disease transmission (Shi and Zhu, 2009). Several studies have shown that various foodborne pathogens including *Escherichia coli* and *Listeria monocytogenes* can survive for hours or even days on utensils and equipment surfaces (Humphrey et al., 2001; Wilks et al., 2005, 2006; Martinon et al., 2012). On the other hand, *L. monocytogenes* and *E. coli* are among the most frequently involved bacterial species in foodborne diseases (Scallan et al., 2011; Oliveira et al., 2012). Consequently, controlling the longevity of microorganisms in surfaces is fundamental in reaching food safety standards and improving food quality and shelf-life (Nitschke et al., 2009).

Several chemical detergents and disinfectants are commonly used and their application depends on their efficacy, safety and toxicity, corrosive effects, ease of removal and the subsequent sensory impact on the final products (Møretrø et al., 2009). Many of these chemicals are corrosive to equipment and toxic to humans if over exposure occurs (Lee and Pascall, 2012). In addition, conventional cleaning and disinfection regimes may also contribute to antimicrobial resistance dissemination (Lunden et al., 2003; Minei et al., 2008; Ryu and Beuchat et al., 2005; Surdeau et al., 2006; Cruz and Fletcher, 2012).

Therefore, new sanitizing strategies based on the use of bio-solutions containing enzymes, phages, inter-species competitions, antimicrobials of microbial origin and natural plant molecules are constantly emerging (Simões et al., 2010; Chorianopoulos et al., 2008). The growing negative consumer perception against synthetic chemical compounds favors the research of such natural alternatives (Davidson, 1997). Essential oils (EOs) are volatile, natural, complex compounds characterized by a strong odor and formed by aromatic plants as secondary metabolites. They have been studied for their antimicrobial activity against many microorganisms, including several pathogens (Dorman and Deans, 2000; Delaquis et al., 2002).

The activity of oils from *Lamiaceae* (Tassou et al., 2000; Gunduz et al., 2010) has been investigated in model and real food systems in order to understand the action of single constituents, their cell targets and to balance their intrinsic variability. Moreover, EOs and their bioactive components have been recently studied also for their antibacterial activity on surface adherent microorganisms in order to evaluate their potential as disinfectants in the food industry (Chorianopoulos et al., 2008; Oliveira et al., 2012) and as promising anti-biofilm agents (Amalaradjou and Venkitanarayanan, 2011). *Origanum vulgare* essen-tial oil has been largely studied for this purpose and its composition, in relation to its geographical origin, dry and extraction methods, has been investigated (Mockute et al., 2001; Teixeira et al., 2013; Figiel et al., 2010). In fact, it is well known that the oil composition, and particularly the presence of phenolic content, can increase its antimicrobial properties. Thus, information regarding the oil composition and the effectiveness of its bioactive components in killing pathogenic species on food contact surfaces is needed to aid in the development of optimal sanitation conditions for food industries.

The aims of this study were:(i) to evaluate the efficacy of killing *L. monocytogenes* and *E. coli* in solution, calculating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oregano essential oil, reported to have antimicrobial activity against a large variety of microorganisms (Marino et al., 2001; Viuda-Martos et al., 2007) and (ii) to evaluate the oregano EO efficacy in reducing pathogenic cell loads on food contact surfaces such as wood and stainless steel. Most of the food processing industry's surfaces such as machinery, pipelines and working surfaces are made of stainless steel. This material is traditionally selected in the kitchen for food preparation because of its mechanical strength, corrosion resistance and longevity (Carrasco et al., 2012). Wood, although less employed in food industry than in domestic food preparation, is often used as cutting boards (Soares et al., 2012). Different contamination levels and contact times were

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Abbreviation: MIC, Minimum inhibitory concentration; **MBC,** minimum bactericidal concentration; **EO,** essential oil; **SPME,** solid phase micro extraction.

assessed for each tested surface.

MATERIALS AND METHODS

Strains

L. monocytogenes Scott A and *E. coli* 555, used in this work, belong to the strain collection of the Department of Agricultural and Food Sciences, University of Bologna. The strains were maintained at -80°C and cultured in brain heart infusion (BHI) broth (Oxoid, Basingstoke, Humpshire, UK) for 24 h at 37°C. Before experiments, the strains were sub-cultured, on BHI broth for 24 h.

Essential oils

In this work, the oregano (*Origanum vulgare)* essential oil was obtained from Flora s.r.l. (Pisa, Italy).

