FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

No induction of antimicrobial resistance in *Staphylococcus aureus* and *Listeria monocytogenes* during continuous exposure to eugenol and citral

Joana Apolónio[1,2], Maria L. Faleiro[1], Maria G. Miguel[3] & Luís Neto[4]

[1]IBB-Centro de Biomedicina Molecular e Estrutural, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal; [2]Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal; [3]IBB-Centro de Biotecnologia Vegetal, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal; and [4]Faculdade de Ciências e Tecnologia, Study Center of Agricultural Sciences and Technology, Universidade do Algarve, Faro, Portugal

Correspondence: Maria Leonor Faleiro, Universidade do Algarve, FCT, IBB-CBME, Campus de Gambelas, 8005-139, Faro, Portugal. Tel.: +351 289800100; fax: +351 289818419; e-mail: mfaleiro@ualg.pt

Received 3 February 2014; revised 26 March 2014; accepted 4 April 2014. Final version published online 24 April 2014.

DOI: 10.1111/1574-6968.12440

Editor: Mark Enright

Keywords
essential oil; adaptation; resistance; virulence.

Abstract

The aim of this study was to evaluate the adaptation response of *Staphylococcus aureus,* methicillin-resistant *S. aureus* (MRSA), and *Listeria monocytogenes* to the essential oil (EO), eugenol, and citral. The minimum inhibitory concentration of eugenol and citral was determined by agar dilution and microdilution. Adaptation to eugenol and citral was done by sequential exposure of the pathogens to increasing concentrations of the essential oils. The M2-A9 standard was used to determine the antibiotic susceptibility. The effect of eugenol and citral on the adherence ability was evaluated by the crystal violet assay. The impact of adaptation to eugenol on virulence was estimated using the *Galleria mellonella* model. No development of resistance to the components and antibiotics was observed in the adapted cells of *S. aureus*, MRSA, and *L. monocytogenes*. Eugenol and citral at subinhibitory concentration reduced the bacterial adherence. Adaptation to subinhibitory concentration of eugenol affected the virulence potential of *S. aureus*, MRSA, and *L. monocytogenes*. Eugenol and citral do not pose a risk of resistance development in a continuous mode of use. These EO components showed a high efficacy as antistaphylococcal and antilisterial biofilm agents. Adaptation at subinhibitory concentration of eugenol protected the larvae against listerial and staphylococcal infection.

Introduction

Either in clinical or in food-processing environment, the bacterial pathogens encounter a myriad of stresses (to name a few; exposure to antimicrobial agents, acids, bases, different osmotic pressures, different pH and water activity values, heat, freezing and thawing). The action of these stresses compromises the cell viability but at the same time, the cells experience a gene expression alteration directed to protect them against each individual stress or even mount a cross-protection response that not only provide the cell with the means to defeat the individual stress but also a complex of stresses injuries (Cebrián *et al.*, 2010; Bergloz *et al.*, 2013). Moreover, cells exposed to stresses can also be armed to beat the host barriers. This type of response is well known in several bacterial pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* and has been known for some time (Davies *et al.*, 1996; Chan *et al.*, 1998; Clements & Foster, 1999; Garner *et al.*, 2006). The threatening emergence of antibiotic-resistant bacteria is of great concern worldwide, and the possibility of food microbiota being reached by antibiotic resistance is greatly disturbing (Divergiliis *et al.*, 2011; Canizalez-Roman *et al.*, 2013; Centers for Disease Control & Prevention, 2013; Wang *et al.*, 2013). The use of natural products such as essential oils (EO) to control foodborne and multiresistant bacteria has been largely investigated and constitutes a promising approach (Hyldgaard *et al.*, 2012; Faleiro & Miguel, 2013). Due to

the multitarget mode of action of essential oils (Faleiro, 2011; Hammer *et al.*, 2012), it is unlikely that bacteria will develop resistance to the natural products. However, this hypothesis cannot be discarded, and several studies with opposite results have emerged (McMahon *et al.*, 2007, 2008; Hammer *et al.*, 2012).

Eugenol, a phenylpropanoid, is the main component of the essential oil of clove (*Syzygium aromaticum*) acting against both Gram-positive and Gram-negative bacteria by affecting the membrane permeability and the uptake and utilization of glucose (Di Pasqua *et al.*, 2006; Devi *et al.*, 2010). Citral (3,7-dimethyl-2,6-octadienal) is a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B) that can be isolated from the leaves and fruits of a large variety of citrus plants and also shows a significant activity against several pathogens (Fisher & Phillips, 2006). Citral acts by disrupting the cytoplasmic and the outer membrane of *Escherichia coli* cells and the sigma factor RpoS, which is involved in *E. coli* response to citral damage (Somolinos *et al.*, 2009). The European Commission accepted both eugenol and citral as flavoring agents in food products (Regulation EU 872/2012).

Taking into account a possible context of continuous exposure of bacterial pathogens to EO components, this study aimed to investigate the impact of adaptation of *S. aureus* and *L. monocytogenes* to eugenol and citral on the development of resistance to antibiotics and to the EO components. The effect of both components on bacterial adherence was investigated. The influence of adaptation to eugenol on virulence was also investigated using a *Galleria mellonella* model.

Materials and methods

Bacterial strains and growth conditions

The culture medium brain-heart infusion (BHI) was purchased from Biokar Diagnostics, Beauvais, and the Mueller-Hinton broth and the tryptic soy broth (TSB) were purchased from Oxoid (Basingstoke, Hampshire, UK). For solid media, agar (VWR) was added at 1.5% w/v. The bacterial strains of *S. aureus* and *L. monocytogenes* used in this study are indicated in Table 1. Bacteria were stored in BHI (*S. aureus*) or TSB (*L. monocytogenes*) supplemented with 25% v/v glycerol at −80 °C and, when necessary, recovered in BHI or TSB. The bacterial cultures maintenance was carried out in the same media supplemented with agar at 4 °C. Prior to use, bacteria were transferred to fresh BHI or tryptic soy agar (TSA) plates and incubated at 37 °C (*S. aureus*) or 30 °C (*L. monocytogenes*) for 24 h.

Table 1. List of bacteria used in the study

Bacteria	Origin and characteristics	Source, Reference
Staphylococcus aureus ATCC 6538	Wound	American Type Culture Collection
Staphylococcus aureus methicillin-resistant 4 (MRSA 4)	Clinical	UAlg, IBB-CBME, Portugal
Staphylococcus aureus methicillin-resistant 12 (MRSA 12)	Clinical	UAlg, IBB-CBME, Portugal
Listeria monocytogenes EGD	Clinical, serovar 1/2a	UL, UK
Listeria monocytogenes ScottA	Clinical, serovar 4b	Dr. J. McLauchlin, Public Health England
Listeria monocytogenes C882	Portuguese cheese, serovar 4b	Faleiro *et al.* (2003)

UAlg: Universidade do Algarve, UL: Department of Infection, Immunity and Inflammation, University of Leicester, United Kingdom.

