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ORIGINAL ARTICLE

Functional and ultrastructural changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Cinnamomum verum* essential oil

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Abstract

Aims: To study cellular damage induced by *Cinnamomum verum* essential oil in *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.

Methods and Results: The effect of cinnamon bark essential oil on these two strains was evaluated by plate counts, potassium leakage, flow cytometry and transmission electron microscopy (TEM). Exposure to this oil induced alterations in the bacterial membrane of *Ps. aeruginosa*, which led to the collapse of membrane potential, as demonstrated by bis-oxonol staining, and loss of membrane-selective permeability, as indicated by efflux of K^+ and propidium iodide accumulation. Thus, respiratory activity was inhibited, leading to cell death. In *Staph. aureus*, cells treated with the oil entered a viable but noncultivable (VNC) state. The oil initially caused a considerable decrease in the metabolic activity and in the replication capacity of these bacterial cells. The loss of membrane integrity appeared later, as indicated by bis-oxonol and Propidium iodide (PI) staining. Data provided by TEM showed various structural effects in response to cinnamon essential oil. In *Ps. aeruginosa* cells, coagulated cytoplasmic material was observed, and intracellular material was seen in the surrounding environment, while oil-treated *Staph. aureus* showed fibres extending from the cell surface.

Conclusions: Cinnamon essential oil damages the cellular membrane of *Ps. aeruginosa*, which leads to cell death. There is evidence of VNC *Staph. aureus* after exposure to the oil.

Significance and Impact of the Study: Cinnamon essential oil shows effective antimicrobial activity and health benefits and is therefore considered a potential food additive. To use this oil as a natural food preservative, especially in combination with other preservation methods, a thorough understanding of the mechanism through which this oil exerts its antibacterial action is required.

Introduction

Microbial spoilage is the most common cause of food deterioration. Thus, one of the major concerns of the food industry is the control of spoilage and pathogenic organisms. Therefore, a broad range of chemicals that inhibit microbial growth are added to food during manufacture to extend shelf-life and ensure food safety and quality. However, although the effectiveness of traditional preservatives is recognized, their safety has been questioned (Türkoglu 2007). Consequently, an increasing number of consumers, concerned about synthetic chemical additives, opt for foodstuffs that contain only natural ingredients. This tendency has contributed to the use of

natural molecules for preservation purposes in the food industry. Several studies have demonstrated the effectiveness of natural products, such as bacteriocins and plant extracts, in controlling food spoilage and food-borne pathogenic bacteria in many food matrices, thus improving their shelf-life and safety (Tsigarida *et al.* 2000; Smith-Palmer *et al.* 2001; Vrinda Menon and Garg 2001; da Silva Malheiros *et al.* 2010).

Essential oils are a complex mixture of secondary metabolites produced by aromatic plants. These oils are responsible for the fragrance of the plants and many of their biological activities (antimicrobial, anti-oxidant). The biological properties of essential oils are exploited in the food industry, for example as flavour additives. Indeed, essential oils and their components also show antimicrobial properties and could thus be useful for the conservation of foodstuffs (Burt 2004). Moreover, many essential oils and their components have been approved by the US Food and Drug Administration for use in food and beverages (USFDA 2009).

Cinnamon (*Cinnamomum zeylanicum* Blume, syn *C. verum*) is a widely used spice that has many applications in the perfumery, food and pharmaceutical industries (Lee and Balick 2005). The essential oil extracted from *C. verum* bark is composed mainly by cinnamaldehyde (Singh *et al.* 2007). Recent *in vitro* studies have shown that *C. verum* essential oil effectively inhibits the food spoilage and the growth of pathogenic bacteria (Oussalah *et al.* 2006; Singh *et al.* 2007; Johny *et al.* 2008). In addition to its antimicrobial activity, this oil has multiple beneficial effects on health (Edris 2007; Mishra *et al.* 2009). It is therefore considered an effective alternative food preservative agent. However, to use cinnamon oil as a food additive, the mechanism through which it exerts its antibacterial activity should be elucidated.

