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A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol

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Aims: The minimum inhibitory concentration (MIC) of oregano essential oil (OEO) and two of its principle components, i.e. thymol and carvacrol, against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was assessed by using an innovative technique. The mechanism of action of the above substances was also investigated.

Methods and Results: The applied technique uses 100-well microtitre plate and collects turbidimetric growth data. To produce the inhibition profiles, a wide range of concentrations were tested for each of the three compounds, as well as for carvacrol–thymol mixtures. Following a specific mathematical analysis of the observed inhibition profiles from all compounds, it was suggested that mixtures of carvacrol and thymol gave an additive effect and that the overall inhibition by OEO can be attributed mainly to the additive antimicrobial action of these two compounds. Addition of low amounts of each additive: (a) increased permeability of cells to the nuclear stain EB, (b) dissipated pH gradients as indicated by the CFDA-SE fluorescent probe irrespective of glucose availability and (c) caused leakage of inorganic ions.

Conclusions: Mixing carvacrol and thymol at proper amounts may exert the total inhibition that is evident by oregano essential oil. Such inhibition is due to damage in membrane integrity, which further affects pH homeostasis and equilibrium of inorganic ions.

Significance and Impact of the Study: The knowledge of extent and mode of inhibition of specific compounds, which are present in plant extracts, may contribute to the successful application of such natural preservatives in foods, since certain combinations of carvacrol–thymol provide as high inhibition as oregano essential oil with a smaller flavour impact.

INTRODUCTION

The antimicrobial action of essential oils in model food systems or in real food is well documented in the literature (Koutsoumanis *et al*. 1998; Skandamis and Nychas 2000; Tsigarida *et al*. 2000). Although the majority of the essential oils are classified as Generally Recognized As Safe (GRAS) (Kabara 1991), their use in foods as preservatives is often limited due to flavour considerations, since effective antimicrobial doses may exceed organoleptically acceptable levels. Therefore, there is an increasing demand for accurate knowledge of the minimum inhibitory (effective) concentrations (MIC) of essential oils to enable a balance between the sensory acceptability and antimicrobial efficacy. This can be achieved with *in vitro* and *in vivo* studies. The former evaluation methods can be divided into groups such as diffusion, dilutions, impedance and optical density methods (Koutsoumanis *et al*. 1998, 1999; Tassou *et al*. 2000). Among these, the dilution method provides more quantitative results (Manou *et al*. 1998) while results obtained with other methods may not be comparable (Skandamis *et al*. 2000; Tassou *et al*. 2000). The limitations within each of the methods should be taken into account. For example, in case of turbidity, interference due to insufficient dissolution of the test compound(s) can be overcome to an extent with

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specific metabolic indicator stains (Chand *et al.* 1994; Mann and Markham 1998). The most recently available techniques for determination of MIC are based mainly on broth microdilution of the tested agents (Carson *et al.* 1995). Lambert and Pearson (2000) introduced and evaluated an innovative technique for assessment of MIC for chemical biocides using the Bioscreen Microbiological Growth Analyser. The advantages of this method rely on the simultaneous examination of multiple preservative concentrations and subsequent determination of MIC based on mathematical processing. To our knowledge this methodology has not been applied to the examination of the MIC of essential oils. However, the determination of possible combinations of the most active compounds of essential oils could be of great importance considering the aforementioned increase in their use. This knowledge could also help to understand the range of effectiveness of these oils with respect to compositional variation brought about through factors such as plant type, geographical location and time of the year (Kokkini *et al.* 1997).

Although the antibacterial activity of essential oils from spices has been reviewed recently (Nychas 1995), their mechanism of action against micro-organisms has not been studied in great detail. For example, little is known about the mechanism of action of oregano essential oil (OEO) on different bacteria, nor about the specific effects of OEO constituents, e.g. carvacrol and thymol (Cox *et al.* 1998; Helander *et al.* 1998; Ultee *et al.* 1999; Skandamis *et al.* 2000; Tassou *et al.* 2000). According to Conner and Beuchat (1984) and Conner *et al.* (1984) the antimicrobial action of essential oils may be due to impairment of a variety of enzyme systems including those involved in energy production and structural component synthesis. In general, studies on the mechanism of action of essential oils have used a common methodology that attempts to illustrate deleterious effects on cellular membranes, i.e. permeability and proton motive force (Denyer and Hugo 1991; Cox *et al.* 1998; Helander *et al.* 1998; Ultee *et al.* 1999; Tassou *et al.* 2000).