Essential oil components

Eugenol (99%) and citral (95%) were purchased from Sigma-Aldrich (Madrid-Spain). A stock solution of each essential oil component at 10% w/v was prepared in 2-propanol and maintained at −20 °C. The required concentrations were obtained from this stock solution.

Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of eugenol and citral was determined according to the Clinical and Laboratory Standards Institute M7-A7 standard (CLSI, 2006). The tested concentrations of eugenol and citral were 0.06, 0.08, 0.1, 0.15, 0.2, and 0.25 mg mL^{-1}. The inoculum was obtained by cultivation of the bacterial strains in BHI agar plates during 24 h at 37 or 30 °C. From each plate, a loop was used to prepare a suspension with a turbidity of 0.5 of the McFarland scale (10^8 CFU mL^{-1}). Decimal dilutions of the bacterial suspension were plated in BHI agar plates with the appropriate EO component concentration using the procedure described by Miles & Misra (1938). The MIC value was determined as the lowest concentration that inhibited the visible bacterial growth. Four replicates for each strain were used. A plate containing 2-propanol (0.54% v/v) and chloramphenicol (30 μg mL^{-1}) was used as control.

The MIC value was also determined by the microdilution method as described by Faleiro *et al.* (2005). The inoculum was prepared as described above. Each well of a flat-bottom microplate (Greiner, Labortechnick, Frichenhausen, Ger-

many) was filled with 180 μL of BHI supplemented with eugenol or citral at appropriate concentration. Twenty microliters of the bacterial suspension prepared with the culture medium supplemented with the EO component at the proper concentration was used to inoculate each well. The growth was followed by spectrophotometry ($OD_{600\ nm}$) in a microplate reader (Tecan Infinite M200, Tecan, Austria). Wells containing the culture medium supplemented with 2-propanol or chloramphenicol were included, as control. A set of wells containing the culture medium with no antimicrobial agent represented the negative control. Three biologic and three technical replicates for each strain were used ($n = 9$). The MIC value was considered the lowest concentration of the essential oil component that caused the inhibition of the bacterial growth (no increase in the $OD_{600\ nm}$ after 24–48 h). The lowest concentration that did not allowed the recovery of cells in agar plates was considered the minimum bactericidal concentration (MBC) (Faleiro *et al.*, 2005).

Adaptation to eugenol and citral

The effect of adaptation (successive passage) of the bacterial strains on the susceptibility to eugenol and citral components was evaluated as described by Hammer *et al.* (2012). The bacterial strains were grown in BHI agar plates during 24 h at 37 or 30 °C. From each plate, a loop was used to inoculate 10 mL of BHI broth, and the culture was incubated overnight. From the overnight, 300 μL culture was centrifuged (2790 × ***g***, 5 min at 4 °C). The pellet was resuspended in 300 μL BHI supplemented with 0.05 mg mL^{-1} eugenol or citral and used to inoculate 10 mL of BHI (supplemented with the same concentration). Cultures were incubated at 37 °C during 18–24 h. The culture was used to inoculate fresh medium supplemented with eugenol or citral (0.05 mg mL^{-1}). During 4 days, bacteria were transferred sequentially at the same concentration. At each serial passage, the bacterial growth was followed by optical density ($OD_{600\ nm}$). Following the four sequential passages, bacteria were transferred to BHI at higher concentration (increment of 0.02 mg mL^{-1}). The process was repeated until bacteria stop growing. Control cultures (bacteria were grown in medium with no EO component) were maintained in parallel. Three independent replicates were carried out.

Influence of adaptation on virulence

The impact of adaptation to eugenol on bacterial virulence was determined using larvae of the Greater Wax Moth *G. mellonella* L. (*Lepidoptera: Pyralidae*) as previously described (Joyce & Gahan, 2010; Schrama *et al.*, 2013). Larvae weighted between 250 and 350 mg and reared during the first and second instars with pollen and wax and afterward an artificial diet prepared with a mixture of glycerol, honey, water, dry dog food, and wheat bran. The development of the insects was carried out in the dark at 30 °C.

Each *S. aureus* or *L. monocytogenes* culture (10^8 CFU mL^{-1}) adapted to eugenol (0.05 mg mL^{-1}) during three consecutive days was centrifuged at 2790 ***g*** for 5 min at 4 °C. The bacterial cells were resuspended in 1 mL of sterile PBS. Ten larvae were infected with 10 μL of the initial suspension (10^6 CFU) by injection on the second right proleg using a microsyringe (50 μL, Sigma, Madrid, Spain). Before injection, larvae were disinfected on the surface with ethanol at 70% v/v. Larvae were placed in sterile Petri dishes at 37 °C during 5 days. Death was checked each 24 h and was considered when no response to touch was observed. Three independent experiments were carried out.

Influence of eugenol and citral on adherence

The impact of eugenol and citral on adherence of *S. aureus* and *L. monocytogenes* was carried out according to Adrião *et al.* (2008). Bacterial suspensions ($OD_{600\ nm}$ = 0.4–0.5) in BHI supplemented with 0.05 and 0.1 μg mL^{-1} of eugenol or citral were allowed to adhere for 30 min. Nonadherent cells were removed by washing the wells twice with sterile phosphate-buffered saline (PBS). Wells were air-dried and adherent cells heat-fixed at 80 °C for 30 min and stained with 0.1% crystal violet (Merck, Germany). After the dissolution of the stain by addition of ethanol–acetone (80 : 20), the $OD_{595\ nm}$ was determined with a microplate reader (Tecan Infinite M200 Model, Austria).

Statistical analysis

Data regarding the effect of adaptation to eugenol and citral on the bacterial growth and on adherence were analyzed for statistical significance by analysis of variance (ANOVA) with the SPSS 21.0 program (Inc., Chicago IL). Statistical significance was set as $P < 0.05$; when the analysis was statistically significant, the Tukey's post hoc test was done. The significant differences on virulence were determined by Student's *t*-test.