This study addresses the effect of *C. verum* essential oil on bacterial cells of *Ps. aeruginosa* and *Staph. aureus* by evaluating the following parameters: potassium leakage, membrane permeability, membrane potential, respiratory activity and ultrastructural damage.

Materials and methods

Antibacterial product and chemicals

Essential oil extracted from *Cinnamomum verum* bark 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 compound of this oil was E-cinnamaldehyde (73·35%; see Table S1 and Figure S1). Polymyxin B sulfate and chlorhexidine dihydrochloride (CHX) were purchased from Sigma (St Louis, MO, USA).

The molecular dyes propidium iodide (PI) and bis-(1,3-dibutylbarbutiric acid) trimethine oxonol (bis-oxonol) were supplied by Molecular Probes Europe BV (Leiden, the Netherlands) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) by Sigma (USA). 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 *Staphylococcus aureus* ATCC 29213 were obtained from the ATCC (Manassas, VA, USA) and sub-cultured weekly on trypticase soy agar (TSA) (Pronadisa, Madrid, Spain). Strains were preserved frozen in cryovials (AES Laboratoire, Combourg, France) at −80°C.

Minimal inhibitory concentration (MIC)

MICs were determined 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 as bacterial growth indicator. First, 50 μl of Mueller Hinton Broth (Oxoid; UK) supplemented with bacteriological agar (0·15% w/v) was distributed from the 2nd to the 12th well of a 96-well polypropylene microtitre plate (Costar; Corning Incorporated, Corning, NY, USA). A dilution of the cinnamon essential oil 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 was added to the first test well of each microtitre line, and then 50 μl of scalar dilution was transferred from the 2nd to the 11th well. The 12th well was considered as growth control, because no essential oil was added. We then added 50 μl of a bacterial suspension 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 was added to each well to assess bacterial growth, as proposed by Mann and Markham (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 was detected by reduction in blue dye resazurin to pink resorufin. A control was carried out to ensure that, at the concentrations tested, the essential oil did not cause a colour change in the resazurin. Experiments were performed 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 TSB at 30°C in a shaking incubator (120 rev min^{-1}). Cells were then washed three times in 0·9% NaCl by centrifugation at 8000 ***g*** for 15 min and re-suspended in 30 ml of 1 mmol l^{-1} glycyl-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 at 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, diluted and filtered through a 0·2-μm pore-size membrane (Sartorius, Goettingen, Germany) to remove 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 to 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 tryptone soy broth (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 re-suspended 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}. Antimicrobials were added to the flasks to reach the MIC (0·125% v/v) or 1·5 × MIC (0·187% v/v) for cinnamon oil and 1·5 × MIC (12 mg l^{-1}) for polymyxin and CHX (1·5 mg l^{-1}). No solubilizing agent was used in the flasks treated by essential oil. The flasks were then incubated at 30°C in a shaker incubator.

The contact times established for flow cytometry (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. They were then washed and re-suspended in 1 ml of filtered Ringer's solution.

For transmission electronic microscopy (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 re-suspended in 1 ml of peptone-buffered water (Oxoid, Basingstoke, 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

The staining protocols for FC experiments were as follows: 10 μl of a 1 mg ml^{-1} stock solution of propidium iodide (PI) in distilled water was added to 1 ml of the bacterial suspension, prepared as described earlier in filtered Ringer's solution (the bacterial concentration was 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.

To evaluate membrane potential, 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 reach a final concentration of 0·5 μmol l^{-1}, and this solution was then incubated for 2 min at room temperature.

To measure respiratory activity, 100 μl of a 50 mmol l^{-1} CTC solution in deionized water filtered through a 0·22-μm pore-size membrane was added to 900 μl of bacterial suspension to reach 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 performed in duplicate.

Flow cytometry

FC experiments were carried out using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). Samples were excited 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) for PI. The results 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 analyzed with SUMMIT® ver. 3.1 software (Cytomation, Fort Collins, CO, USA).

Bacterial count

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

$1 - N_T/N_C \times 100$; N_T, bacterial count in treated sample; N_C, bacterial count in control sample.