The present study sought to investigate (1) the MIC of OEO and two of its constituents, i.e thymol and carvacrol, which are believed to be the principal inhibitory components of the oil, (2) the hypothesis of whether the antimicrobial effect of OEO can be predicted based on the knowledge of an additive, synergistic or an antagonistic effect of thymol and carvacrol and (3) the mechanism of action of OEO.

MATERIALS AND METHODS

Micro-organisms and suspension media

Staphylococcus aureus (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 2730), were grown overnight in flasks containing 80 ml of Tryptone Soya Broth (TSB, Oxoid), with shaking, at 30°C. The culture was centrifuged at 4000 r.p.m. (510*g*, Sigma, Harz, Germany; model 3K-1) for 10 min. The resulting cell pellets were pooled and resuspended in 0·1% peptone water. The approximate level of the inoculum used was assessed from an investigation of O.D. readings and plate count numbers.

Water and a non-fluorescence bacto-yeasts nitrogen base (3·4 g/740 ml) media with amino acids were used as suspension liquids for the experiments. The composition of the latter was: adenine (40 mg l^{-1}), arginine (20 mg l^{-1}), tyrosine (30 mg l^{-1}), isoleucine (30 mg l^{-1}), phenylananine (50 mg l^{-1}), glutamic acid (100 mg l^{-1}), aspartic acid (100 mg l^{-1}), threonine (200 mg l^{-1}), serine (200 mg l^{-1}), valine (150 mg l^{-1}), methionine (150 mg l^{-1}), lysine (180 mg l^{-1}), uracile (40 mg l^{-1}), L-histidine (60 mg l^{-1}), $(NH_4)_2SO_4$ (10 g/740 ml) and 1% glucose. In all cases, the pH was adjusted with HCl.

Preparation of preservatives

Thymol and carvacrol were obtained from Fluka-Aldrich (Poole, Dorset, UK) and used as received. Oregano essential oil (OEO) was obtained from the University of Athens. Stock solutions of the testing agents were prepared in TSB. From the stock solutions, 'principle' dilutions, e.g. 1–0·2 fractional dilutions, were made using TSB as the medium.

Loading of bacterial cells for measurements of internal pH

An overnight culture of *Staphylococcus aureus* was harvested by centrifugation, as described above, resuspended in 100 mM citric/phosphate buffer (100 mM citric acid; 50 mM sodium dihydrogen orthophosphate; 50 mM potassium hydroxide; pH 5·75) and loaded overnight at 30°C with a 100μM concentration of the fluorescent probe, carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) (Lambda Fluorescence Technologie, Graz, Austria). After being washed twice with 100 mM citric phosphate buffer (pH 5·75), cells were incubated with 10 mM glucose for 30 min at 30°C to eliminate non-conjugated carboxyfluorescein diacetate succinimidyl ester. The cells were subsequently washed twice and resuspended in 100 mM citric/phosphate buffer.

Determination of pH_{in}

The pH_i of the bacterial suspensions was analysed similarly to the method proposed by Breeuwer *et al.* (1996). In particular, 1 ml was removed from flasks containing 10^8 cells ml^{-1} treated with the fluorescent probe and OEO (18–36 μl) at progressively decreasing pH (from 5·8 to 4)

with HCl and placed in a 3-ml optical clear quartz cuvette (Helma; Fisher Scientific, UK). The cuvette was placed in a stirred, thermostatic cuvette holder of a Shimadzu RF-1501 spectrofluorometer (Shimadzu UK, Haverhill, Suffolk, UK). Fluorescence readings followed an excitation scan between 400 nm and 500 nm. The emission wavelength was 525 nm, and the excitation and emission slit widths were 5 and 10 nm, respectively. The intracellular pH was calculated by the ratio of fluorescence intensities at excitation wavelengths of 495 nm (pH-dependent point) and 435 nm (pH-dependent point) at 30°C. At the end of each assay, the extracellular fluorescence signal (background) was determined by filtration of the cell suspension through a 0·22μm pore size membrane filter and measuring the cell-free filtrate. The 495–435 nm ratios were corrected for these background signals.