Results

Susceptibility to eugenol and citral

The observed MIC values for eugenol and citral determined using the agar dilution and the microdilution methods are indicated in Table 2. Differences were observed between the MIC values either of eugenol or of citral, in particular for *S. aureus* strains with trend to a lower MIC values obtained by the microdilution method;

the range of the MIC value for eugenol was 0.10–0.15 mg mL^{-1} for *S. aureus* strains determined by the agar dilution method, whereas the MIC value determined by the microdilution method ranged from 0.06 to 0.08 mg mL^{-1}. The susceptibility of *S. aureus* strains to citral was similar to eugenol (Table 2). In contrast to *S. aureus*, no differences were observed in the MIC values of eugenol for *L. monocytogenes* determined by the two methods (Table 2). However, for citral, the *L. monocytogenes* strains showed lower MIC values when microdilution method was used: by the agar dilution, the MIC value ranged between 0.08 and 0.1 mg mL^{-1}, whereas by the microdilution, the range was between 0.06 and 0.08 mg mL^{-1}. The MBC value determined by the microdilution was 0.1 mg mL^{-1} for all bacterial strains except for *L. monocytogenes* Scott A that was 0.08 mg mL^{-1}.

Adaptation to subinhibitory concentrations of citral and eugenol does not induce resistance to oil components or antibiotics

To evaluate the impact of continuous exposure of the bacterial pathogens *S. aureus* and *L. monocytogenes* to eugenol and citral on the acquisition of resistance to the oil components, the bacterial strains were subjected to sequential passages in the presence of subinhibitory to lethal concentrations of eugenol and citral.

The ability of the bacterial strains to overcome the lethal concentration of the oil components and antibiotics after the previous sequential exposure to subinhibitory concentrations is indicated in Table 3. None of the *S. aureus* or *L. monocytogenes* was able to mount a resistance response after the adaptation exposure to subinhibitory concentrations of eugenol and citral (Table 3). The *S. aureus* ATCC 6538 and MRSA 4 adapted cells were able to overcome just three passages at the MBC concentration of eugenol (0.1 mg mL^{-1}) after the sequential passages at 0.06 and 0.08 mg mL^{-1}, whereas the MRSA 12 adapted cells were capable to overcome four passages at the MBC concentration but failed to overcome the following lethal concentration of 0.015 mg mL^{-1} after the first passage. The *L. monocytogenes* EGD and ScottA adapted cells were affected by the sequential exposure to the subinhibitory concentration of 0.06 mg mL^{-1} of eugenol, as were not able to overcome the first passage at the MIC concentration (0.08 mg mL^{-1}). In contrast, the *L. monocytogenes* C882 adapted cells evidenced to be more robust being able to overcome the four passages at subinhibitory and the MIC concentration but failed to overcome the first passage at the MBC concentration.

The MRSA 4 adapted cells were less affected by the adaptation at subinhibitory concentrations of citral being able to overcome the four passages at 0.08 mg mL^{-1} but did not succeed the MBC concentration of citral (0.1 mg mL^{-1}) after the first passage (Table 3). In contrast, the *S. aureus* ATCC 6538 and MRSA 12 adapted cells were incapable to overcome the four passages at 0.08 mg mL^{-1} of citral. The *L. monocytogenes* EGD and C882 adapted cells were capable to survive the four passages at 0.08 mg mL^{-1} but failed the first passage at 0.1 mg mL^{-1} (MBC concentration). The *L. monocytogenes* ScottA adapted cells were only able to overcome three passages at 0.08 mg mL^{-1} of citral (MBC concentration).

The influence of adaptation to subinhibitory concentration (0.05 mg mL^{-1}) of eugenol and citral on the susceptibility to the antibiotics penicillin, chloramphenicol, kanamycin, vancomycin and erythromycin was evaluated. The sequential passages of all tested bacteria to eugenol or citral did not induce resistance to the antibiotics tested (data not shown). Using the standard M100-S17, the MRSA 12 showed an intermediate susceptible profile to kanamycin and a resistant profile to erythromycin and penicillin. The MRSA 4 strain showed a resistant profile to penicillin and erythromycin, but a susceptible profile to kanamycin (Table 4).

The three *L. monocytogenes* strains were resistant to penicillin but were susceptible to the remaining tested antibiotics (Table 4).

Table 2. Minimum inhibitory and minimum bactericidal concentrations of eugenol and citral

	Eugenol			Citral		
	MIC (mg mL^{-1})		MBC	MIC (mg mL^{-1})		MBC
Bacteria	Agar dilution	Microdilution	(mg mL^{-1})	Agar dilution	Microdilution	(mg mL^{-1})
Staphylococcus aureus ATCC 6538	0.1	0.06***	0.1	0.1	0.08***	0.1
Staphylococcus aureus methicillin-resistant 4 (MRSA 4)	0.1	0.08***	0.1	0.1	0.06***	0.1
Staphylococcus aureus methicillin-resistant 12 (MRSA 12)	0.15	0.08***	0.1	0.15	0.08***	0.1
Listeria monocytogenes EGD	0.08	0.08	0.1	0.1	0.08***	0.1
Listeria monocytogenes ScottA	0.08	0.08	0.1	0.08	0.06***	0.08
Listeria monocytogenes C882	0.08	0.08	0.1	0.1	0.08***	0.1

Data represent the mean of four (agar dilution) and three (microdilution) determinations. For each oil, data with *** are significantly different ($P < 0.0001$).

Table 3. Fold change* in growth at subinhibitory and lethal concentrations of eugenol and citral

