Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Investigation of functional and morphological changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Origanum compactum* essential oil

S. Bouhdid[1], J. Abrini[1], A. Zhiri[2], M.J. Espuny[3] and A. Manresa[3]

1 Laboratoire de Biologie et Santé, Equipe de Biotechnologies et Microbiologie Appliquée, Département de Biologie, Faculté des Sciences, Université Abdelmalek Essaâdi, Tétouan, Morocco
2 PRANAROM INTERNATIONAL S. A. 37, Ghislenghien, Belgium
3 Laboratori de Microbiologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain

Keywords
Origanum compactum essential oil, antimicrobial activity, flow cytometry, transmission electron microscopy, potassium leakage.

Correspondence
Angeles Manresa, Laboratori de Microbiologia, Facultat de Farmàcia, Universitat de Barcelona, c/ Joan XXIII s/n, 08028 Barcelona, Spain.
E-mail: amanresa@ub.edu

2008/0972: received 9 June 2008, revised and accepted 16 October 2008

doi:10.1111/j.1365-2672.2008.04124.x

Abstract

Aims: Evaluation of the cellular effects of *Origanum compactum* essential oil on *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.
Methods and Results: The damage induced by *O. compactum* essential oil on these two strains has been studied using different techniques: plate count, potassium leakage, flow cytometry (FC) and transmission electron microscopy (TEM). The results showed that oil treatment led to reduction of cells viability and dissipated potassium ion gradients. Flow cytometric analysis showed that oil treatment promoted the accumulation of bis-oxonol and the membrane-impermeable nucleic acid stain propidium iodide (PI), indicating the loss of membrane potential and permeability. The ability to reduce 5-cyano-2,3-ditolyl tetrazolium chloride was inhibited. Unlike in *Ps. aeruginosa*, membrane potential and membrane permeability in *Staph. aureus* cells were affected by oil concentration and contact time. Finally, TEM showed various structural effects. Mesosome-like structures were seen in oil-treated *Staph. aureus* cells whereas in *Ps. aeruginosa*, coagulated cytoplasmic material and liberation of membrane vesicles were observed, and intracellular material was seen in the surrounding environment. Both FC and TEM revealed that the effects in *Ps. aeruginosa* were greater than in *Staph. aureus*.
Conclusions: Oregano essential oil induces membrane damage showed by the leakage of potassium and uptake of PI and bis-oxonol. Ultrastructural alterations and the loss of cell viability were observed.
Significance and Impact of the Study: Understanding the mode of antibacterial effect of the oil studied is of a great interest in it further application as natural preservative in food or pharmaceutical industries.

Introduction

Over the last few years many research groups have been interested in the functional role of natural products, especially plant extracts. Essential oils are a complex mixture of volatile molecules produced by the secondary metabolism of aromatic plants and are extracted by distillation from different plant parts. Some essential oils are classified as generally recognized as safe by the US Food and Drug Administration. They are appreciated for their bioactive efficacy as fungicides (Pina-Vaz *et al.* 2004), bactericides (Kotzekidou *et al.* 2008), antioxidants (Eminagaoglu *et al.* 2007), antigenotoxics (Bakkali *et al.* 2006) and many other biological activities. Such activities are ascribed to the chemical complexity of these products; the resultant activity may be the consequence of the synergistic effect of two or more components acting on different cellular targets (Burt 2004).

Despite the large number of studies that have highlighted the antibacterial activity of essential oils against a wide range of microbial strains, the mechanisms involved are still not fully understood. Understanding the mode of antibacterial action of essential oils is of great interest in their application in food or pharmaceutical industries. Previous studies have reported that the antibacterial activity of essential oil components appears to be due to their lipophilic nature, which allows their accumulation in biological membranes (Sikkema *et al.* 1994). Consequently, they can cause degradation of the outer membrane of Gram-negative bacteria, leading to the loss of outer membrane-associated material (Helander *et al.* 1998), disruption of the cytoplasmic membrane (Ultee *et al.* 2002) and thus increasing its permeability to protons and other cells contents (Fitzgerald *et al.* 2004; Gill and Holley 2006; Cristani *et al.* 2007), and inactivation of membrane-embedded enzymes (Ultee *et al.* 1999).

Origanum compactum is an endemic specie growing in the north of Morocco where it is traditionally used in culinary and medical preparations (Bellakhdar 1997). The essential oil extracted from this plant is mainly constituted by carvacrol, thymol, γ-terpinene and *p*-cymene (Bakkali *et al.* 2005; Mezzoug *et al.* 2007). A previous study reported that the antibacterial activity of these four monoterpene compounds is related to their ability to perturb membrane structure (Cristani *et al.* 2007).

In this study, we evaluated the action of *O. compactum* essential oil on *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Diverse techniques may be used to assess the effects of biocides on cellular functions and structure. Here, the leakage of intracellular potassium was assessed as it is considered an early indication of membrane disruption (Lambert and Hammond 1973). Flow cytometry (FC) together with specific fluorescent dyes was applied to investigate the effect on some membrane functions. Propidium iodide (PI), which is a membrane-impermeable dye, was used to evaluate the effect on membrane permeability. Membrane potential was assessed using bis-1,3-dibutylbarbutiric acid (bis-oxonol) and respiratory activity was assessed using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Ultrastructural alterations were examined by transmission electron microscopy (TEM).

Material and methods

Antibacterial product and chemicals

Essential oil extracted from flower heads of *O. compactum* was provided by PRANARÔM (B-7822 Ghislenghien, Belgique). According to the data of the gas chromatography analysis of the essential oil provided by the manufacturer, the main compounds of this oil were carvacrol (37·77%), thymol (19·79%), γ-terpinene (17·01%) and *p*-cymene (11·31%) (see supporting information Fig. S1 and Table S1). Polymixin B sulfate and chlorhexidine dihydrochloride (CHX) were purchased from Sigma (St Louis, MO, USA).

The molecular dyes PI and bis-(1,3-dibutylbarbutiric acid) trimethine oxonol (bis-oxonol) were supplied by Molecular Probes Europe BV (Leiden, the Netherlands) and CTC by Sigma. Solvents and reagents were of analytical grade. Ultra pure water, produced by a Nanopure purification system coupled to a Milli-Q water purification system, resistivity = 18·2 MΩ cm, was used for the aqueous solutions.

Micro-organisms

Pseudomonas aeruginosa ATCC 27853 and *Staph. aureus* ATCC 29213 were obtained from the ATCC (Manassas, VA, USA) and subcultured weekly on Trypticase Soy Agar (Pronadisa, Madrid, Spain). Strains were preserved frozen in cryovials (AES Laboratoire, Combourg, France) at −80°C.