Transmission electron microscopy (TEM)

After treatment of cell suspensions of each micro-organism for 30 min with the corresponding MIC of the oil, the bacterial pellets were rinsed with buffered water peptone (pH 7) and washed three times. *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. *Ps. aeruginosa* samples were cryofixed 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}). 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 the two micro-organisms were then embedded in L.R. (London Resin Co. Ltd, London, UK) white resin. Ultrathin sections were prepared and stained with 1% uranyl acetate and sodium citrate. Microscopy was performed with a Phillips EM 30 (Eindhoven, Holland) microscope with an acceleration of 60 kV.

Results

Cinnamomum verum essential oil showed an MIC of 0·125% (v/v) for *Staph. aureus* and *Ps. aeruginosa*. To inspect the cellular damage produced by the oil on the bacteria, we worked at the MIC and 1·5 × MIC. Polymyxin and chlorhexidine were used as markers of antimicrobial activity on Gram-negative and Gram-positive bacteria, respectively.

Potassium leakage

To determine changes in cell membrane permeability, K^+ leakage from the two bacterial strains was examined over the exposure time. In response to the oil, *Ps. aeruginosa* cells showed some leakage of intracellular potassium. The amount of extracellular potassium reached 0·059 ± 0·023 and 0·138 ± 0·027 ppm after 120 min of contact with the MIC and 1·5 × MIC, respectively (Fig. 1a). Heat-treated cells showed more marked effect on K^+ leakage. In the case of *Staph. aureus*, the oil induced a marked leakage of

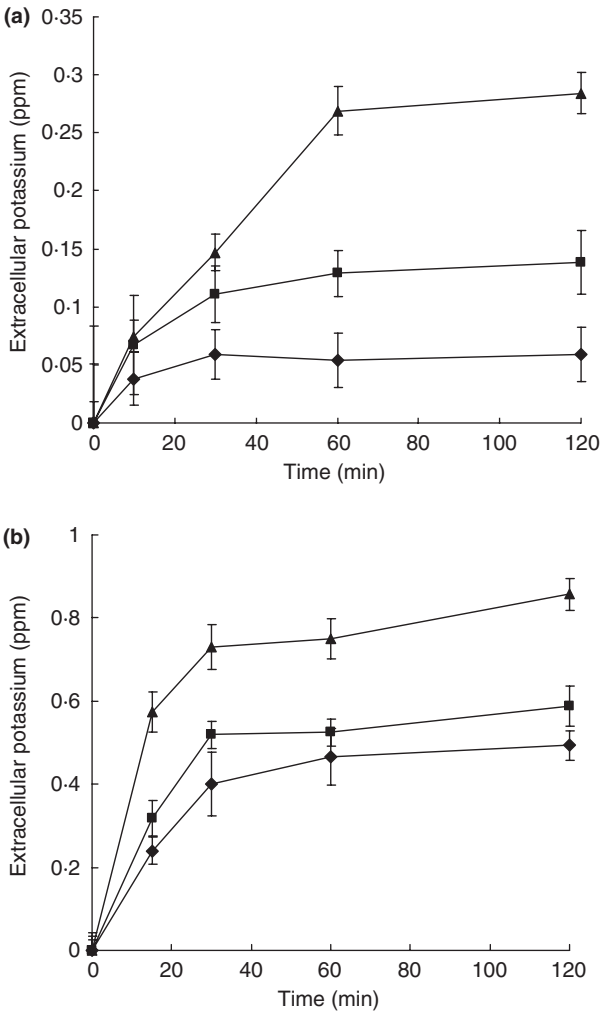

Figure 1 Potassium leakage of the cell suspension of *Pseudomonas aeruginosa* (a) and *Staphylococcus aureus* (b) exposed to MIC (filled rhombus (◆) and 1·5 × MIC (filled squares (■)) of *Cinnamomum verum* essential oil. Exposure to 70°C (filled triangle (▲)) acted as positive control. The amount of 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.

intracellular potassium (Fig. 1b), attaining 0·493 ± 0·035 ppm and 0·588 ± 0·048 ppm after 120 min of treatment with the MIC and 1·5 × MIC, respectively. Heat treatment produced greater K^+ leakage (0·857 ± 0·039 ppm after 120 min of exposure).