Calibration curves of CFDA-SE were made in water and bacto-yeasts nitrogen base media buffered with 25 mM citric-phosphate with pH values ranging from 3 to 10 adjusted with HCl. The calibration curve was determined as described by Coote *et al*. 1998.

Determination of potassium and phosphate ions efflux

Overnight cultures of *Staph. aureus* ATCC 6538 and *Ps. aeruginosa* ATCC 2730 in TSB were harvested and washed twice in deionized water. Cells were treated with the OEO (18–36 μl) and the amounts of extracellular potassium and phosphate ions were measured with an ion selective electrode (Russell) and a phosphorus inorganic kit 670-A (Sigma Diagnostics), respectively. Values were compared with standard calibration curves of KCl and HPO_4, for potassium and phosphate ions, respectively. To test the viability of cells both prior to resuspension in water and during the incubation period (at 30°C) in the absence of the antimicrobial, 50 μl were transferred to 250 μl TSB and changes in O.D were recorded with the bioscreen.

Evaluation of membrane integrity

Membrane permeability was measured by capturing confocal scanning laser microscopy (CSLM) images of the uptake of the fluorescent nuclear stain ethidium bromide (EB), a positively charged, sparingly membrane permeable, mutagenic dye (Ueckert *et al*. 1995). The general principle is that viable cells, having an intact membrane, do not allow penetration of EB and thus are not stained, whereas dead cells or cells with permeable and disrupted membranes give a fluorescent signal due to the penetration of EB and intercalation into cellular DNA and RNA (Ueckert *et al*. 1995; Breeuwer and Abee 2000). The examination of membrane integrity was based on the method described by Coote *et al*. (1998). Late-exponential phase cells were harvested and resuspended in TSB (pH 7·0). Following this, 10 μg of EB per ml was added to 200 μl aliquots of these cells, and this was followed by the addition of 0·1% OEO, carvacrol or thymol. After incubation at 30°C for 10 min, treated and untreated cells were visualized with a Bio-Rad MRC 600 confocal laser microscope fitted with a 20 mW krypton argon mixed gas laser (Bio-Rad). Objective-magnification of × 20 (Nikon × 20, 0·75 numerical aperture) was used. By capturing such magnification, single-channel epifluorescent images (up to 100–150 cells per field of view), it was possible to count the number of fluorescent cells in the population. The method was used to calculate the percentage of fluorescent cells in the population treated with the test preservatives. At least four random, independent images were captured and the frequency of occurrence of fluorescent or permeabilized cells was calculated. Each datum point represents the mean and standard deviation acquired from the counting of at least 20 cells.

Determination of susceptibility

Oregano oil was mixed with TSB and the MIC (minimum inhibitory concentration) against *Staph. aureus* and *Ps. aeruginosa* determined using the method of Lambert and Pearson (2000). For combinations of the two preservatives, a 10×10 grid of concentrations was used, using half-fold dilutions within the Bioscreen plate. For these combination experiments eqn 1 (Lambert, unpublished) was used to fit the observed data.

$$fa = \exp\left(-\left(\frac{thymol}{m_1} + \frac{carvacrol}{m_2}\right)^P\right) \qquad (1)$$

where fa is the fractional area (area under the test O.D./time curve relative to the area under the control O.D./time curve), m_1 and m_2 are the concentrations of the inflexion points of the log concentration/fa curve for thymol and carvacrol, respectively, and P is a slope parameter determined from the log concentration/fa curve.

Statistical analysis

Non-linear regression analysis was used to determine the parameters of eqn 1. The method essentially minimizes the sum of the squares of the errors (eqn 2).

$$SSE = \sum_{i=1}^{n}\left\{y_i - \exp\left(-\left(\frac{x}{m_1} + \frac{y}{m_2}\right)^P\right)\right\} \qquad (2)$$

However, the fitting procedure assumes the homogeneity of variance. In general, the variance is not homogeneous but increases to a maximum at the inflexion point of a log concentration/fa curve. To be able to deal with this type of

variance, essentially preventing points for which we have less confidence having more influence on the procedure, a re-iterative weighting procedure should be used, where appropriate.