Minimal inhibitory concentration (MIC)

The MICs were determined by using the broth microdilution assay as previously described (Ismaili *et al.* 2004) with a slight modification: agar at 0·15% (w/v) was used as stabilizer of the oil–water mixture and resazurin was used as bacterial growth indicator. In a first step, 50 μl of Mueller Hinton Broth (Oxoid, UK) supplemented with bacteriological agar (0·15% w/v) were distributed from the second to the 12th well of a 96-well polypropylene microtitre plate (Costar; Corning Inc, Corning, NY, USA). Essential oil dilution was prepared in Mueller Hinton Broth supplemented with bacteriological agar (0·15% w/v), to reach a final concentration of 4%; 100 μl of these suspensions were added to the first test well of each microtitre line, and then 50 μl of scalar dilution were transferred from the second to the 11th well. The 12th tube was considered as growth control, because no essential oil was added. Then, 50 μl of a bacterial suspension were added to each well at a final concentration of approximately 10^5 CFU ml^{-1}. The final concentration of the essential oil was between 2 and 0·0019% (v/v). Plates were incubated at 37°C for 18 h. After incubation, 5 μl of resazurin were added to each well to assess bacterial growth as suggested by Mann and Markham (1998). After further incubation at 37°C for 2 h, the MIC was determined as the lowest essential oil concentration that prevented a change in resazurin colour. Bacterial growth is detected by reduction of blue dye resazurin to pink resorufin. Control was carried out to assure that, at used

concentrations, the essential oil do not cause a colour change in the resazurin. Experiments were conducted in triplicate and modal values were selected.

Potassium leakage

Potassium leakage was determined as previously described (Rodriguez *et al.* 2004) with a slight modification. Briefly, micro-organisms were grown overnight on tryptone soy broth (TSB) at 30°C in a shaking incubator (120 rev min^{-1}). Cells were then washed thrice with 0·9% NaCl by centrifugation at 8000 *g* for 15 min and re-suspended in 30 ml of 1 mmol l^{-1} glycil–glycine (Sigma, USA) buffer solution pH 6·8, to obtain a cell density of $7{\cdot}4 \times 10^7$ CFU ml^{-1} for *Ps. aeruginosa* and $8{\cdot}8 \times 10^7$ CFU ml^{-1} for *Staph. aureus*.

The bacteria were treated with the essential oil at the MIC and 1·5× MIC and incubated at 30°C in a shaking incubator. Samples (5 ml) of cell suspension were removed at 0, 10, 15, 30, 60 and 120 min, and diluted and filtered through 0·2 μm pore-size membrane (Sartorius, Goettingen, Germany) to eliminate bacteria. Oil-free controls were prepared in the same conditions to determine normal K^+ flux over the time course of the experiment. Heat treatment was also conducted by incubating the cell suspension in a water bath at 70°C. The potassium concentration in the supernatant was measured using an atomic absorption spectrophotometer UNICAM 939/959 model (Cambridge, UK). The instrumental parameters were as follows: potassium hollow cathode lamp, wavelength of 766·5 nm, bandpass of 0·5 nm, air-acetylene flame and fuel flow rate of 1 l min^{-1}. Absorbance values were converted into potassium ion concentration (ppm) by reference to a curve previously established using standard potassium ion solutions of 0, 0·05, 0·1, 0·3, 0·5 and 1 ppm concentrations. Experiments were conducted in triplicate and means and standard deviations were calculated.

Exposure of micro-organisms to biocides

Suspensions of the micro-organisms were obtained from an overnight culture of each strain on TSB (Oxoid, USA) at 30°C in a shaker incubator. Cultures were then centrifuged at 8000 ***g*** for 15 min, washed twice in sterile Ringer's solution (Scharlau, Barcelona, Spain) and resuspended in peptone water (Oxoid, UK). Five millilitres of this cell suspension was used to inoculate flasks containing 45 ml of peptone water to obtain a cell density of about 10^7–10^8 CFU ml^{-1}. Essential oil was added to the flasks to give a final concentration of 1 and 1·5 of the MIC. No solubilizing agent was used. The inoculated flasks were incubated at 30°C in a shaker incubator.

The contact times established for FC were 30 min and 1 h. At each time-point, 20 ml samples were diluted 1 : 2 and centrifuged at 8000 ***g*** for 30 min, washed and resuspended in 1 ml of filtered Ringer's solution.

For TEM observations, the contact time was 30 min; 20 ml samples were taken, diluted 1 : 2 and centrifuged at 8000 ***g*** for 30 min. The pellet was resuspended in 1 ml of peptone-buffered water (Oxoid, UK).

In all cases, control experiments were carried out in parallel; cells were incubated in oil-free buffer solution and treated under the same conditions.

Staining procedure

Staining protocols for FC experiments were as follows: 10 μl of a 1 mg ml^{-1} stock solution of PI in distilled water were added to 1 ml of the bacterial suspension prepared as described above in filtered Ringer's solution (the bacterial concentration was of about 10^7–10^8 CFU ml^{-1}). The *Staph. aureus* and *Ps. aeruginosa* suspensions were incubated with the dyes for 5 and 30 min, respectively. Staining was carried out at room temperature before the FC analysis.

For membrane potential dye, 2 μl of a 250 μmol l^{-1} stock solution of bis-oxonol in ethanol was added to 1 ml of the bacterial suspension to a final concentration of 0·5 mmol l^{-1} and incubated for 2 min at room temperature.

In the case of respiratory activity staining, 100 μl of a 50 mmol l^{-1} CTC solution in deionized water filtered through 0·22 μm pore-size membrane were added to 900 μl of bacterial suspension to a final concentration of 5 mmol l^{-1}. Incubation was for 30 min at 30°C in a shaking incubator.

Heat-killed cells (30 min at 70°C) were used as a positive control for PI and bis-oxonol staining protocols and as a negative control for CTC staining. Experiments were conducted in duplicate.

Flow cytometry

Flow cytometric experiments were carried out using a Cytomics FC500 MPL flow cytometer (Beckman Coulter Inc, Fullerton, CA, USA). Excitation of the sample was done using a 488 nm air-cooled argon-ion laser at 15 mW power. The instrument was set up with the standard configuration: forward scatter (FS), side scatter (SS), green (525 nm) fluorescence for bis-oxonol, red (620 nm) for CTC and red (675 nm) fluorescence for PI were collected on logarithmic scales. Optical alignment was checked using 10 nm fluorescent beads (Flow-Check fluorospheres; Beckman Coulter). The cell population was selected by gating in a FS *vs* SS dot plot, excluding aggregates and cell debris. Fluorescence histograms were represented in single-parameter histograms (1024 channels).