Membrane permeability

To assess whether the essential oil affects bacterial membrane permeability, we used PI staining coupled to FC analysis. The fluorescence profiles obtained were referred to two controls, one of untreated cells indicative of membrane integrity (intact cells, R1) and the other of heat-treated cells indicative of permeabilized membrane (dead cells, R2) (Fig. 2).

In the untreated population of *Ps. aeruginosa*, only 8·01% had permeabilized membranes. Treatment with the oil at the MIC increased the PI-stained population to 58·00 and 64·29% after 30 and 60 min of contact, respectively (Table 1), while for 1·5 × MIC percentages of 66·58 and 75·93% were registered after 30 and 60 min, respectively. The effect on membrane permeability was stronger when cells were exposed to polymyxin (1·5 × MIC). In this case, the fraction of permeabilized cells reached 89·40 and 94·15% after 30 and 60 min of contact, respectively.

In *Staph. aureus*, the control population was impermeable to PI (0·65% PI-stained cells). Exposure to the oil at the MIC had no notable effect on membrane permeability. Indeed, the proportion of PI-positive cells was 4·18 and 4·99% after 30 and 60 min, respectively. The increase

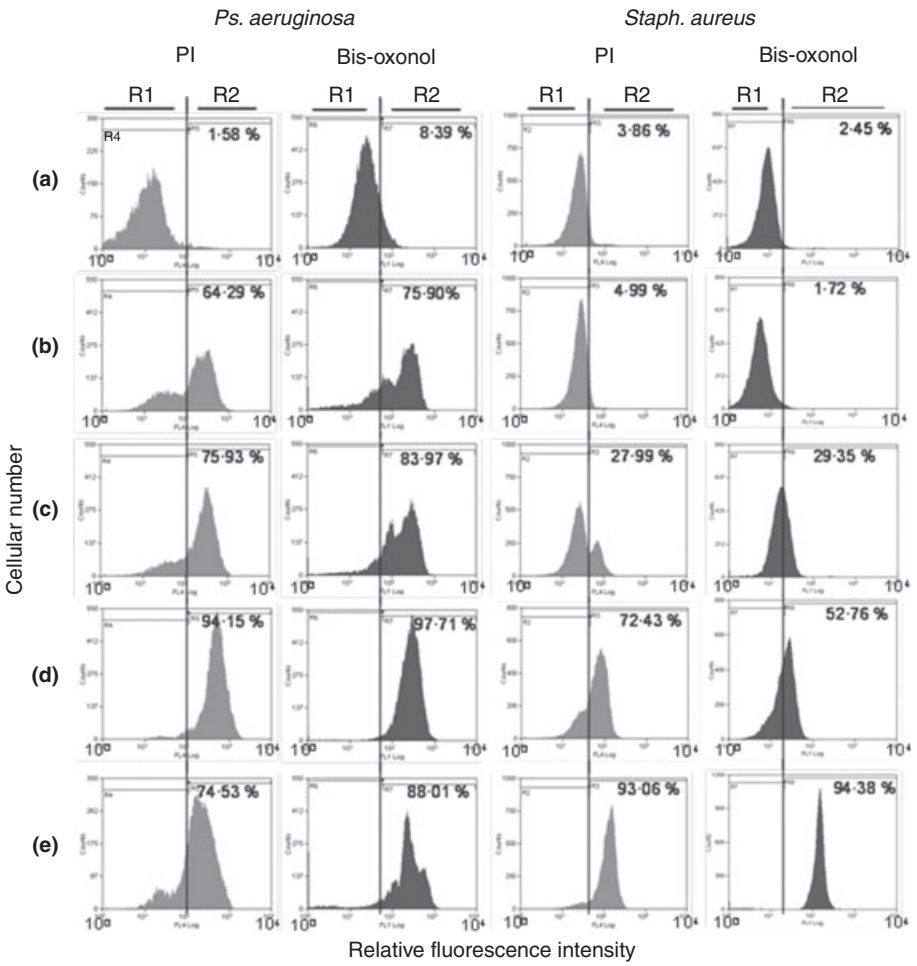

Figure 2 Effect on the membrane permeability and the membrane potential of *Pseudomonas aeruginosa* and *Staphylococcus aureus* caused by exposure to the essential oil of *Cinnamomum verum,* polymyxin and CHX revealed by PI and bis-oxonol staining. The relative fluorescence intensities within the R1 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 polymyxin (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.

Table 1 Percentage of stained cells measured by flow cytometry analysis and viability reduction in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells treated with Cinnamon essential oil, polymyxin and CHX

			Ps. aeruginosa				*Staph. aureus*			
			% of stained cells				% of stained cells			
	Conc.	Contact time (min)	PI	Bis-oxonol	Tetrazolium chloride	% 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	58·00	74·50	5·90	40·77	4·18	1·79	12·36	–
		60	64·29	75·90	3·95	84·62	4·99	1·72	9·89	42·86
	1·5 × MIC	30	66·58	84·07	4·42	100	24·61	6·50	10·95	79·53
		60	75·93	83·97	2·68	100	27·99	29·35	9·12	81·90
Polymyxin	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

in oil concentration to 1·5 × MIC enhanced cell permeabilization, which reached 24·61 and 27·99% after 30 and 60 min of contact, respectively. The treatment by CHX (1·5 × MIC) produced a stronger effect on membrane permeability, with 53·45 and 72·43% of PI-stained cells after 30 and 60 min, respectively.