Characterization of oregano essential oil using GC/MS-solid phase micro extraction (SPME)

Oregano EO in amount of 0.5 mL was placed into a 10 mL vial and sealed through a PTFE/silicon septum. Three different samples were prepared for each EO. The samples were conditioned for 30 min at 25°C. An SPME fiber covered by 50 mm divinylbenzene-carboxen-poly (dimethylsiloxane)- (DVB/CARBOXEN/PDMS StableFlex) (Supelco, Steiheim, Germany) was exposed to each sample at room temperature (25°C) for 20 min, and finally, the adsorbed molecules were desorbed in the GC for 10 min. For peak detection, an Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett-Packard, Geneva, Switzerland) and a Varian (50 m×320 µm×1.2 µm) fused silica capillary column were used. The temperature program, starting from 50°C, increased to 230°C at 3°C/min, this temperature was maintained for 1 min. Injector, interface, and ion source temperatures were 200, 200 and 230°C, respectively. Injections were performed with a split ratio of 30:1 and helium as carrier gas (1 mL/min). Compounds were identified by the use of the Agilent Hewlett-Packard NIST 98 mass spectral database.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of oregano EO against *L. monocytogenes* and *E. coli*

For the determination of MIC values, 150 µL of BHI broth inoculated at three different levels (2, 4 or 6 log cfu/mL) of the tested pathogens (*L. monocytogenes* and *E. coli*), were added to 200 µL microtiter wells (Corning Incorporated, NY, USA). Oregano essential oil was properly diluted in ethanol 96% (VWR international, PROLABO, France) and 50 µL of the different dilutions were added in the microtiter wells, in order to obtain oregano EO concentrations ranging between 50 and 400 mg/L. Microtiter plates were incubated at 37°C and checked after 48 h. The MBC were determined by spotting 10 µL of each well after 48 h, onto BHI agar plates. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound preventing visible growth of the inoculated cells after 48 h (MIC48h). The MBC was defined as the lowest concentration of the compound that caused the death of the inoculated cells and therefore there was no growth after 48 h of incubation at 37°C of a 10 µL spot plated onto BHI agar.

Sanitization tests on surfaces

Stainless steel and wood surfaces were used for decontamination experiment with EOs. The sizes of the surfaces were 1 and 2.25 cm^2 for stainless steel and wood, respectively. Before use, the surfaces were sterilized by autoclave at 121°C for 15 min.

The target microorganisms chosen for this experiment were *E. coli* and *L. monocytogenes*. Both target microorganisms were inoculated at a concentration of 6.2 log cfu/cm^2 for wood and 7 log cfu/cm^2 for stainless steel. The inoculum was prepared from the pre-inoculum by making serial dilution in aphysiological solution, and the surfaces were inoculated with 10 (stainless steel) or 100 µL (wood). The inoculated surfaces were dried at room temperature for 0, 15, 30 and 60 min before treatments with Oregano EO. The treatments were performed by the immersion of the surfaces in 20 mL of Oregano EO solutions used at concentration of 125 mg/L for the treatment of the surfaces inoculated with *L. monocytogenes*, and 250 mg/L for the surfaces inoculated with *E. coli*. Oregano EO was delivered through 1% of ethanol. The duration of treatments was 10 min and the surfaces were removed from the solutions and placed into 10 mL of physiological solution, to determine viable bacteria by plate counting. *E. coli* was determined on Violet Red Bile Agar (VRBA, Oxoid, Basingstoke, Hants, England) with addition of MUG (Oxoid) supplement while Listeria Selective Agar based (Oxford formuladion) (Oxoid, Basingstoke, Hants, England) was used to detect *L. monocytogenes*.

Data processing and statistical analysis

The cell load data were analyzed by means of ANOVA one way by using Statistica for Windows.

RESULTS AND DISCUSSION

GC/MS-SPME characterization oregano essential oil

Preliminarily, oregano EO was characterized using GC/MS-SPME. This technique was chosen because it gives a measure of the volatile molecules of the oil and the preliminary condition for the antimicrobial effects of EO is the contact between the antimicrobial molecule and the target cells. The contact is favored if the molecules are in their vapor phase, that corresponds to their most hydrophobic state, because this improves their partition in the cell membranes. In addition, this technique provides a volatile profile fingerprinting fundamental to standardize the EO composition in terms of the most effective molecules and consequently to standardize antimicrobial activity of the essential oils. In fact, the EO composition, and consequently the volatile molecule profile, can notably vary with plant variety and origin, extraction modality, agronomic practices, etc (Nannapaneni et al., 2009). Table 1 shows the total area of the GC peaks and the percentage (on the basis of the relative peak area) of each compound present in the headspace of the oregano

Table 1. GC/MS-SPME characterization of oregano *(O. vulgare)* essential oil.