The windows used to calculate percentages were set using living and dead cell populations for each strain. Data were analysed with SUMMIT® version 3.1 software (Cytomation, Fort Collins, CO, USA).

Bacterial count

Viable counts (CFU ml^{-1}) were obtained on trypticase soy agar. After an appropriate dilution in Ringer's solution, 100 μl samples were inoculated on plates and incubated at 30°C for 24 h. Cell counting was performed in triplicate and mean was calculated. To measure growth inhibition, viability reduction was calculated from this data as follows:

$$(1 - N_T/N_C) \times 100$$

where N_T is the bacterial count in treated sample and N_C is the bacterial count in control sample.

Transmission electron microscopy

After treatment of cell suspensions for 30 min with the oil at the MIC for each micro-organism, the bacterial pellets were rinsed with buffered water peptone (pH 7) and washed thrice. *Staphylococcus aureus* samples were fixed with 2·5% buffered glutaraldehyde for 1 h at 4°C. The cells were then postfixed in 1% buffered osmium tetroxide for 1 h, stained with 1% uranyl acetate and dehydrated in a graded series of ethanol. *Pseudomonas aeruginosa* samples were cryofixed by high pressure with a pressure of about 2100 bars and a reduction in temperature of 8°C s^{-1}. The cryofixed cells were kept in liquid nitrogen and cryosubstituted in pure acetone containing 2% of osmium tetroxide and 0·1% of uranyl acetate for 72 h at −90°C. The temperature was gradually increased to 4°C (5°C h^{-1}). The samples were kept at this temperature for 2 h, followed by 2 h at room temperature, and were then washed twice with acetone. The fixed cells of both micro-organisms were then embedded in L.R. (London Resin Co. Ltd, London, UK) white resin. Ultra-thin sections were prepared and stained with 1% uranyl acetate and sodium citrate. Microscopy was performed with a Philips EM 301 (Eindhoven, Holland) microscope with an acceleration of 60 kV.

Results

The essential oil of *O. compactum* was active against *Staph. aureus* and *Ps. aeruginosa* at MICs of 0·031% and 1% (v/v), respectively. To investigate the cellular injury produced by the oil treatment on the bacterial population, we worked at the MIC and 1·5× MIC concentrations (Table 1). Polymixin and clorhexidine were used as markers of antimicrobial activity on Gram-negative and Gram-positive bacteria, respectively.

Table 1 Antibacterial concentrations tested

	Pseudomonas aeruginosa		*Staphylococcus aureus*	
Compound	MIC*	1·5× MIC	MIC	1·5× MIC
Origanum compactum essential oil	1	1·5	0·031	0·046
Polymixin	8	12	–	–
CHX	–	–	1	1·5

*The concentrations are expressed in % v/v for essential oil and in mg l^{-1} for polymixin and CHX.

Potassium leakage

To determine the change in cell membrane permeability, K^+ leakage from the two strains studied was examined over the exposure time. After treatment of cell suspensions with essential oil at the MIC, K^+ immediately started to leak from the *Ps. aeruginosa* cells within the 10 first minutes to reach 0·09 ppm, and remained constant until the end of the experiment. In the case of 1·5× MIC treated cells, leakage started immediately and gradually increased with incubation time to reach 0·24 ppm after 120 min of contact (Fig. 1a). The addition of oregano essential oil increased the K^+ extracellular concentration in *Staph. aureus* cell suspensions (Fig. 1b), reaching 0·64 and 0·77 ppm after 120 min of treatment with the MIC and 1·5× MIC, respectively.

Membrane permeability

Propidium iodide staining was used to assess the change in membrane permeability of bacterial cells of *Ps. aeruginosa* and *Staph. aureus* (Table 2). Samples were analysed by FC.

Two intervals of PI relative fluorescence were considered (Fig. 2), depending on the intensity of fluorescence emitted by negative (nontreated cells) and positive controls (heat-treated cells). Thus, we can distinguish two cell states: cells with intact membrane, which emit a low relative fluorescence (intact cells, region R1), and cells with permeabilized membrane, which emit a high relative fluorescence (dead cells, region R2).

In the control population of *Ps. aeruginosa* no more than 8·80% was stained with PI (cells with damaged membrane). The PI-labelled population increased considerably after 30 min of treatment with the oil at the MIC and 1·5× MIC to reach 92·44% and 94·99%, respectively. No remarkable differences in the proportion of PI-stained cells were observed after increasing the contact time to 60 min. In accordance with these results, the viability reduction calcu-

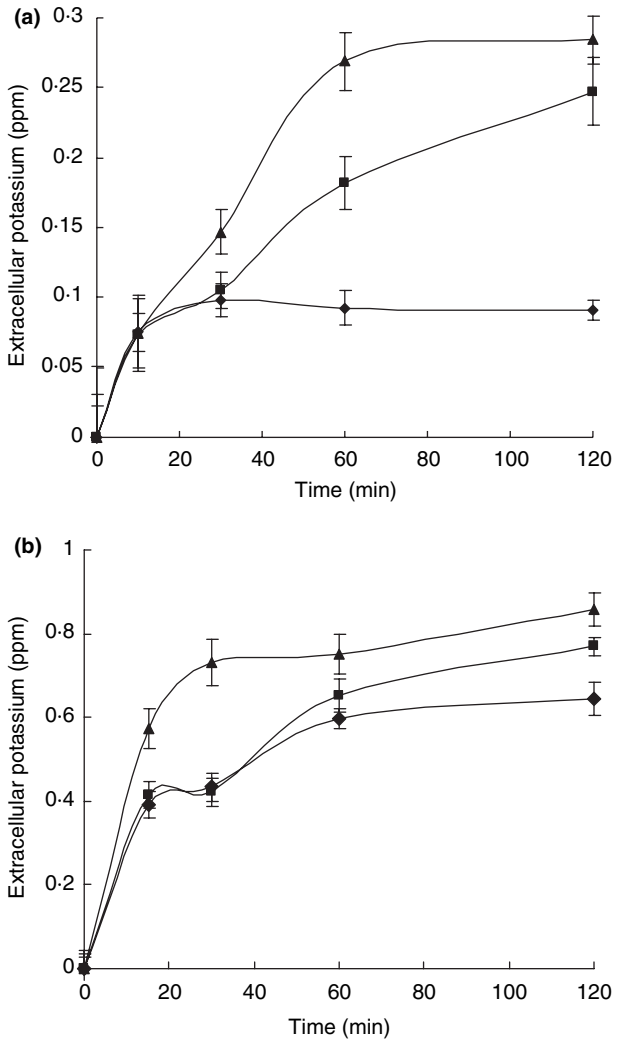

Figure 1 Potassium leakage of the cell suspension of *Pseudomonas aeruginosa* (a) and *Staphylococcus aureus* (b) exposed to MIC (◆) and 1·5× MIC (■) of *Origanum compactum* essential oil. Exposure to 70°C (▲) acted as positive control. The amount of extracellular potassium of untreated cells (0·225 ppm for *Ps. aeruginosa* and 0·5 ppm for *Staph. aureus*) was subtracted at each time-point from that of treated cells. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.