Bacteria/	Eugenol				Citral			
Passage	0.06 mg mL^{-1}	0.08 mg mL^{-1}	0.1 mg mL^{-1}	0.15 mg mL^{-1}	0.05 mg mL^{-1}	0.06 mg mL^{-1}	0.08 mg mL^{-1}	0.1 mg mL^{-1}
S. aureus ATCC 6538								
1°	39.11 ± 2.80^{a}	40.32 ± 0.66^{a}	39.25 ± 4.99^{b}		32.52 ± 5.47^{b}	13.90 ± 0.66^{a}	12.86 ± 1.64^{a}	
2°	50.00 ± 1.99^{b}	56.89 ± 7.28^{ab}	68.40 ± 1.66^{c}		18.55 ± 1.89^{ab}	19.13 ± 0.95^{a}	15.41 ± 2.40^{a}	
3°	37.09 ± 1.73^{a}	42.56 ± 3.00^{a}	6.01 ± 1.35^{a}	NG	14.44 ± 1.01^{a}	13.38 ± 2.39^{a}	14.61 ± 0.96^{a}	NG
4°	48.43 ± 1.26^{b}	63.09 ± 5.65^{b}	0.76 ± 0.08^{a}		22.06 ± 3.16^{ab}	16.28 ± 1.02^{a}	0.76 ± 0.16^{b}	
MRSA 4								
1°	31.30 ± 3.91^{a}	43.36 ± 8.39^{a}	29.72 ± 9.86^{a}		59.07 ± 5.09^{b}	40.47 ± 6.11^{b}	27.19 ± 4.23^{a}	0.79 ± 0.13
2°	35.31 ± 2.72^{a}	60.64 ± 4.93^{a}	5.63 ± 4.16^{b}		30.31 ± 0.48^{a}	18.16 ± 1.30^{a}	16.37 ± 1.41^{a}	
3°	33.68 ± 3.30^{a}	45.85 ± 6.25^{a}	0.82 ± 0.29^{b}		54.60 ± 3.23^{b}	23.02 ± 3.51^{a}	23.02 ± 3.51^{a}	NG
4°	58.08 ± 1.35^{b}	53.20 ± 7.19^{a}	NG		26.54 ± 3.68^{a}	19.04 ± 3.36^{a}	16.66 ± 1.74^{a}	
MRSA 12								
1°	39.54 ± 3.52^{a}	43.53 ± 1.86^{a}	55.47 ± 7.50^{a}	0.54 ± 0.16	46.52 ± 2.14^{b}	21.44 ± 3.59^{a}	18.88 ± 3.82^{a}	
2°	44.07 ± 2.34^{ab}	55.74 ± 1.80^{b}	47.88 ± 13.29^{a}	NG	35.41 ± 3.05^{ab}	18.64 ± 0.38^{a}	17.16 ± 0.62^{a}	NG
3°	37.54 ± 1.05^{a}	44.53 ± 0.89^{ab}	43.14 ± 1.49^{a}		25.63 ± 2.64^{a}	15.75 ± 1.35^{a}	0.78 ± 0.063^{b}	
4°	53.56 ± 1.42^{b}	55.88 ± 4.46^{b}	48.18 ± 13.36^{a}		28.58 ± 2.39^{a}	19.43 ± 0.98^{a}	NG	

Bacteria/	Eugenol			Citral			
Passage	0.06 mg mL^{-1}	0.08 mg mL^{-1}	0.1 mg mL^{-1}	0.05 mg mL^{-1}	0.06 mg mL^{-1}	0.08 mg mL^{-1}	0.1 mg mL^{-1}
L. monocytogenes EGD							
1°	28.53 ± 1.28^{a}	0.77 ± 0.20		29.35 ± 1.50^{a}	30.91 ± 1.22^{a}	11.87 ± 0.59^{a}	1.10 ± 0.20
2°	33.11 ± 0.55^{a}			22.41 ± 2.29^{a}	19.98 ± 2.34^{a}	13.08 ± 1.06^{ab}	
3°	25.50 ± 1.78^{a}	NG	NA	21.31 ± 1.61^{a}	23.13 ± 3.01^{a}	16.09 ± 1.48^{ab}	NG
4°	27.69 ± 5.2^{a}			24.60 ± 3.45^{a}	28.04 ± 3.59^{a}	18.94 ± 2.20^{b}	
L. monocytogenes ScottA							
1°	16.88 ± 1.70^{a}	0.95 ± 0.15		30.14 ± 1.68^{ab}	29.11 ± 2.06^{a}	21.76 ± 1.64^{a}	
2°	24.30 ± 1.92^{a}			21.05 ± 0.25^{c}	24.41 ± 1.35^{a}	18.85 ± 1.12^{b}	
3°	22.24 ± 1.10^{a}	NG	NA	24.14 ± 1.29^{bc}	32.66 ± 2.67^{a}	16.83 ± 0.72^{b}	NG
4°	16.47 ± 2.41^{a}			34.60 ± 2.48^{a}	32.53 ± 2.22^{a}	1.18 ± 0.26^{c}	
L. monocytogenes C882							
1°	31.50 ± 0.13^{a}	10.57 ± 0.24^{a}	1.50 ± 0.37	28.11 ± 1.11^{ab}	24.96 ± 0.84^{a}	21.41 ± 3.24^{a}	1.50 ± 0.47
2°	36.63 ± 2.14^{ab}	15.89 ± 1.20^{a}		22.80 ± 0.58^{a}	21.71 ± 1.02^{a}	16.67 ± 0.28^{a}	
3°	32.92 ± 1.69^{ab}	12.28 ± 2.36^{a}	NG	23.88 ± 1.17^{a}	24.52 ± 3.86^{a}	18.87 ± 1.35^{a}	NG
4°	39.47 ± 1.60^{b}	13.28 ± 1.57^{a}		36.15 ± 4.21^{b}	28.18 ± 2.27^{a}	21.02 ± 2.37^{a}	

*Fold change in growth at each passage is indicated as the ratio between the OD at T_{24} and the OD at T_0 (OD_{T24}/OD_{T0}), NG–No Growth. NA–Not Applied. Data are representative of three independent replicates ± standard error. For each strain, data in the column with different superscript letters are significantly different ($P < 0.05$).

Table 4. The susceptibility of the bacterial strains and the equivalent MIC value (μg mL^{-1}) for the tested antibiotics. The standard susceptibility criteria M100-S17 (CLSI, 2007) were used

	PEN		CLO		ERI		KAN		VAN	
Bacteria	R/I/S	MIC	R/I/S	MIC	R/I/S	MIC	R/I/S	MIC	R/I/S	MIC
S. aureus										
ATCC 6538	S	≤ 0.12	S	≤ 8	S	≤ 0.5	S	≤ 6	S	≤ 2
MRSA12	R	-	S	≤ 8	R	≥ 8	I	-	S	≤ 2
MRSA4	R	-	S	≤ 8	R	≥ 8	S	≤ 6	S	≤ 2
L. monocytogenes										
EGD	R	-	S	≤ 8	S	≤ 0.5	S	≤ 6	S	≤ 2
ScottA	R	-	S	≤ 8	S	≤ 0.5	S	≤ 6	S	≤ 2
C882	R	-	S	≤ 8	S	≤ 0.5	S	≤ 6	S	≤ 2

PEN, penicillin; CLO, chloramphenicol; ERY, erythromycin; KAN, kanamycin; VAN, vancomycin.

Inhibition of adherence

The *S. aureus* strains showed different ($P < 0.05$) ability to adhere to polystyrene (Fig. 1a and b). The MRSA 12 was the strain that showed the highest ability to adhere ($P < 0.05$) followed by MRSA 4. The strain *S. aureus* ATCC 6538 showed the lowest ability to adhere ($P < 0.05$) (Fig. 1a and b). As observed for *S. aureus*, also *L. monocytogenes* strains showed different adherence abilities ($P < 0.05$). The *L. monocytogenes* C882 and Scott A strains showed a similar adherence ability ($P > 0.05$) and higher than the *L. monocytogenes* EGD ($P < 0.05$) (Fig. 1c).