Membrane potential

The effect of the oil on the bacterial membrane was also assessed by using bis-oxonol dye, which accumulates in cells with depolarized membrane. As shown in Fig. 2, the membrane potential of *Ps. aeruginosa* cells was considerably disturbed after the oil exposure. Thus, the percentage of cells that accumulated bis-oxonol reached 74·50 and 84·07% after 30 min of contact with this oil at the MIC and 1·5 × MIC, respectively (Table 1). An increase in the contact time to 60 min did not affect the proportion of depolarized cells. Treatment with polymyxin had a stronger effect on the membrane potential of *Ps. aeruginosa*. Indeed, 95·42 and 97·71% of these cells were marked by the bis-oxonol after 30 and 60 min of exposure, respectively.

The untreated population of *Staph. aureus* did not show any significant membrane depolarization (0·89% stained with bis-oxonol). At the MIC, the oil had no effect on membrane potential, which dissipated after 60 min of exposure to this oil at 1·5 × MIC in 29·35% of cells. The effect on membrane potential was influenced by oil concentration and contact time. The action of CHX was strongly affected by the contact time. Indeed, the proportion of bis-oxonol-positive cells increased from 3·52% after 30 min to 52·15% after 60 min of contact.

Respiratory activity

CTC was used to examine the effect of the oil on respiratory enzymes. In control suspension *Ps. aeruginosa*, a high proportion of cells reduced the CTC (66·94%). After treatment with the oil, a significant reduction in the capacity of the cell population to reduce CTC was observed for the two concentrations tested (5·90 and 4·42% after 30 min of contact). This result was not affected by the prolongation of contact time to 60 min. Exposure to polymyxin considerably affected the CTC-reducing capacity of cells (Table 1). Thus, the proportion of CTC-positive cells decreased to 0·44 and 0·46% after 30 and 60 min of contact, respectively.

With regard to *Staph. aureus*, the respiratory activity of the oil-treated suspension was significantly reduced. Indeed, the proportion of the cells that reduced CTC was 12·36% after 30 min and 9·89% after 60 min of contact with the oil at the MIC. No substantial differences were observed when the oil concentration was increased to 1·5 × MIC. Exposure to CHX dramatically reduced the proportion of active respiring cells (0·73 and 0·64% after 30 and 60 min, respectively).