Molecule	Total peak area	Area (%)
α-Pinene	29616709	3.43
Camphene	3431254	0.40
β-Pinene	1664439	0.19
3-Carene	1693569	0.20
β-Myrcene	22216747	2.57
α-Phellandrene	2456150	0.28
α-Terpinene	33644575	3.89
Limonene	8547408	0.99
β-Thujene	5079423	0.59
γ-Terpinene	72693569	8.41
p-Cymene	309885246	35.86
Terpinolene	3166331	0.37
Ylangene	1739261	0.20
α-Cubebene	7410171	0.86
β-Bourbonene	3635866	0.42
Linalol	322546	0.04
Caryophyllene	49654406	5.75
(+)-Aromadendrene	1836300	0.21
Carvone	122563	0.01
α-Caryophyllene	1293689	0.15
γ -Muurolene	2910065	0.34
α-Terpineol	140525	0.02
Borneol	1637263	0.19
Copaene	311791	0.04
β-Farnesene	1325112	0.15
α-Muurolene	237200	0.03
δ-Cadinene	3114797	0.36
γ-Cadinene	1189108	0.14
Anetol	529966	0.06
Calamenene	525772	0.06
p-Cymen-8-ol	244657	0.03
p-Timol	1092970	0.13
Thymol	41459717	4.80
Carvacrol	249347302	28.85

EO, as well as the cumulative percentages of the classes of compounds (monoterpenes, sesquiterpenes, oxygenated monoterpenes, aliphatic alcohols, aliphatic aldehydes, esters and ketones). The volatile profiles of the used oregano essential oil was characterized by the presence of 34 identified molecules belonging to different chemical classes. The main components of this type of oregano were terpinolene, carvacrol and p-cymene accounting for about 70% of the total area of the identified molecules. These data are in agreement with the data of Ortega-Nieblas et al. (2011), Russo et al. (1997) and Bisht (2009) who found carvacrol as one of the major components. Also, according to Teixeira et al. (2013), who studied the composition of oregano essential oil from Portuguese origin, carvacrol, terpinene and thymol were the main components. This is positive because a wide literature attributed to carvacrol and to monoterpenes the great antibacterial activity of oregano EO (Burt, 2004; Gutierrez et al., 2008; Oussalah et al., 2006). In fact, such molecules can interact with some cellular structures causing the inhibition of cell growth or cell death. However, according to Caccioni et al. (1998), to evaluate the antimicrobial activity of an EO it is fundamental to use a holistic approach due to

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oregano *(Origanum vulgare)* essential oil against *L. monocytogenes* and *E. coli* in relation to the inoculum level.

Microorganism	Cell concentration (log cfu/mL)					
	6 log cfu/mL		4 log cfu/mL		2 log cfu/mL	
	MIC 24 h (mg/L)	MBC (mg/L)	MIC 24 h (mg/L)	MBC (mg/L)	MIC 24 h (mg/L)	MBC (mg/L)
L. monocytogenes	175	225	175	225	125	150
E. coli	350	350	300	325	250	250

synergistic or antagonistic actions among the different EO components.

MIC and MBC determination

The MICs and the MBCs of the oregano EO against *L. monocytogenes* Scott A and *E. coli*555 were assessed after incubation at 37°C with three levels of the target microorganisms (Table 2). Differences in the MICs and MBCs were observed in relation to species and the inoculum level taken into consideration. In fact, increasing the inoculation level increased the MIC and MCB values for both microorganisms considered. This data are in agreement with literature (Belletti et al., 2010). Oregano EO showed the highest antimicrobial activity against *L. monocytogenes* with respect to *E. coli*. In fact, the *L. monocytogenes* MIC ranged between 125 and 200 mg/L while those for *E. coli* ranged between 250 and 350 mg/L. This behavior for Gram-negative bacteria can be due to the presence of the outer membrane, which acts as an efficient permeability barrier against macromolecules and hydrophobic substances, as well as to the high content in cyclopropane fatty acids of the inner membrane (Chang and Cronan, 1999).

Effects of oregano EO in decontaminating stainless steel and wood surfaces inoculated with *L. monocytogenes* and *E. coli*

To evaluate the decontamination efficacy of oregano EO, stainless steel and wood coupons previously sterilized were inoculated at level of 7 and 6.2 log cfu/cm^2 with *L. monocytogenes* and *E. coli*, respectively. Immediately after the inoculation and after 15, 30, 60 min at room temperature (about 25°C), the coupons were treated with 20 ml of oregano EO treatment solutions at concentration of 125 ppm for *L. monocytogenes*, or 250 ppm for *E. coli*., corresponding to the MIC values previously determined in antimicrobial assay. After 10 min of contact between the coupons and the EO solution, the surfaces were removed from treatment solutions and were placed into 10 ml of physiological solution, which was used for the determination of the surviving *L. monocytogenes* and *E. coli* cells. In Figures 1 and 2, the results obtained for *E. coli* and *L.* monocytogenes, respectively, are shown. A decrease of viability over time was observed independently of microorganisms and oregano EO supplementation. The viability decreases were more pronounced on wood material than in steel coupons, independently of the treatment time and EO supplementation. 60 min after inoculation, *E. coli* and *L. monocytogenes* were present on the control steel coupons (untreated) at cell loads of 6.6 and 5.9 log cfu/cm^2, respectively. Significantly lower counts (3.6 and 4.2 log cfu/cm^2, for *E. coli* and *L. monocytogenes,* respectively) were recorded in the control wood coupons 60 min after inoculation. Earlier research indicate that survival of microorganisms on surfaces is affected by many factors including temperature, microbial species (Rusin et al., 2002), nature of surfaces (Gill and Jones, 2002), time lapsed post-inoculation, moisture level and inoculum size (Monville and Schaffner, 2003).