Both eugenol and citral at the subinhibitory concentration (0.05 mg mL^{-1}) significantly inhibited ($P < 0.05$) the adherence ability of *S. aureus* and *L. monocytogenes* strains (Fig. 1a and b). The exposure of bacterial cells at the MBC concentration of eugenol or citral during 30 min strongly reduced the adherence of all tested bacteria ($P < 0.05$).

The chlorhexidine (0.2% w/v) did not inhibit the adherence of *S. aureus* or *L. monocytogenes* strains; instead, the bacterial strains exposed to this disinfectant adhered equally well ($P > 0.05$) in comparison with the control cells (Fig. 1), except for *L. monocytogenes* C882, which adherence ability was promoted ($P < 0.05$) in the presence of chlorhexidine (Fig 1c and d). The adherence of the bacterial cells exposed to 2-propanol, the solvent used for eugenol and citral, was similar ($P > 0.05$) to the control culture (Fig. 1 a–d).

Adaptation to eugenol affects the bacterial virulence

The effect of adaptation to eugenol on the virulence of the strains of *S. aureus* ATCC6538, MRSA 12, *L. monocytogenes* Scott A, and *L. monocytogenes* C882 was evaluated using the *G. mellonella* model.

The survival of larvae injected with *S. aureus* ATCC6538 adapted cells was higher ($P < 0.05$) in comparison with the control cells until the fourth day. The injection of larvae with MRSA 12 adapted cells also resulted in a higher survival of larvae ($P < 0.05$); however, the highest survival was observed only until the second day (Fig. 2) evidencing a higher virulence of MRSA 12 in comparison with *S. aureus* ATCC 6538.

The adaptation of *L. monocytogenes* strains to eugenol also affected their virulence potential (Fig. 2). The larvae injected with Scott A adapted cells survived significantly better ($P < 0.05$) than the larvae injected with nonadapted cells along 4 days. At day five, no significantly differences ($P > 0.05$) between survival of larvae injected with adapted and nonadapted cells were found. Also the virulence of *L. monocytogenes* C882 was diminished by the

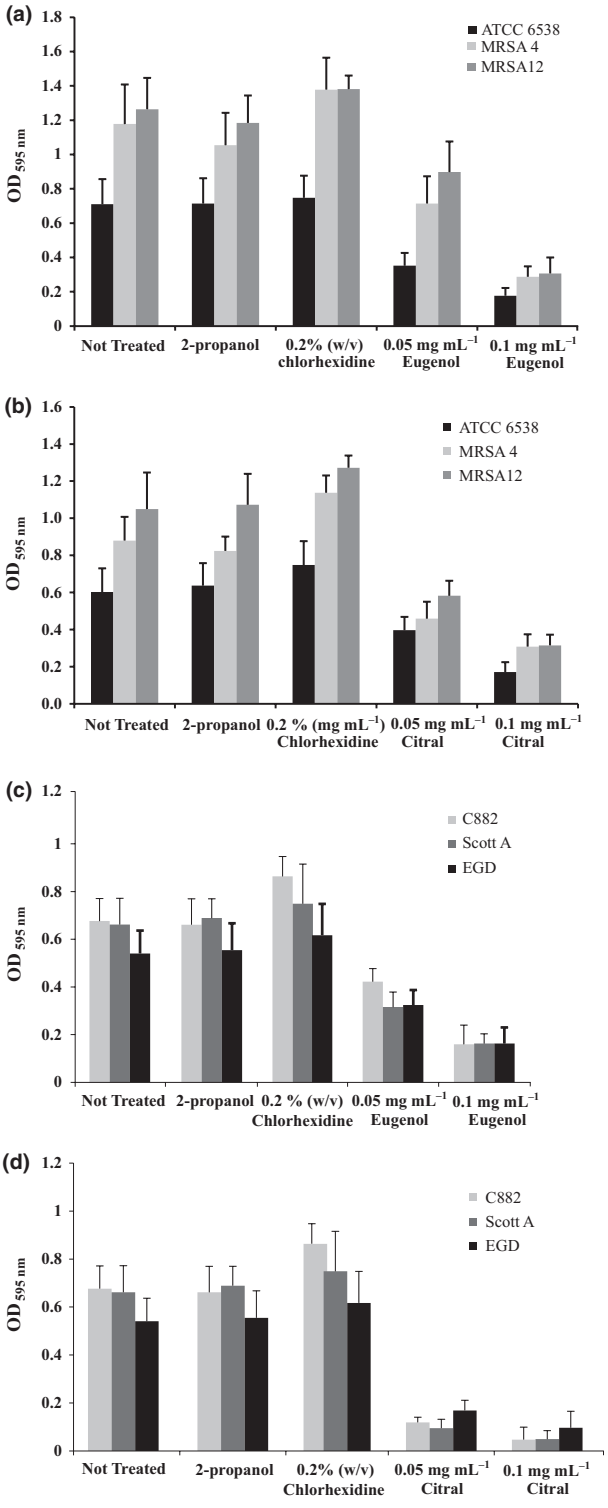

Fig. 1. Impact of eugenol and citral components on the adherence ability of *Staphylococcus aureus* ATCC 6538, MRSA 12, MRSA 4 (a) and (b), and *Listeria monocytogenes* EGD, Scott A, and C882, (c) and (d). Data are the mean of three independent experiments ($n = 12$). Error bars represent the standard deviation.