Transmission electron microscopy (TEM)

The effect of the oil treatment on bacterial ultrastructure was assessed using TEM. The oil caused major structural changes in *Ps. aeruginosa* cells (Fig. 3). In fact, treated bacteria showed coagulated material, especially close to the cell envelope (white arrows) and projections from the cell wall (black arrows). Moreover, a significant amount of cytoplasmic material was observed outside the cells.

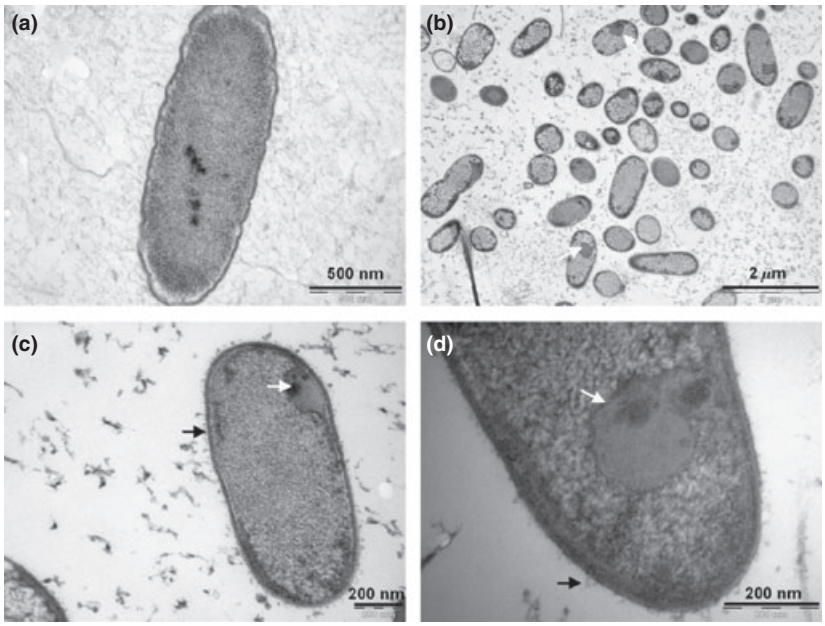

Figure 3 Electron micrograph of nontreated (a) and oil-treated (b, c and d) *Pseudomonas aeruginosa* cells. White arrows signal coagulated material. Black arrows indicate projections from cell wall. Time of contact was 30 min, and essential oil concentration was MIC.

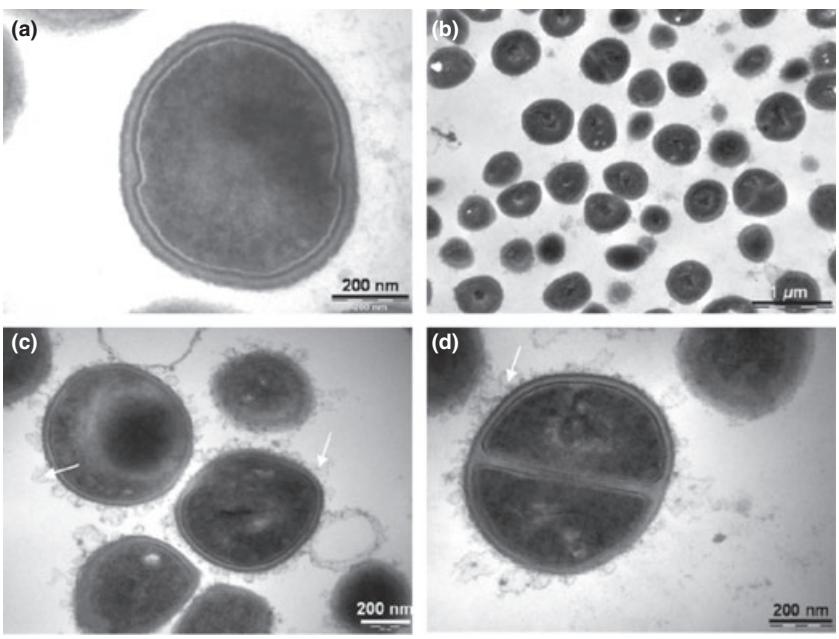

Figure 4 Electron micrograph of nontreated (a) and oil-treated (b, c and d) *Staphylococcus aureus* cells. Arrows indicate fibres extending from cell surface. Time of contact was 30 min, and essential oil concentration was MIC.