lated from viable counts was of 99·99% (Table 2). Similar results were observed in the polymixin-treated population (1·5× MIC) with 89·40% and 94·15% PI-stained cells after 30 and 60 min, respectively. A total of 74·53% of heat-treated cells (70°C for 30 min) was permeable to PI.

In *Staph. aureus* (Table 2), the whole control population was impermeable to PI (0·65% PI-stained cells). After 30 min of contact with the essential oil at the MIC, the proportion of PI-stained cells was slightly augmented (5·93%) and increased to 8·89% when the contact time was prolonged to 60 min. Viability was reduced by 19·05% and 50·48% for the two contact times. Treatment with 1·5× MIC appeared to have a more marked effect on membrane permeability. Indeed, a clear bimodal distribution was observed (Fig. 2). The PI-labelled population was augmented to 39·66% and 53·44% after 30 and 60 min of contact, respectively. Exposure to CHX showed a higher level of action on cell membrane permeability with 53·45% and 72·43% PI-stained cells after 30 and 60 min, respectively.

Membrane potential

Loss of membrane potential was assessed using bis-oxonol dye, which accumulates in cells with depolarized membranes. Exposure to the MIC and 1·5× MIC for 30 min led to the loss of membrane potential in 62·28% and 75·49% of the *Ps. aeruginosa* population, respectively (Table 2). This stained proportion did not appear to change by increasing contact time to 60 min. Moreover, no substantial modifications were observed in bis-oxonol stained cells between the MIC and 1·5× MIC treated cells. Polymixin-treated cells showed a more marked effect on membrane potential. The proportions stained with bis-oxonol were 95·42% and 97·71% after 30 and 60 min of contact, respectively.

In *Staph. aureus* cells, the entire control population showed intact membrane potential (0·89% stained with bis-oxonol). Exposure to the oil at the MIC had no significant effect on cells membrane potential (Table 2); a very small fraction of the treated cells had incorporated bis-oxonol (1·95% and 1·97% after 30 and 60 min, respectively). Treatment with the oil at 1·5× MIC resulted in the loss of membrane potential of 44·66% and 66·23% of cells after 30 and 60 min of exposure respectively. In this case, the increase in essential oil concentration did affect the proportion of bis-oxonol stained cells (Fig. 2). The action of CHX on membrane potential seemed to be strongly dependent on the time of exposure; the proportion bis-oxonol stained-cells was 3·52% after 30 min increasing to 52·15% after 60 min of contact.

Respiratory activity

Bacterial cells with active respiratory activity are able to reduce CTC to formazan. This stain was used to assess the effect of the oil on respiratory enzymes.

The control cell suspension of *Ps. aeruginosa* contained a high proportion of CTC-reducing bacteria (66·94%). Significant inhibition of formazan production by electron transfer system associated dehydrogenases was observed after treatment of suspensions of *Ps. aeruginosa* by the essential oil. This is demonstrated by the decrease in CTC-reducing cells from 66·94 to 15·62% and 1·53% after 30 min exposure to the MIC and 1·5× MIC, respec-

Table 2 Percentage of stained cells measured by flow cytometry analysis and viability reduction of *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells treated with oregano essential oil, polymixin and CHX

			Ps. aeruginosa				*Staph. aureus*			
			% of stained cells				% of stained cells			
	Conc.	Contact time (min)	PI	Bis-oxonol	CTC	% viability reduction	PI	Bis-oxonol	CTC	% viability reduction
Control	–	30	8·01	1·78	66·94	–	0·65	0·89	79·05	–
		60	1·58	8·39	57·02	–	3·86	2·45	70·76	–
Essential oil	MIC	30	92·44	62·28	15·62	99·99	5·93	1·95	30·40	19·05
		60	92·36	61·91	11·74	99·99	8·89	1·97	17·09	50·48
	1·5× MIC	30	94·99	75·49	1·53	99·99	39·66	44·66	14·60	99·67
		60	94·01	77·63	1·14	99·99	53·44	66·23	13·76	99·96
Polymixin	1·5× MIC	30	89·40	95·42	0·44	100	–	–	–	–
		60	94·15	97·71	0·46	100	–	–	–	–
CHX	1·5× MIC	30	–	–	–	–	53,45	3·52	0·73	99·98
		60	–	–	–	–	72·43	52·15	0·64	99·99
70°C		30	74·53	88·01	0·80	100	93·06	94·38	1·29	100

tively. There was no further significant change after 60 min of treatment, the proportion of CTC-stained cells being 11·74% for the MIC-treated cells and 1·14% for 1·5× MIC-treated cells (Table 2). Polymixin dramatically decreased the ability to reduce CTC among treated cells. The CTC-reducing proportion decreased to 0·44% and 0·46% after 30 min and 60 min contact time, respectively.

A high proportion of *Staph. aureus* control cells reduced CTC (79·05%), while exposure to the MIC of oregano oil reduced this population to 30·40% after 30 min of contact and to 17·09% after 60 min. Treatment with the oil at 1·5× MIC led to a decrease in CTC-stained cells to 14·60%. This proportion was not affected by increasing contact time to 60 min (13·76%). Contact with CHX resulted in reduction of respiring bacteria to 6·54% and 8·48% after 30 and 60 min, respectively.

Transmission electron microscopy

To check for morphological changes resulting from oil treatment, TEM was performed on thin sections of bacteria that had been treated with the essential oil for 30 min. Significant morphological changes occurred in *Ps. aeruginosa* cells exposed to the oil (Fig. 3). Numerous projections from the cell wall and liberation of membrane vesicles were observed (black arrows). Coagulated material was seen inside the treated cells especially close to the cell's envelope (white arrows). Likewise, a significant amount of cytoplasmic material was observed in the surrounding environment.