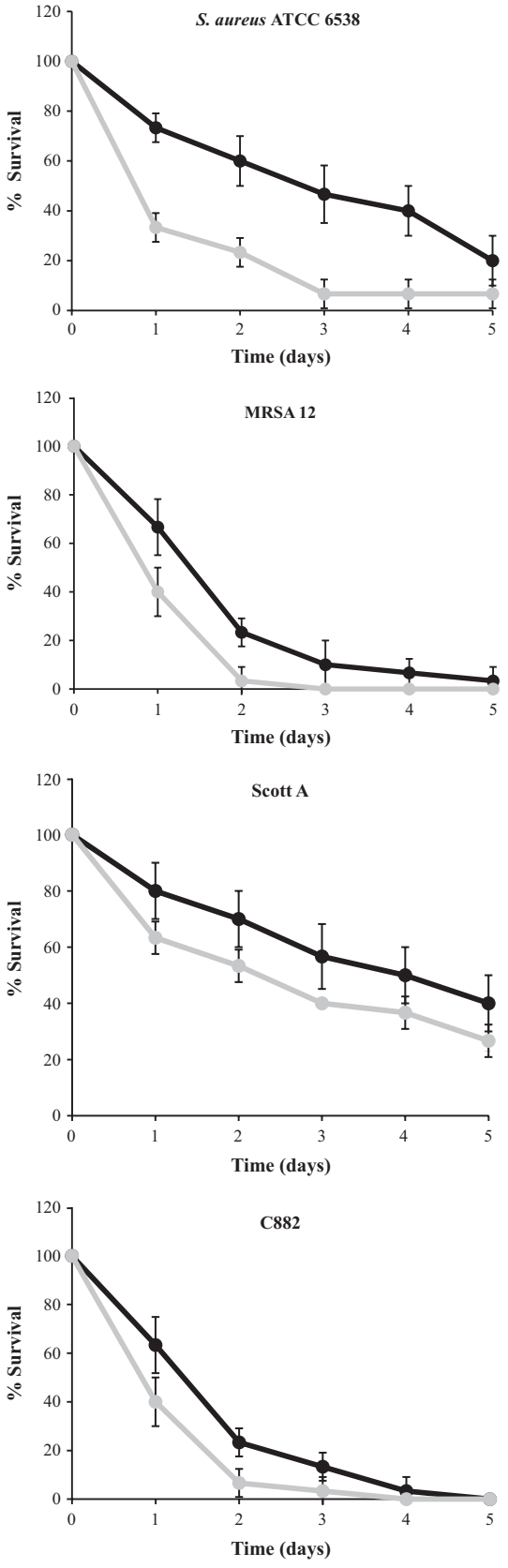

Fig. 2. Survival of insects after injection with (●) adapted (serial passage at 0.05% mg mL^{-1} of eugenol) and (●) nonadapted cells (serial passage in BHI in the absence of eugenol, control passage). Data are the mean of three independent experiments ($n = 30$). Error bars represent the standard deviation.

eugenol sequential exposure, the larvae injected with C882 adapted cells survived significantly better ($P < 0.05$) during the first 2 days in comparison with nonadapted cells. *L. monocytogenes* C882 demonstrated a higher virulence potential in comparison with the Scott A strain, after 3 days, no larvae survival was observed for either adapted or nonadapted cells.

Larvae injected with PBS achieved a 100% survival during the 5 days.

Discussion

The testing of antibacterial activity of essential oils or their components is still lacking standardization (Burt, 2004). In virtue of this is important to validate the determination of MIC values by more than one method. In the present study, the agar dilution and the microdilution method were used to determine the MIC value of eugenol and citral against *S. aureus* and *L. monocytogenes* strains. The differences observed between the MIC values obtained by the two methods suggest that for these two EO components, the bacterial cells were more susceptible when microdilution method was used, except for *L. monocytogenes* cells that showed a similar susceptibility to eugenol tested by the two methods. The difference between the two methods may be associated with the lower diffusion of the components on the agar matrix, as observed in the study of Skandamis & Nychas (2000), and the cells inside the colony can gain protection from the surrounding cells (Burt, 2004).

The MIC values of eugenol for Gram-positive and Gram-negative bacteria that have been reported are in the range of 0.125–0.75% (v/v) (Oyedemi *et al.*, 2009; Ali *et al.*, 2013; Devi *et al.*, 2013). The observed MIC values for eugenol in our study seem to be quite similar to the previous reported. A higher susceptibility of *E. coli* to citral in comparison with *L. monocytogenes* has been reported (Somolinos *et al.*, 2008, 2009). In our study, the outbreak strain *L. monocytogenes* Scott A was more susceptible to citral than the cheese isolate *L. monocytogenes* C882 and *L. monocytogenes* EGD suggesting that different susceptibilities to this EO component can be found among *L. monocytogenes* strains. The *S. aureus* strains showed a similar susceptibility pattern to citral, as observed for *L. monocytogenes* strains.

The development of antimicrobial resistance is a worldwide concern, being the MRSA strains one of the

foremost examples of this concern (80 461 infections per year from which 11 285 result in death) (CDC, 2013). However, the emergence of antibiotic resistance in other pathogens, such as *L. monocytogenes* will represent a serious health problem due to the high mortality rate, is fatal in 20–30% of cases (Farber & Peterkin, 1991; Swaminathan & Gerner-Smidt, 2007).

The number of studies on the use of essential oils against clinical, including multiresistant bacteria and foodborne pathogens, has been increasing and has shown the high potential of EO to control a large number of pathogens (Burt, 2004; Hyldgaard *et al.*, 2012; Faleiro & Miguel, 2013). It is now important to clarify the impact of a continuous exposure of pathogens to EOs and their components on the development of resistance, either to EO or components and antibiotics. In our study, the sequential exposure of *S. aureus* and *L. monocytogenes* strains to eugenol or citral did not result on the development of resistance to the oil components itself or to antibiotics. These findings have been reported in the few studies that have investigated the induction of resistance to essential oils or their components (Hammer *et al.*, 2012; Luz *et al.*, 2012; Ali *et al.*, 2013; Thomsen *et al.*, 2013). A very few studies have reported a reduced susceptibility of bacterial strains continued exposed to tea tree oil (TTO) (McMahon *et al.*, 2007, 2008). In those studies, the described diminished antibiotic susceptibility of TTO adapted cultures showed to be reversible as when the cells were subcultured in the absence of TTO, the previous susceptibility profile was reverted. The observed diminished susceptibility can be associated with the selection of a portion of the bacterial population that become more resistant (persisters cells) during the adaptation process or a phenotypic adaptation that assigns a low tolerance level usually linked to reversible modifications of the lipidic composition of the cellular membrane or to changes in the efflux pumps (Keren *et al.*, 2004; Papadopoulos *et al.*, 2008; Hammer *et al.*, 2012). The no development of resistance in conditions of continuous exposure to EO or their components can be associated with the multitarget mode of action of essential oils (Faleiro, 2011; Hyldgaard *et al.*, 2012).

EOs and their components are effective against biofilms, which are microbial cell structured aggregates displaying tight adherence to surfaces (Niu & Gilbert, 2004). Biofilm cells are of particular concern by virtue of exhibit a high resistance to different antimicrobial agents in comparison with their planktonic counterpart (Gilbert *et al.*, 2002; Ito *et al.*, 2009).

Recently, Zhou *et al.* (2013) showed that eugenol at 400 μM inhibited the biofilm formation of *Pseudomonas aeruginosa* PAO1 due to the quorum-sensing system (QS) disruption. The adherence ability of all tested strains in our study was impaired by the exposure to subinhibitory concentration of eugenol or citral. How eugenol and citral modulate the biofilm formation mechanisms of *S. aureus* and *L. monocytogenes* needs to be further investigated.