The MIC values are given by eqn 3.

$$MIC_i = m_i \exp\left(\frac{1}{P}\right) \qquad (3)$$

A note on previous experimental methodology

When dealing with essential oils it has become common practice to disperse them throughout the growth medium using a solvent, detergent or emulsifying agent. The MIC values obtained tend to be a function of the dispersion agent used (Remmal *et al*. 1993a), the MIC being lower when such agents are either not used or are used sparingly. This is simply a confirmation that these types of agents 'quench' the action of the antimicrobial. The use of a 0·2% agar solution has been suggested as a more appropriate method (Remmal *et al*. 1993b).

In this work, the oils and compounds were freely mixed with TSB with no added emulsifying agents used to disperse them.

RESULTS

Minimum inhibitory concentration (MIC) of thymol, carvacrol and oregano essential oil against *Staph. aureus*, and *Ps. aeruginosa*

The analyses of inhibition of mixtures of thymol and carvacrol for *Staph. aureus* and *Ps. aeruginosa* are given in Figs 1 and 2, respectively. It was evident (Table 1) that *Ps. aeruginosa* was less sensitive to the action of thymol and carvacrol than *Staph. aureus* (Figs 1, 2 and 3; Table 1). When the experimental data was modelled using eqn 1, the pattern of inhibition observed was very similar to that, based on the calculation of a purely additive effect, of thymol and carvacrol (Table 1). It needs to be noted, however, that the concentrations giving the MIC of the mixtures were close to the region of increasing chemical turbidity. Indeed, at higher concentrations of thymol and carvacrol, the media becomes turbid due to the insolubility of the oils. At such concentrations (> 800 mg l^{-1} each) the model used will begin to fail unless this effect is taken into account. The model can be compensated by the removal of data in this extreme region, as was employed here, or by the addition of a precipitation/chemical turbidity model to eqn 1. The observed inhibitory effect of OEO is shown in Fig. 3a,b for *Staph. aureus* and *Ps. aeruginosa*, respectively, while Table 1 lists the parameters obtained from the log concentration/*fa* analyses.

The hypothesis which was originally put forth was 'do the combined effect of thymol and carvacrol give the inhibition observed with OEO?'. Essentially, we were asking if these

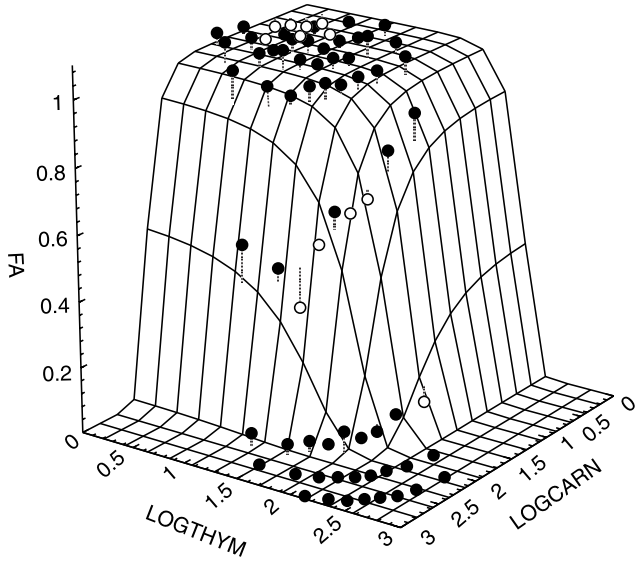

Fig. 1 Observed and predicted fractional areas of mixtures of thymol and carvacrol against *Staphylococcus aureus*. The data were fitted to eqn 1, with $m_1 = 91{\cdot}5$, $m_2 = 105{\cdot}2$ and $P = 3{\cdot}19$, the standard deviation of the residuals was 0·0187. Filled symbols represent points above the calculated surface, open symbols are on, or below, the surface