Staphylococcus aureus electron micrograph showed an accumulation of membranous structures in the cytoplasm of the oil-treated cells (arrows in Fig. 4b–d), while no ultrastructural alterations were seen in the untreated cells (Fig. 4a). The electron micrographs did not show any cell lysis in either strain.

Discussion

Origanum compactum essential oil is constituted by a complex mixture of volatile molecules dominated by four monoterpene compounds [carvacrol (37·77%), thymol (19·79%), γ-terpinene (17·01%) and *p*-cymene (11·31%)] that are known for their antibacterial activity. The involvement of less abundant constituents in the antibacterial effect of the whole oil should be considered. Moreover, possible antagonistic and synergistic effects may occur between the oil components depending on the microorganism tested (Cox *et al.* 2001). In this study, we report the effect of oregano essential oil on some cell functions and structures in *Ps. aeruginosa* and *Staph. aureus*.

Potassium is a major intracellular cation that plays a crucial role in the generation of membrane potential, activating cytoplasmic enzymes and maintaining turgor pressure in cells. The gradient of potassium across the cytoplasmic membrane is maintained by membrane-embedded energy-dependent pumps. Generally, leakage of potassium is an early sign of an increase in cytoplasmic membrane permeability (Lambert and Hammond 1973). Therefore, K^+ release during exposure to the oil was measured in both strains, and was found to be induced at all concentrations tested. This result is consistent with previous studies that demonstrated that essential oils and some of their components interact with cellular membranes, changing their permeability to cations like H^+ and K^+. Indeed, carvacrol and thymol, which are the major components of oregano essential oil were found to dissipate the potassium gradient in *Bacillus cereus* (Ultee *et al.* 1999), *Staph. aureus* and *E. coli* (Walsh *et al.* 2003). Other

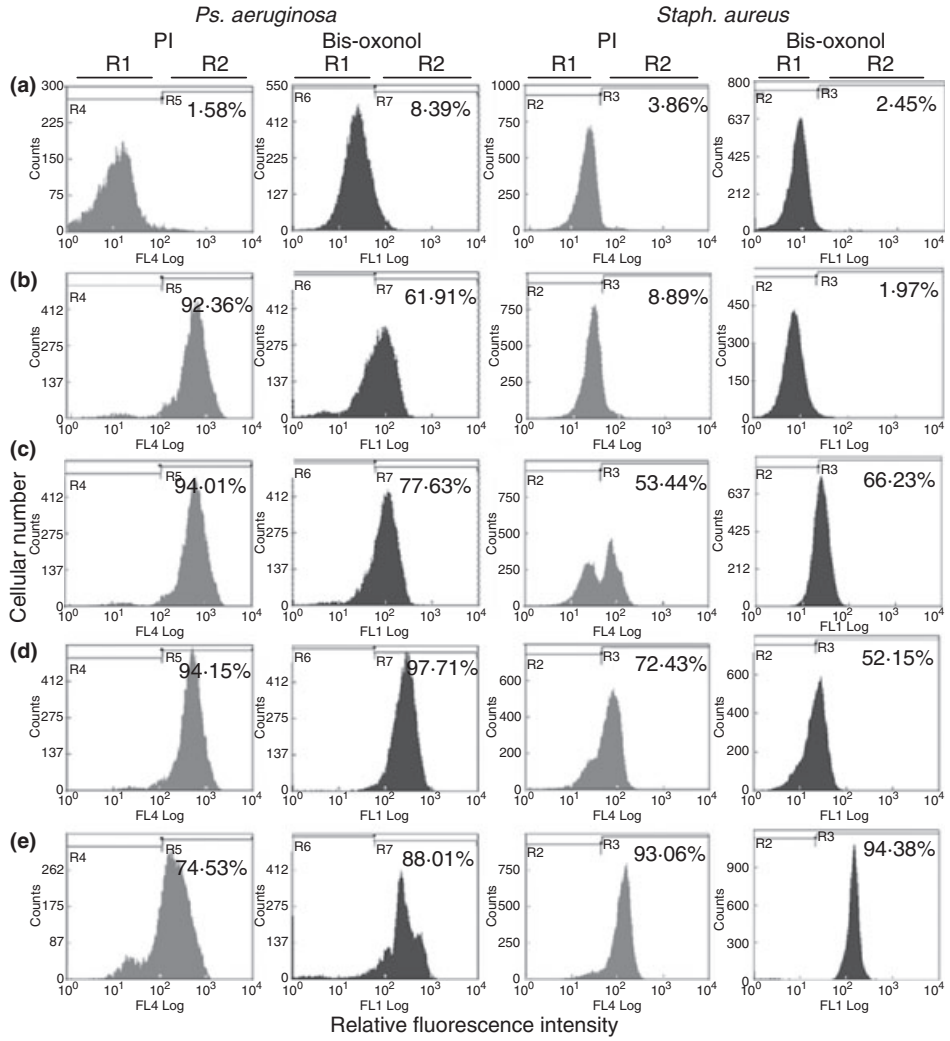

Figure 2 Effect on the membrane permeability and the membrane potential of *Pseudomonas aeruginosa* and *Staphylococcus aureus* caused by exposure to the essential oil of *Origanum compactum*, polymixin and CHX, revealed by PI and bis-oxonol staining. The relative fluorescence intensities within the R1 regions were taken as live cells, and those within the R2 regions were taken as dead cells (logarithmic scale). (a) Untreated cells control, (b) cells treated with essential oil at MIC, (c) cells treated with essential oil at 1·5× MIC, (d) cells treated with polymixin (for *Ps. aeruginosa*) at 1·5× MIC and with CHX (for *Staph. aureus*), (e) cells heated at 70°C. In all cases, time of contact was 1 h.

authors reported that treatment with tea tree oil resulted in K^+ leakage from *Staph. aureus* (Hada *et al.* 2003) and *E. coli* cells (Cox *et al.* 1998). The amount of potassium ions released from 1·5× MIC-treated cells was 3·2 times higher in *Staph. aureus* suspensions than in *Ps. aeruginosa*. Similarly, potassium ions released from *Staph. aureus* exposed to the monohydrochloride of L-arginine, *N*α-lauroyl ethyl ester was 2·5 times higher than in *S. typhimurium* (Rodriguez *et al.* 2004).