It is noteworthy that chlorhexidine (0.2% w/v) did not inhibit the adherence ability of all tested strains; in fact the adherence of the *L. monocytogenes* C882 was promoted. The low capacity of chlorhexidine to combat biofilm formation has been reported (Bonez *et al.*, 2013).

The bacterial virulence potential can be challenge during exposure to stress conditions (Conte *et al.*, 2000; Garner *et al.*, 2006; Shaw *et al.*, 2008). To clarify the impact of adaptation on virulence, adapted *S. aureus* ATCC 6538, MRSA 12, and *L. monocytogenes* Scott A and C882 cells to subinhibitory eugenol concentration were injected in *G. mellonella* larvae, and their survival was monitored. The *G. mellonella* model has been used to evaluate the virulence potential of several human pathogens, including *S. aureus* and *L. monocytogenes* (Joyce & Gahan, 2010; Mukherjee *et al.*, 2010; Desbois & Coote, 2011). The action of antimicrobial agents and response to stress conditions on bacterial virulence also have been investigated using the larvae model (Desbois & Coote, 2011; Gibreel & Upton, 2013; Schrama *et al.*, 2013). Sequential exposure of *S. aureus*, MRSA, and *L. monocytogenes* strains to subinhibitory eugenol concentration affected their virulence evidencing the great potential of eugenol to combat these human pathogens either by inhibiting their ability to form biofilm and diminishing their virulence potential. The exact mode of action of eugenol on the virulence mechanisms of these pathogens still needs to be clarified. However, it can be anticipated that eugenol by acting on cellular membrane and nutrient uptake will cause the disruption of essential cell functions including the secretion of toxins impairing the virulence potential (Di Pasqua *et al.*, 2006; Devi *et al.*, 2010; Yossa *et al.*, 2012; Zhou *et al.*, 2013).

Conclusions

In conclusion, eugenol and citral did not induce the development of resistance to antibiotics or to the components itself in *S. aureus*, MRSA, and *L. monocytogenes* strains. At subinhibitory concentration, eugenol and citral significantly inhibit the biofilm formation of the tested strains. Sequential adaptation to subinhibitory concentration of eugenol resulted in a diminished virulence in the tested pathogens. The findings observed in this study show that eugenol and citral are effective antimicrobial agents without the risk of developing resistance and affecting important bacterial abilities, such as biofilm formation and virulence.

Acknowledgements

This work was supported by FCT – Fundação para a Ciência e Tecnologia (PEst-OE/EQB/LA0023/2013).

Conflict of interest

The authors report no conflict of interest.

References

Adrião A, Vieira M, Fernandes I *et al.* (2008) Marked intra-strain variation in response of *Listeria monocytogenes* dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces. *Int J Food Microbiol* **123**: 142–150.

Ali SM, Khan AA, Ahmed I, Musaddiq M, Ahmed KS, Polasa H, Rao LV, Habibullah CM, Sechi LA & Ahmed N (2013) Antimicrobial activities of eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. *Ann Clin Microbiol Antimicrob* **4**: 20.

Bergloz TM, Tang S, Wiedmann M & Boor K (2013) Nisin resistance of *Listeria monocytogenes* is increased by exposure to salt stress and is mediated via LiaR. *Appl Environ Microbiol* **79**: 5682–5688.

Bonez PC, Alves CFS, Dalmolin TV, Agertt VA, Mizdal CR, Flores VC, Marques JB, Santos RCV & de Campos MMA (2013) Chlorhexidine activity against bacterial biofilms. *Am J Infect Control* **41**: e119–e122.

Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods - a review. *Int J Food Microbiol* **94**: 223–253.

Canizalez-Roman A, Gonzalez-Nuñez E, Vidal JE, Flores-Villaseñor H & León-Sicairos N (2013) Prevalence and antibiotic resistance profiles of diarrheagenic *Escherichia coli* strains isolated from food items in northwestern México. *Int J Food Microbiol* **164**: 36–45.

Cebrián G, Sagarzazu N, Pagán R, Condón S & Mañas P (2010) Development of stress resistance in *Staphylococcus aureus* after exposure to sublethal environmental conditions. *Int J Food Microbiol* **140**: 26–33.

Centers for Disease Control and Prevention (2013) Antibiotic resistant threats in the United States, 2013.

Chan PF, Foster SJ, Ingham E & Clements MO (1998) *Staphylococcus aureus* alternative sigma factor sigma B controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. *J Bacteriol* **180**: 6082–6089.

Clements MO & Foster SJ (1999) Stress resistance in *Staphylococcus aureus*. *Trends Microbiol* **7**: 458–462.

Clinical and Laboratory Standards Institute (2006) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition document M7–A7*. CLSI, Wayne, PA.

Clinical and Laboratory Standards Institute (2007) Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI document M100-S17. CLSI, Wayne, PA.

Conte MP, Petrone G, Di Biase AM, Ammendolia MG, Superti F & Seganti L (2000) Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. *Microb Pathog* **29**: 137–144.

Davies MJ, Coote PJ & O'Byrne CP (1996) Acid tolerance in *Listeria monocytogenes*; the adaptative acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology* **142**: 2975–2982.

Desbois AP & Coote PJ (2011) Wax moth larva (*Galleria mellonella*): an *in vivo* model for assessing the efficacy of antistaphylococcal agents. *J Antimicrob Chemother* **66**: 1785–1790.

Devi KP, Nisha SA, Sakthivel R & Pandian SK (2010) Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella* typhi by disrupting the cellular membrane. *J Ethnopharmacol* **130**: 107–115.

Devi KP, Sakthivel R, Nisha SA, Suganthy N & Pandian SK (2013) Eugenol alters the integrity of cell membrane and acts against the nosocomial pathogen *Proteus mirabilis*. *Arch Pharm Res* **36**: 282–292.

Divergiliis G, Barile S & Perozzi G (2011) Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes Nutr* **6**: 275–284.

Di Pasqua R, Hoskins N, Betts G & Mauriello G (2006) Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J Agric Food Chem* **54**: 2745–2749.

Faleiro ML (2011) The mode of antibacterial action of essential oils. Science against microbial pathogens: communicating current research and technological advances, Vol. 2, Formatex Research Center Badajoz, Spain.

Faleiro ML & Miguel MG (2013) Use of essential oils and their components against multidrug-resistant bacteria. *Fighting Multidrug Resistance With herbal Extracts, Essential Oils and Their Components*. Academic Press, Elsevier, USA.

Faleiro ML, Andrew PW & Power D (2003) Stress response of *Listeria monocytogenes* isolated from cheese and other foods. *Int J Food Microbiol* **84**: 207–216.