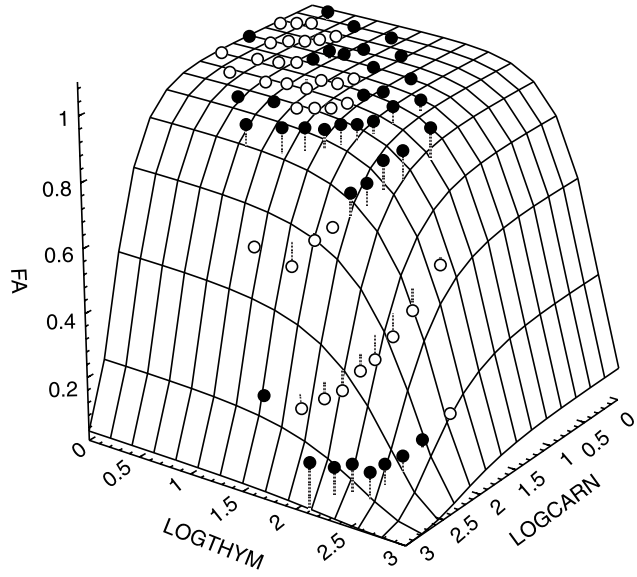

Fig. 2 Observed and predicted fractional areas of mixtures of thymol and carvacrol against *Pseudomonas aeruginosa*. The data were fitted to eqn 1, with $m_1 = 255{\cdot}3$, $m_2 = 278{\cdot}0$ and $P = 1{\cdot}57$, the standard deviation of the residuals was 0·048. Filled symbols represent points above the calculated surface, open symbols are on, or below, the surface

Table 1 Inhibition parameters for OEO and mixtures of thymol and carvacrol in TSB against *Staph. aureus* and *Ps. aeruginosa* obtained from eqn 1

	Staph.aureus		*Ps. aeruginosa*	
Parameter	OEO	Thymol/carvacrol mixture	OEO	Thymol/carvacrol mixture
m_1 (thymol)/mg l^{-1}	472	91·5	1181	255·3
m_2 (carvacrol)/mg l^{-1}		105·3		278·0
P	4·82	3·188	3·00	1·574
RMSE	0·0244	0·0187	0·042	0·048
Calculated MIC/mg l^{-1} of thymol in mixture		125 (140)*		482 (385)*
Calculated MIC/mg l^{-1} of carvacrol in mixture		144 (175)*		525 (450)*
MIC/mg l^{-1} OEO	575		1648	

*Values obtained for the MIC of thymol and carvacrol individually.

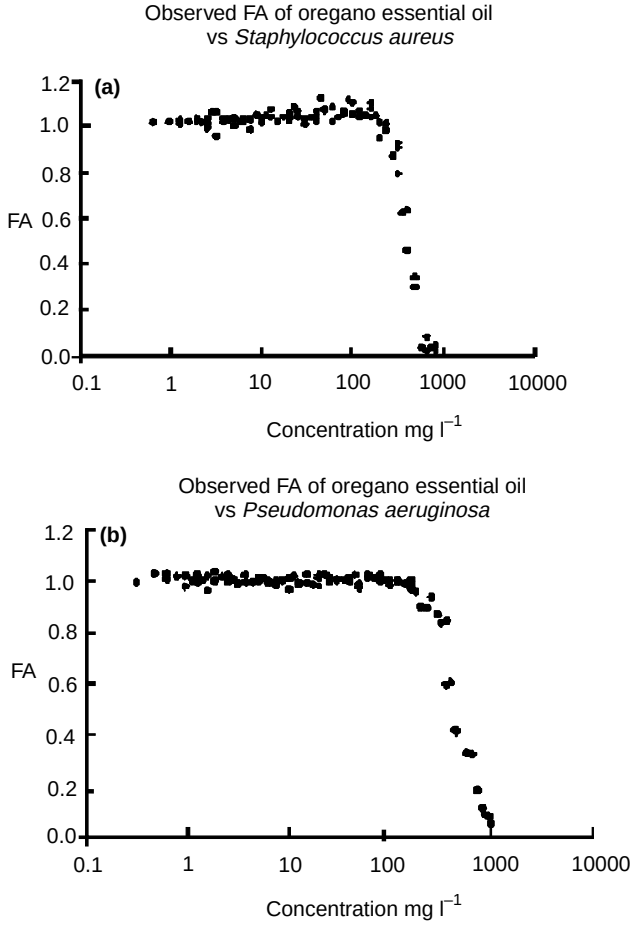

Fig. 3 The inhibition profile of oregano essential oil against *Staphylococcus aureus* (a) and *Pseudomonas aeruginosa* (b)

two compounds acting together can account for the majority of the inhibition observed with OEO. To test this hypothesis requires equating the inhibition of OEO with the combined inhibitory effects of thymol and carvacrol.