Flow cytometric analysis was performed with different staining protocols to assess the effect on different cell activities. The effect on membrane permeability was evaluated using PI, a cationic nucleic acid dye that is excluded by intact plasma membranes but can penetrate into cells with damaged membranes and intercalates with double-stranded DNA or RNA. The fluorescence conferred by this probe is generally associated with cells that have lost their membrane integrity. The effect on membrane potential was assessed by bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$]. This anionic lipophilic dye has a low binding affinity for intact membranes and is limited to the outer regions of the cell membrane in living bacteria. Membrane depolarization leads to the incorporation of the dye into the cell where it binds to lipid-rich intracellular compounds resulting in an increasing fluorescence signal detected by FC (Vives-Rego *et al.* 2000). This stain has previously been used with FC to assess antibacterial activity (Mason *et al.* 1995; Nuding

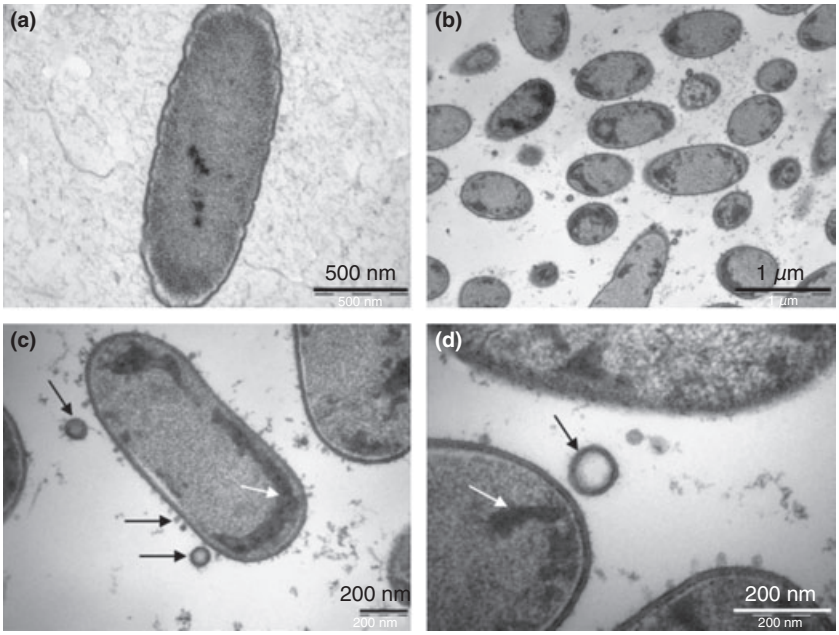

Figure 3 Electron micrograph of nontreated (a) and oil treated (b–d) *Pseudomonas aeruginosa* cells. Vesicles protruding from cell enveloped are signalled by black arrows. White arrows indicate coagulated material. Time of contact was 30 min and essential oil concentration was MIC.

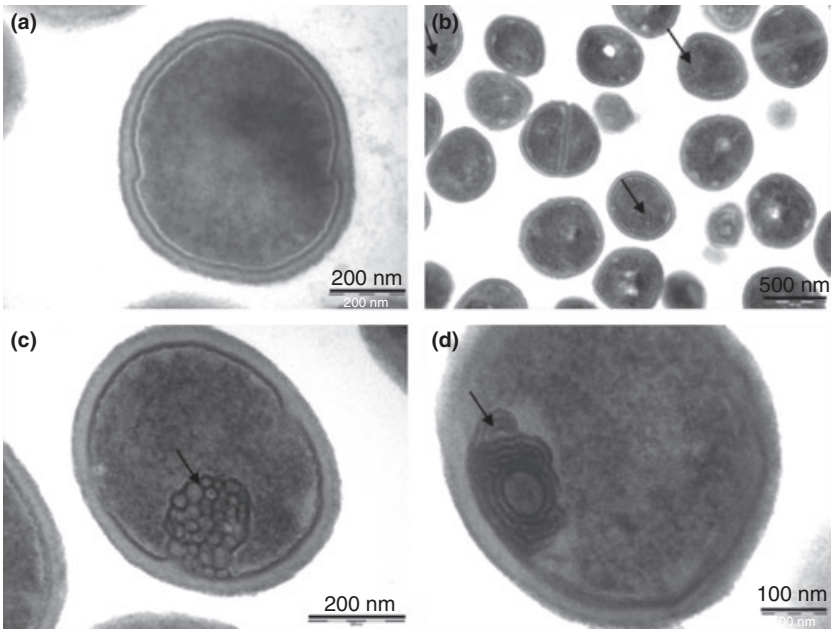

Figure 4 Electron micrograph of nontreated (a) and oil treated (b–d) *Staphylococcus aureus* cells. Arrows indicate the intracellular membranes formation. Time of contact was 30 min and essential oil concentration was MIC.

et al. 2006). The redox dye CTC is reduced by the respiratory electron transport chain to an insoluble fluorescent formazan, which accumulates in actively respiring cells. Previous studies have reported that the intracellular reduction of CTC is achieved directly by electron transfer system-associated dehydrogenase enzymes in *Micrococcus luteus* (Kaprelyants and Kell 1993) and by NAD(P)H in *E. coli* (López-Amorós *et al.* 1995). This dye has gained wide application in recent years (Yamaguchi and Nasu 1997; Berman *et al.* 2001; Proctor and Souza 2001).

In *Ps. aeruginosa*, both oil concentrations (Table 2) caused complete growth inhibition (99·99%), accompanied by a significant decrease in respiratory activity (15·62–1·14%), collapse of cell membrane potential (62·28–77·63%) and loss of membrane permeability (92·44–94·01%). These results are close to those produced by polymixin used as a positive control. The correlation between loss of membrane potential, membrane permeability, the ability to reduce CTC and reduction of cell viability indicate that oregano oil kills *Ps. aeruginosa* cells

by disrupting membrane function, leading to a loss of cytosolic constituents, as observed with K^+ ion leakage. Similar conclusions were reached by other authors attempting to explain the antibacterial action of essential oils. Tea tree oil treatment of *Staph. aureus* and *E. coli* inhibits respiration and increases the permeability of bacterial cytoplasmic membrane to potassium and other cytosolic constituents (Cox *et al.* 2000). It has been reported that the antibacterial action of essential oils of *Thymus vulgaris*, *Ocimum gratissimum* and *Cymbopogon citratus* was caused by the permeabilization of the cytoplasmic membrane of *Listeria innocua* (Nguefack *et al.* 2004). Carvacrol and thymol were also found to permeabilize and depolarize the cytoplasmic membrane of *E. coli* and to reduce it intracellular ATP pool (Helander *et al.* 1998; Xu *et al.* 2008).