Faleiro L, Miguel G, Gomes S, Costa L, Venâncio F, Teixeira A, Figueiredo AC, Barroso JA & Pedro LG (2005) Antibacterial and antioxidant activities of essential oils isolated from *Thymbra capitata* L. (Cav.) and *Origanum vulgare* L. *J Agric Food Chem* **53**: 8162–8168.

Farber JM & Peterkin PI (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* **55**: 476–511.

Fisher K & Phillips CA (2006) The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus in vitro* and in food systems. *J Appl Microbiol* **101**: 1232–1240.

Garner MR, James KE, Callahan MC, Wiedmann M & Boor KJ (2006) Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. *Appl Environ Microbiol* **72**: 5384–5395.

Gibreel TM & Upton M (2013) Synthetic epidermicin NI01 can protect *Galleria mellonella* larvae from infection with *Staphylococcus aureus*. *J Antimicrob Chemother* **68**: 2269–2273.

Gilbert P, Allison DG & McBain AJ (2002) Biofilm *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *J Appl Microbiol* **92**: 98S–110S.

Hammer KA, Carson CF & Riley TV (2012) Effects of *Melaleuca alternifolia* (Tea Tree) essential oil and the major monoterpene component terpinen-4-ol on the development of single- and multistep antibiotic resistance and antimicrobial susceptibility. *Antimicrob Agents Chemother* **56**: 909–915.

Hyldgaard M, Mygind T & Meyer RL (2012) Essential oils in food preservation: mode of action, and interactions with food matrix components. *Front Microbiol* **3**: 12.

Ito A, Taniuchi A, May T, Kawata K & Okabe S (2009) Increased antibiotic resistance of *Escherichia coli* in mature biofilms. *Appl Environ Microbiol* **75**: 4093–4100.

Joyce SA & Gahan CGM (2010) Molecular pathogenesis of *Listeria monocytogenes* in the alternative model host *Galleria mellonella*. *Microbiology* **156**: 3456–3468.

Keren I, Kaldalu N, Spoering A, Yipeng W & Lewis K (2004) Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230**: 13–18.

Luz IS, Neto NJG, Tavares AG, Nunes PC, Magnani M & de Sousa EL (2012) Evidence for lack of acquisition of tolerance in *Salmonella enterica* serovar Typhimurium ATCC 14028 after exposure to subinhibitory amounts of *Origanum vulgare* L. essential oil and carvacrol. *Appl Environ Microbiol* **78**: 5021–5024.

McMahon M, Blair I, Moore J & McDowell DA (2007) Habituation to sublethal concentrations of tea tree oil (*Melaleuca alternifolia*) is associated with reduced susceptibility to antibiotics in human pathogens. *J Antimicrob Chemother* **59**: 125–127.

McMahon M, Tunney MM, Moore JE, Blair IS, Gilpin DF & McDowell DA (2008) Changes in antibiotic susceptibility in staphylococci habituated to sublethal concentrations of tea tree oil (*Melaleuca alternifolia*). *Lett Appl Microbiol* **47**: 263–268.

Miles AA & Misra SS (1938) The estimation of the bactericidal power of blood. *J Hygiene* **38**: 732–749.

Mukherjee K, Altincicek B, Hain T, Domann E, Vilcinskas A & Chakraborty T (2010) *Galleria mellonella* as a model system for studying *Listeria* pathogenesis. *Appl Environ Microbiol* **76**: 310–317.

Niu C & Gilbert ES (2004) Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. *Appl Environ Microbiol* **70**: 6951–6956.

Oyedemi SO, Okoh AI, Mabinya LV, Pironcheva G & Afolayan AJ (2009) The proposed mechanism of bactericidal action of eugenol, α-terpineol and γ- terpinene against *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli*. *Afr J Biotechnol* **8**: 1280–1286.

Papadopoulos CJ, Carson CF, Chang B & Riley TV (2008) Role of the MexAB-OprM efflux pump of *Pseudomonas aeruginosa* in tolerance to tea tree (*Melaleuca alternifolia*) oil and its monoterpene components terpinen-4-ol, 1,8-cineole, and α-Terpineol. *Appl Environ Microbiol* **74**: 1932–1935.

Schrama D, Helliwell N, Neto L & Faleiro ML (2013) Adaptation of *Listeria monocytogenes* in a simulated cheese medium: effects on virulence using the *Galleria mellonella* infection model. *Lett Appl Microbiol* **56**: 421–427.

Shaw LN, Lindholm C, Prajsnar TK, Miller HK, Brown MC, Golonka E, Stewart GC, Tarkowski A & Potempa J (2008) Identification and characterization of σ^s, a novel component of the *Staphylococcus aureus* stress and virulence responses. *PLoS ONE* **3**: e3844.

Skandamis PN & Nychas G-JE (2000) Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. *Appl Environ Microbiol* **66**: 1646–1653.

Somolinos M, García D, Pagan R & Mackey B (2008) Relationship between sublethal injury and microbial inactivation by the combination of high hydrostatic pressure and citral or tert-butyl hydroquinone. *Appl Environ Microbiol* **74**: 7570–7577.

Somolinos M, García D, Condón S, Mackey B, Pagan R (2009) Inactivation of *Escherichia coli* by citral. *J Appl Microbiol* **108**: 1928–1939.

Swaminathan B & Gerner-Smidt P (2007) The epidemiology of human listeriosis. *Microb Infect* **9**: 1236–1243.

Thomsen NA, Hammer KA, Riley TV, Van Belkum A & Carson CF (2013) Effect of habituation to tea tree (*Melaleuca alternifolia*) oil on the subsequent susceptibility of *Staphylococcus* spp. to antimicrobials, triclosan, tea tree oil, terpinen-4-ol and carvacrol. *Int J Antimicrob Agents* **41**: 343–351.

Wang X-M, Lu X-F, Yin L, Liu H-F, Zhang W-J, Si W, Yu S-Y, Shao M-L & Liu S-G (2013) Occurrence and antimicrobial susceptibility of *Listeria* isolates from retail raw foods. *Food Control* **32**: 153–158.

Yossa N, Patel J, Macarisin D, Millner P & Murph C (2012) Antibacterial activity of cinnamaldehyde and sporan against *Escherichia coli* 0157:H7 and *Salmonella*. *J Food Process Preserv*. doi:10.1111/jfpp.12026.

Zhou L, Zheng H, Tang Y, Yu W & Gong Q (2013) Eugenol inhibits quorum sensing at sub-inhibitory concentrations. *Biotechnol Lett* **35**: 631–637.