Thymol and carvacrol are present in OEO at a given percentage each. If the calculated inhibitory effect of a combined thymol–carvacrol mixture is equal to that of oregano oil which contains the same thymol–carvacrol content, then we can assert that the combined effect of thymol and carvacrol accounts for the inhibitory effect found with OEO. This would simply state that the inhibitory effect of OEO is principally that of the thymol-carvacrol mixture. For a mixture of inhibitors, if the components act independently from one another, i.e. no synergy nor antagonism is present, then the sum of the inhibitory capacities of each component must be equal to the inhibition ability of the mixture itself, eqn 4.

$$\frac{1}{MIC_{mixture}} = \sum_{i=1}^{n} \frac{f(i)}{MIC_i} \quad (4)$$

where: *f(i)* is the fraction of component *i* in the mixture containing *n* components. Using eqn 4 for OEO, we can define an equation based on the assumption of independent activity: eqn 5.

$$\frac{1}{MIC_{OEO}} = \frac{f(thymol)}{MIC_{thymol}} + \frac{f(carvacrol)}{MIC_{carvacrol}} + \sum_{i=1}^{n-2} + \frac{f(i)}{MIC_i} \quad (5)$$

where: $\sum_{i=1}^{n-2} \frac{f(i)}{MIC_i}$ is the inhibition due to all the other components of the essential oil.

The oregano oil used in this study contained 5·00% ± 0·009 thymol and 25·00% ± 0·017 carvacrol, as determined by analysis of 75 oregano extracts from the original market with gas chromatography (results not shown).

From the parameters in Table 1 and eqn 5, we obtain for *Staph. aureus* and *Ps. aeruginosa* eqns 6 and 7, respectively.

$$\text{Staph. aureus: } \frac{1}{575} = \frac{f(thymol)}{125} + \frac{f(carvacrol)}{144} + \sum_{i=1}^{n-2} \frac{f(i)}{MIC_i} \quad (6)$$

$$\text{Ps. aeruginosa: } \frac{1}{1648} = \frac{f(thymol)}{482} + \frac{f(carvacrol)}{525} + \sum_{i=1}^{n-2} \frac{f(i)}{MIC_i} \quad (7)$$

Substituting the values for the composition of OEO, it was found that for *Staph. aureus* the combined effect of thymol and carvacrol should be greater than that observed (a calculated MIC of 468 mg l^{-1} compared with the observed MIC of 575 mg l^{-1}). Since it was already shown that there was no antagonism between thymol and carvacrol (Figs 1 and 2), the reason for this difference either lies within the errors of the technique else the term $\sum_{i=1}^{n-2} \frac{f(i)}{MIC_i}$ is not additive but antagonistic.

For *Ps. aeruginosa*, we obtain a value for the combined effect of thymol and carvacrol as 1/1724. This suggests that, for this organism, 96% of the inhibition observed with oregano oil can be attributed to the additive effect of thymol and carvacrol with the remaining 4% from the other components.

Mechanism of activity

The mechanism of activity of OEO as well as its constituents (thymol and carvacrol) against these two pathogens for their membrane integrity, ion leakage and internal pH was also investigated.

The penetration of EB into dead or injured cells (Fig. 4b) was studied with confocal laser microscope (phase-contrast; Fig. 4a). Fluorescence due to EB was neglible and did not change in the control suspension of bacterial cells throughout the incubation period (Fig. 4). On the contrary, addition of 0·1% of antimicrobial, i.e. OEO, thymol or carvacrol, resulted in a high percentage (90% ± 0·7) of fluorescent cells of *Ps. aeruginosa* (Fig. 4). Similar results were obtained for *Staph. aureus* (results not shown). Such observations indicate that OEO and its components impaired the cell membrane and rendered the intracellular nucleic acids accesible to EB.

The effect of the addition of OEO on the potassium and phosphate leakage of both *Staph. aureus* and *Ps. aeruginosa* is shown in Figs 5 and 6, respectively. A sample of OEO (18 μl for *Staph. aureus* and 36 μl for *Ps. aeruginosa*) was administered to a culture of approximately 1×10^9 cfu ml^{-1}. Relative to the control, leakage was observed after addition of the essential oil. The results simply suggest that increased membrane permeability is a factor in the mechanism of antimicrobial action.