When *Staph. aureus* was exposed to the oil at the MIC, no significant effect was observed on membrane potential (1·95–1·97%) or permeability (5·93–8·89%) whereas respiratory activity (30·40–17·09%) and viability (19·05–50·48% reduction) appeared to be affected. Increasing the oil concentration to 1·5× MIC resulted in a critical reduction in viability (99·67–99·96%), a significant reduction in membrane potential (44·66–66·23%) and membrane integrity (39·66–53·44%), but only a slight effect on respiratory activity (14·60–13·76%) compared with the MIC-treated cells (Table 2). The difference between the results obtained by bacterial count and the PI staining protocol indicate the presence of cells that have maintained membrane integrity but are not able to grow, suggesting that the membrane is not the only target of antibacterial action of oregano oil in *Staph. aureus*. Different mechanisms may be involved in the antibacterial activity of an essential oil depending on its composition and nature of the micro-organism studied. The difference observed here between the two strains studied may be due to the difference in membrane structure and composition. The cell wall of Gram-positive bacteria contains much more peptidoglycan than that of Gram-negative cells, providing increased rigidity (Wu *et al.* 2008).

The different responses to the oil treatment observed between the two strains studied by FC measurement were supported by electron micrographs. Cells exposed to oregano oil revealed morphological alterations that were more evident in *Ps. aeruginosa* than in *Staph. aureus* (Figs 3 and 4). In treated *Ps. aeruginosa* cells, intracytoplasmic coagulated material was seen close to the cell wall. A similar effect was observed in *E. coli* cells after addition of oregano oil (Becerril *et al.* 2007) or tea tree oil (Gustafson *et al.* 1998). This coagulated material was thought to be a precipitate of abnormal proteins or denatured membranes (Gustafson *et al.* 1998; Becerril *et al.* 2007). In addition, numerous projections from the cell wall and membrane vesicles were observed. Similar results were seen in *Ps. aeruginosa* treated with polymixin (Koike *et al.* 1969) and gentamicin (Kadurugamuwa and Beveridge 1997).

Some of the oil-treated *Staph. aureus* cells contained mesosome-like structures that were not seen in untreated cells. Such intracytoplasmic membrane inclusions are regarded as being indicative of cytoplasmic membrane alteration. Similar alterations were seen in *Staph. aureus* cells exposed to terpinen-4-ol (Carson *et al.* 2002), indolicidin derivative (Friedrich *et al.* 2001) and defensin (Shimoda *et al.* 1995). No apparent lysis or gross leakage of cellular cytoplasmic contents was observed.

Conclusions

Our results demonstrate that despite the high MIC value for *Ps. aeruginosa* in comparison with *Staph. aureus*, the ultrastructural and functional changes that occurred were greater in *Ps. aeruginosa* than in *Staph. aureus* when both strains were exposed to the corresponding MIC and 1·5× MIC values. Oregano essential oil induces membrane damage (leakage of potassium and uptake of PI and bis-oxonol), ultrastructural alterations and the loss of cell viability.

Acknowledgements

The financial support of the Comissió Interdepartamental de Recerca i Tecnologia CIRIT project 2005GR00143 and the Ministerio de Ciencia e Innovación, project CTQ2007-60749/PPQ is gratefully acknowledged. S.B. is grateful to the Agencia Española de Cooperación Internacional (AECI) for the fellowship.

References

Bakkali, F., Averbeck, S., Averbeck, D., Zhiri, A. and Idaomar, M. (2005) Cytotoxicity and gene induction by some essential oils in the yeast *Saccharomyces cerevisiae*. *Mutat Res* **585**, 1–13.

Bakkali, F., Averbeck, S., Averbeck, D., Zhiri, A., Baudoux, D. and Idaomar, M. (2006) Antigenotoxic effects of three essential oils in diploid yeast (*Saccharomyces cerevisiae*) after treatments with UVC radiation, 8-MOP plus UVA and MMS. *Mutat Res* **606**, 27–38.

Becerril, R., Gómez-Lus, R., Goñi, P., López, P. and Nerín, C. (2007) Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon or oregano against *E. coli* and *S. aureus*. *Anal Bioanal Chem* **388**, 1003–1011.

Bellakhdar, J. (1997) *La pharmacopée Marocaine traditionnelle. Médecine arabe ancienne et savoirs populaires*. Paris: Ibis Press.

Berman, T., Kaplan, B., Chava, S., Viner, Y., Sherr, B.F. and Sherr, E.B. (2001) Metabolically active bacteria in Lake Kinneret. *Aquat Microb Ecol* **23**, 213–224.

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

Carson, C.F., Mee, B.J. and Riley, T.V. (2002) Mechanism of Action of *Melaleuca alternifolia* (Tea Tree) Oil on *Staphylococcus aureus* Determined by Time-Kill, Lysis, Leakage, and Salt Tolerance Assays and Electron Microscopy. *Antimicrob Agents Chemother* **46**, 1914–1920.

Cox, S.D., Gustafson, J.S., Mann, C.M., Markham, J.L., Liew, Y.C., Hartland, R.P., Bell, H.C., Warmington, J.R. *et al.* (1998) Tea tree oil causes K+ leakage and inhibits respiration in *Escherichia coli*. *Appl Environ Microbiol* **26**, 355–358.

Cox, S.D., Mann, C.M., Markham, J.L., Bell, H.C., Gustafson, J.E., Warmington, J.R. and Wyllie, S.G. (2000) The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol* **88**, 170–175.

Cox, S.D., Mann, C.M. and Markham, J.L. (2001) Interactions between components of the essential oil of *Melaleuca alternifolia*. *J Appl Microbiol* **91**, 492–497.

Cristani, M., D'arrigo, M., Mandalari, G., Castelli, F., Sarpietro, M.G., Micieli, D., Venuti, V., Bisignano, G. *et al.* (2007) Interaction of four monoterpenes contained in essential oils with model membranes: implications for their antibacterial activity. *J Agric Food Chem* **55**, 6300–6308.

Eminagaoglu, O., Tepe, B., Yumrutas, O., Akpulat, A., Daferera, D., Polissiou, M. and Sokmen, A. (2007) The in vitro antioxidative properties of the essential oils and methanol extracts of *Satureja spicigera* (K. Koch.) Boiss. and Satureja cuneifolia ten. *Food Chem* **100**, 339–343.

Fitzgerald, D.J., Stratford, M., Gasson, M.J., Ueckert, J., Bos, A. and Narbad, A. (2004) Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. *J Appl Microbiol* **97**, 104–113.