The addition of oregano EO speed up the viability decrease of both microorganisms. The treatment with EO, at the concentration used, reduced, after 10 min of contact, *E. coli* cell loads of 1.9 and 1.2 log cfu/cm^2 in steel and wood, respectively while *L. monocytogenes,* immediately after the inoculation on steel and wood coupons reduced its counts of about 2 and 1 log cfu/cm^2, respectively.

When the treatment with the EO was performed after 30 and 60 min from the inoculation of the coupons, lower microbial counts were recorded with respect to treatment carried out immediately after the inoculation. This phenomenon was more pronounced in wood in comparison with steel.

This result can be due to the porosity of the wood where the microbial cells might penetrate under the surface of the wood. On the other hand, several authors make remarks on the problem of recovery of microorganisms from porous or damaged surfaces (De Vere and Purchase, 2003). Earlier research indicates the decreased number of microorganisms over time deliberately inoculated on wood surfaces (Carpentier, 1997). For example Abrishami et al. (1994) observed a reduction of 98% 2 h after inoculation of new wood by *E. coli*,

Figure 1. Recovery of *Escherichia coli* cell loads (log cfu/cm^2) inoculated onto stainless steel (washed or not with oregano essential oil) and wood coupons (washed with oregano essential oil or not). The treatment with oregano essential oil was performed for 10 min after that the recovery of the pathogenic strain was performed immediately after treatment (0), after 15, 30, 60 min. For each group considered, different letter represent significant differences ($p<0.005$).

Figure 2. Recovery of *Listeria monocytogenes* cell loads (log cfu/cm^2) inoculated onto stainless steel (washed or not with oregano essential oil) and wood coupons (washed with oregano essential oil or not). The treatment with oregano essential oil was performed for 10 min after that the recovery of the pathogenic strain was performed immediately after treatment (0), after 15, 30, 60 min. For each group considered, different letter represent significant differences ($p<0.005$).

while Ak et al. (1994) observed a reduction of 99.9% of *L. monocytogenes* after 2 h. Also Milling et al. (2005) showed a consistent viability loss of the inoculated micro-organism on wood surfaces. These authors showed that the survival of the bacteria on wood was dependent on various factors such as the wood species, the type of the inoculated bacterium, the ambient temperature, and humidity and attributed it to the better hygienic perfor-mances of pine and oak with respect to plastic in combination with the hygroscopic properties of wood and the effect of wood extractives. Similar results were observed by Gehrig et al. (2002) and Schonwalder et al.

(2002) who concluded with the possibility that bacteria are transferred into the wood surface by absorption with no evidence of a subsequent release.

Conclusion

This research shows the good potential of the used oregano essential oil to inhibit pathogenic microorganisms both when tested as planctonic cells and when inoculated onto surfaces of industrial interest. In particular, the trials of surface decontamination have highlighted the ability of this type of oregano essential oil to inactivate *L. monocytogenes* and *E. coli* after just 10 min of contact, independently of the surface considered. The reductions obtained, representing more than 90% of the population, are very promising, also taking into account that the inoculation levels tested exceeded significantly those present on industrial surfaces. The American Public Health Association recommends that chemical sanitizers are able to reduce the pathogenic species and mesophilic bacteria of stainless steel surfaces up to 0.3 log cfu/cm^2. The trials we performed inoculating *L. monocytogenes* and *E. coli* at level of 10-100 cfu/cm^2 of surface and treating with oregano essential oil permitted reaching cell loads under the detection limit after 10 min of contact. According to APHA, the sanification level is acceptable when the coliform cell loads are under 5 cfu/cm^2 and acceptable when ranging between 5-100 cfu/cm^2. Moreover, according to Lelieveld e al. (2003), an ideal sanitizer should have characteristics such as wide action spectrum, environmental resistance, toxicity and corrosiveness absence. In our opinion, oregano essential oil could be considered as new tool to prevent or delay colonization of food contact surfaces. However, its use at industrial level still requires additional investigations on the ability of removing it and on its organoleptic impact.

Conflict of Interest

The authors did not declare any conflict of interest.

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