Changes in the internal pH of the tested cells were also observed (Fig. 7). In the absence of glucose, from an initial external pH of 5·95, the pH was adjusted to 5·05. After 2 h the control culture of *Staph. aureus* reached an internal pH of 5·05, whereas the addition of 0·05% OEO reduced the

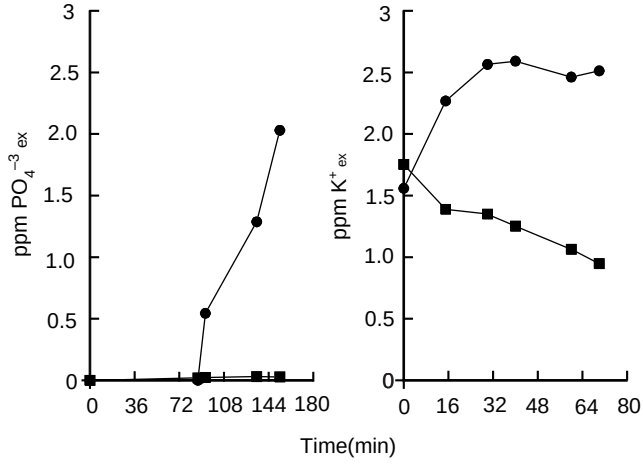

Fig. 5 Extracellular concentration of potassium and phosphate ions in aliquots of *Staphylococcus aureus* without (■) and with 18 μl oregano essential oil (●). Data-points represent results from two independent experiments (coefficient of variation < 5%)

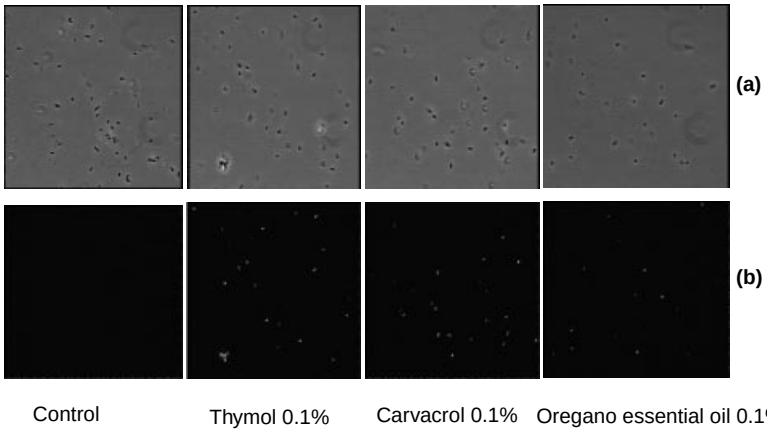

Fig. 4 Results illustrating the effect of exposure to 0·1% oregano essential oil, thymol and carvacrol on the permeability of the membrane of individual cells of *Pseudomonas aeruginosa* in TSB (pH 7·0) by CSLM; phase contrast (a) and epigluorescent images (b) – excitation line, 488 nm. Preservatives induced membrane disruption was visualized by influx of the fluorescent nuclear stain ethidium bromide (10 μg ml^{-1}). The captured images are representative of a typical result

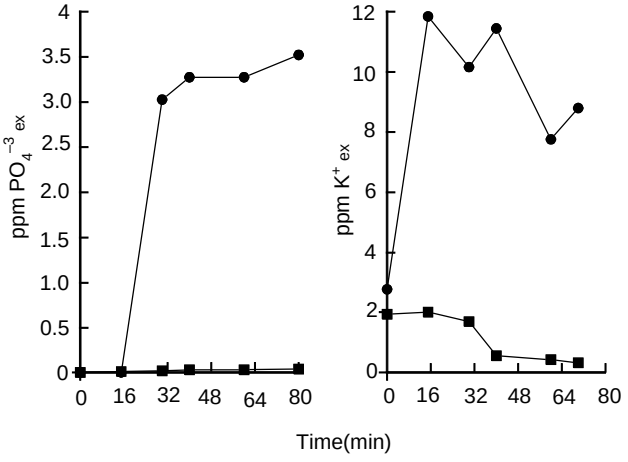

Fig. 6 Extracellular concentration of potassium and phosphate ions in aliquots of *Pseudomonas aeruginosa*, without (■) and with 36 μl oregano essential oil (●). Data-points represent results from two independent experiments (coefficient of variation < 5%)