Friedrich, C.L., Rozek, A., Patrzykat, A. and Hancock, R.E.W. (2001) Structure and mechanism of action of an indolicidin peptide derivative with improved activity against gram-positive bacteria. *J Biol Chem* **276**, 24015–24022.

Gill, A.O. and Holley, R.A. (2006) Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *Int J Food Microbiol* **108**, 1–9.

Gustafson, J.E., Liew, Y.C., Markham, J., Bell, H.C., Wyllie, S.G. and Warmington, J.R. (1998) Effects of tea tree oil on *Esherichia coli*. *Lett Appl Microbiol* **26**, 194–198.

Hada, T., Inoue, Y., Shiraishi, A. and Hamashima, H. (2003) Leakage of K+ ions from *Staphylococcus aureus* in response to tea tree oil. *J Microbiol Methods* **53**, 309–312.

Helander, I.M., Alakomi, H.-L., Latva-Kala, K.s., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M. and Wright, A.v. (1998) Characterization of the action of selected essential oil components on gram-negative bacteria. *J Agric Food Chem* **46**, 3590–3595.

Ismaili, H., Milella, L., Fkih-Tetouani, S., Ilidrissi, A., Camporese, A., Sosa, S., Altinier, G., Loggia, R.D. *et al.* (2004) In vivo topical anti-inflammatory and in vitro antioxidant activities of two extracts of *Thymus satureioides* leaves. *J Ethnopharmacol* **91**, 31–36.

Kadurugamuwa, J.L. and Beveridge, T.J. (1997) Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of minoglycoside antibiotics on their release. *J Antimicrob Chemother* **40**, 615–621.

Kaprelyants, A.S. and Kell, D.B. (1993) The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for the visualisation of respiratory activity in individual cells of *Micrococcus luteus*. *J Microbiol Methods* **17**, 115–122.

Koike, M., Iida, K. and Matsuo, T. (1969) Electron microscopic studies on mode of action of polymyxin. *J Bacteriol* **97**, 448–452.

Kotzekidou, P., Giannakidis, P. and Boulamatsis, A. (2008) Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens in vitro and on the fate of inoculated pathogens in chocolate. *LWT-Food Sci Technol* **41**, 119–127.

Lambert, P.A. and Hammond, S.M. (1973) Potassiom fluxes. First indications of membrane damage in microorganisms. *Biochem Biophys Res Commun* **54**, 796–799.

López-Amorós, R., Mason, D.J. and Lloyd, D. (1995) Use of two oxonols and a fluorescent tetrazolium dye to monitor starvation of *Escherichia coli* in seawater by flow cytometry. *J Microbiol Methods* **22**, 165–176.

Mann, C.M. and Markham, J.L. (1998) A new method for determining the minimum inhibitory concentration of essential oils. *J Appl Microbiol* **84**, 538–544.

Mason, D.J., Power, E.G.M., Talsania, H., Phillips, I. and Gant, V.A. (1995) Antibacterial action of ciprofloxacin. *Antimicrob Agents Chemother* **39**, 2752–2758.

Mezzoug, N., Elhadri, A., Dallouh, A., Amkiss, S., Skali, N.S., Abrini, J., Zhiri, A., Baudoux, D. *et al.* (2007) Investigation of the mutagenic and antimutagenic effects of *Origanum compactum* essential oil and some of its constituents. *Mutat Res* **629**, 100–110.

Nguefack, J., Budde, B.B. and Jakobsen, M. (2004) Five essential oils from aromatic plants of Cameroon: their antibacterial activity and ability to permeabilize the cytoplasmic membrane of *Listeria innocua* examined by flow cytometry. *Lett Appl Microbiol* **39**, 395–400.

Nuding, S., Fellermann, K., Wehkamp, J., Mueller, H.A.G. and Stange, E.F. (2006) A flow cytometric assay to monitor antimicrobial activity of defensins and cationic tissue extracts. *J Microbiol Methods* **65**, 335–345.

Pina-Vaz, C., Rodrigues, A.G., Pinto, E., Costa-de-Oliveira, S., Tavares, C., Salgueiro, L., Cavaleiro, C., Gonçalves, M. *et al.* (2004) Antifungal activity of *Thymus* oils and their major compounds. *J Eur Acad Dermatol Venereol* **18**, 73–78.

Proctor, L.M. and Souza, A.C. (2001) Method for enumeration of 5-cyano-2,3-ditoyl tetrazolium chloride (CTC)-active cells and cell-specific CTC activity of benthic bacteria in riverine, estuarine and coastal sediments. *J Microbiol Methods* **43**, 213–222.

Rodriguez, E., Seguer, J., Rocabayera, X. and Manresa, A. (2004) Cellular effects of monohydrochloride of L-arginine, Na-lauroyl ethylester (LAE) on exposure to *Salmonella typhimurium* and *Staphylococcus aureus*. *J Appl Microbiol* **96**, 903–912.

Shimoda, M., Ohki, K., Shimamoto, Y. and Kohashi, O. (1995) Morphology of defensin-treated *Staphylococcus aureus*. *Infect Immun* **63**, 2886–2891.

Sikkema, J., de Bont, J.A. and Poolman, B. (1994) Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem* **269**, 8022–8028.

Ultee, A., Kets, E.P.W. and Smid, E.J. (1999) Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **65**, 4606–4610.

Ultee, A., Bennik, M.H.J. and Moezelaar, R. (2002) The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **68**, 1561–1568.

Vives-Rego, J., Lebaron, P. and Caron, G.N.-V. (2000) Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiol Rev* **24**, 429–448.

Walsh, S.E., Maillard, J.-Y., Russell, A.D., Catrenich, C.E., Charbonneau, D.L. and Bartolo, R.G. (2003) Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria. *J Appl Microbiol* **94**, 240–247.

Wu, V.C.-H., Qiu, X., Bushway, A. and Harper, L. (2008) Antibacterial effects of American cranberry (*Vaccinium macrocarpon*) concentrate on foodborne pathogens. *LWT Food Sci Technol* **41**, 1834–1841.

Xu, J., Zhou, F., Ji, B.-P., Pei, R.-S. and Xu, N., II (2008) The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. *Lett Appl Microbiol* **47**, 174–179.

Yamaguchi, N. and Nasu, M. (1997) Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *J Appl Microbiol* **83**, 43–52.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Chromatographic profile of *Origanum compactum* essential oil.

Table S1 Chemical composition of *Origanum compactum* essential oil according to the data provided by the manufacturer.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