For *Staph. aureus*, electron micrographs showed the presence of fibres extending from surface of oil-treated cells (Fig. 4).

Discussion

Cinnamomum verum essential oil showed a strong antibacterial activity against *Staph. aureus* and *Ps. aeruginosa*. This activity may be associated with the presence of cinnamaldehyde, which is the major component of the oil (73·35%). Indeed, cinnamaldehyde is active against a range of Gram-positive and Gram-negative bacteria (Ali *et al.* 2005). To establish whether this inhibitory activity causes cell membrane injury, we first measured the release of potassium cation from treated cells. This approach is used to study the effect of antimicrobial activity on membrane integrity (Orlov *et al.* 2002). K^+ is the most abundant intracellular cation in all living organisms, including bacteria, and is essential for many essential cellular functions. Dissipation of the K^+ gradient across cell membrane is a consequence of membrane damage and breakdown of the permeability barrier. Our results

demonstrated that cinnamon essential oil induces leakage of intracellular K^+ from cells of *Staph. aureus* and *Ps. aeruginosa*. These results are consistent with previous studies that reported the capacity of essential oils and components to alter the permeability of bacterial cells to cations like K^+ (Cox *et al.* 1998; Ultee *et al.* 1999; Hada *et al.* 2003; Walsh *et al.* 2003; Inoue *et al.* 2004; Bouhdid *et al.* 2009).

The effect of cinnamon oil on membrane functions was also evaluated using FC coupled to specific fluorescent dyes, a valuable approach to study damage induced by antibacterial activity. The alteration of membrane integrity was then assessed using PI, a dye that can cross only damaged membranes and that binds to DNA or RNA, thereby conferring red fluorescence. This stain has been used to assess the antibiotic susceptibility of bacteria (Braga *et al.* 2003). Bis-oxonol (bis-(1,3-dibutylbarbituric acid) trimethine oxonol) was used to examine the effect of the oil on membrane potential. This anionic probe has a high voltage affinity, penetrates depolarized membranes and binds to lipid-rich intracellular components, thereby conferring green fluorescence (Vives-Rego *et al.* 2000). The action of the oil on metabolic activity was evaluated using CTC, which is reduced by the respiratory electron transport chain to an insoluble fluorescent formazan, which accumulates inside cells and confers red fluorescence to active respiring cells (Suller and Lloyd 1999).

From the obtained results, we propose that cinnamon oil induces alterations in the bacterial membrane of *Ps. aeruginosa.* These alterations led to the loss of membrane-selective permeability and thus the inhibition of respiratory activity, and also the loss of other essential enzymatic activities. All these changes led to cell death. Our results are in accordance with previous studies that demonstrated that essential oils and their components disturb the bacterial membrane, thereby leading to the loss of cytoplasmic material (Fitzgerald *et al.* 2004; Gill and Holley 2006a) and the inhibition of membrane-bound enzymes (Ultee *et al.* 1999; Gill and Holley 2006b). Thus, it has been reported that cinnamaldehyde, the major component of cinnamon oil, alters the membrane lipidic profile of *Escherichia coli*, *Staph. aureus*, *Salmonella typhimurium*, *Ps. fluorescens* and *Bacillus thermosphacta,* thereby causing a strong decrease in unsaturated fatty acids (Di Pasqua *et al.* 2007). This compound also inhibits carboxylase activity of *Enterobacter aerogenes* (Wendakoon and Sakaguchi 1995).

In the case of *Staph. aureus*, the methods used here allowed us to distinguish between various physiological states of cells treated with cinnamon essential oil. Plate count allowed the detection of viable and cultivable cells. Cells stained with PI have irreversibly damaged membrane and correspond to dead cells. CTC staining allowed the detection of active respiring bacteria (CTC-positive cells). CTC-negative cells are not necessarily nonviable cells but inactive ones, including dormant and moribund cells (Caruso *et al.* 2003). Indeed, cells that have a low respiratory activity are not detected as CTC-positive (Sieracki *et al.* 1999).

On the basis of our findings, we propose that cinnamon essential oil initially causes potassium ion leakage, a considerable reduction in metabolic activity and in the replication capacity of *Staph. aureus*. Membrane integrity did not appear to be the first target of the antibacterial effect of the oil, as indicated by bis-oxonol and PI staining. This observation indicates that treatment of this bacterial strain with this essential oil induces damage to the bacterial membrane, thereby increasing its permeability to small ions like potassium. The efflux of potassium ions caused impaired enzymatic activity, as shown by the inhibition of respiration. However, this membrane alteration was not sufficient to allow PI uptake. Similar results were obtained for tea tree oil. Indeed, when *Staph. aureus* was exposed to tea tree oil, the reduction in cell viability was close to the inhibition of respiration and K^+ ion efflux while the percentage of PI-stained cells was significantly lower (Cox *et al.* 2001).

Divergence between the results obtained by plate count and CTC staining and those of PI and bis-oxonol staining indicated the presence of cells that maintained membrane integrity but whose respiratory activity was restricted and replication capacity on growth media lost. The literature refers to this state as viable but noncultivable (VNC). Under these conditions, a number of major metabolic changes occur, including reductions in nutrient transport, respiration rates and macromolecular synthesis (Oliver 2005). It has been proposed that the VNC state is a survival strategy adopted by many bacteria in response to stress (Rowan 2004). The VNC state may also be regarded as transitory physiological stage before cell death (Coutard *et al.* 2007). When in this state, bacteria maintain pathogenicity factors/genes; and in some cases, the VNC state has been reversed in response to a return to favourable environmental conditions (Lleò *et al.* 2001; Baffone *et al.* 2003). VNC bacteria are a serious problem for food hygiene and public health because their detection requires sophisticated equipment that is usually unavailable in most routine food and clinical microbiology laboratories.

Our findings provide further evidence that plate count, a conventional analytical method widely used to assess bacterial viability, underestimates the population of viable cells. Here, we show that the use of FC, associated with specific fluorochromes, allows the differentiation of cells in various physiological states and therefore provides a

reliable estimation of decreases in viability in response to antibacterial agents.

The various cellular effects detected by FC were supported by the electronic micrographs. Indeed, the bacterial cells exposed to the essential oil showed ultrastructural changes. These were more marked in *Ps. aeruginosa*. In fact, coagulated cytoplasmic material was observed near the cell wall of treated *Ps. aeruginosa*. A similar alteration was observed in *Ps. aeruginosa* exposed to *Origanum compactum* essential oil (Bouhdid *et al.* 2009) and in *E. coli* cells treated with oregano (Becerril *et al.* 2007) and tea tree oil (Gustafson *et al.* 1998). This coagulated material is thought to be a precipitate of abnormal proteins or denatured membrane (Gustafson *et al.* 1998; Becerril *et al.* 2007). The treated cells also showed numerous projections extending from external membrane. Similar observations have been reported as morphological evidence of outer membrane damage by membrane-acting antibiotics such as polymyxin B and gentamicin (Koike *et al.* 1969; Kadurugamuwa and Beveridge 1997). With respect to *Staph. aureus*, microscopic observation of oil-treated cells showed the presence of fibres extending from cell surface while no significant damage was observed inside cells. These findings support the results obtained by FC for cells treated with the oil at the MIC.

Conclusions

Our findings demonstrate that cinnamon bark essential oil damages the cellular membrane of *Ps. aeruginosa*, which leads to cell death. Evidence of VNC bacterial cells was found in *Staph. aureus* after treatment with the oil.

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Supporting information

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

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

Figure S1 Chromatographic profile of *Cinnamomum verum* essential oil.

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