internal pH to 5·05 within 30 min (Fig. 7a). In another experiment, in the presence of glucose, the external pH was reduced from 5·8 to 4·05. The control reached a minimum internal pH of 4·6 after 2 h, whereas in the presence of 0·05% OEO an internal pH of 4·05 was obtained in the same time period, indicating the protective role of glucose in pH homeostasis (Fig. 7b). In all cases, dissipation of the pH gradient occurred by the time of addition of antimicrobial agents. Similar results were obtained with *Ps. aeruginosa*.

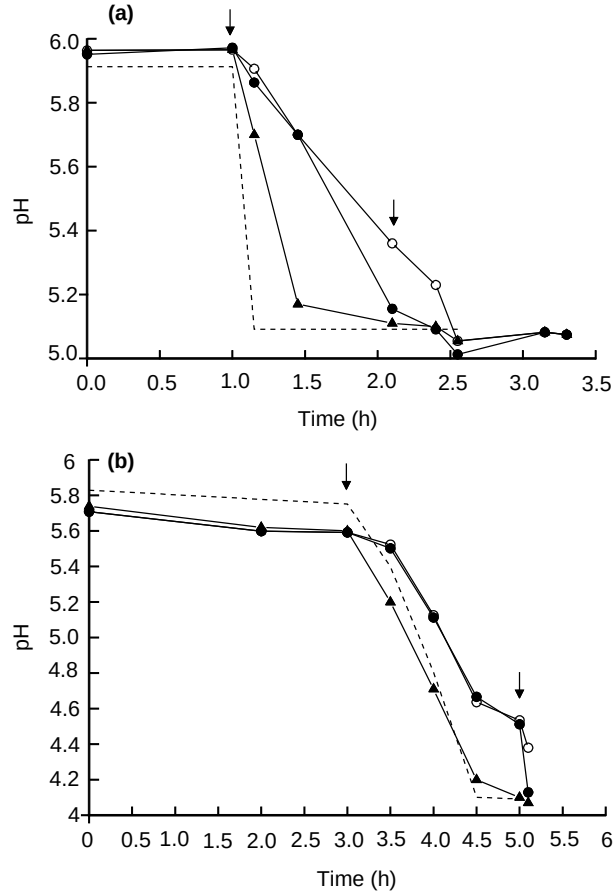

Fig. 7 Changes in cytoplasmic pH (pH_i) of *Staphylococcus aureus* against decreasing extracellular pH (–), without oregano essential oil (○) and adding 18μl oregano essential oil after 1 h (▲ – top arrow) or after 2 h (● – bottom arrow) incubation at 30°C, in 100 mM citric phosphate buffer without glucose (a) or in yeasts nitrogen base with amino acids and 1% glucose (b); pHi was measured by determining the intracellular pH-dependent fluorescence of CF-SE. Data-points represent results from two independent experiments (coefficient of variation < 5%)

DISCUSSION

The potential use of essential oils as natural antimicrobial agents is less exploited than their uses as flavouring and antioxidant compounds. Among these OEO has a long history, used mainly as a flavouring as well as a preservative. Focusing on the latter, numerous studies in broth model systems, as well as in real foods, have revealed the bacteriostatic or bactericidal activity of this essential oil on pathogens such as *Staph. aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Ps. aeruginosa*, *Bacillus subtilis*, spoilage bacteria *Brochothrix thermosphacta*, *P. fluorescens*, *Serratia liquefaciens*, *Lactobacillus carvatus*, *Lact. sake* and moulds *Rhizobium leguminosarum*, *Aspergillus niger*, *Asp. flavus*, *Asp. ochraceus*, *Fusarium oxysporum* and *Penicillium* spp. in broth (Conner and Beuchat 1984; Galli *et al.* 1985; Paster *et al.* 1990, 1995; Daouk *et al.* 1995; Sivropoulou *et al.* 1996; Quattara *et al.* 1997; Manou *et al.* 1998), as well as in real foods (Tassou *et al.* 1996; Koutsoumanis *et al.* 1999; Skandamis and Nychas 2000; Tsigarida *et al.* 2000). However, the limited application of this compound as a food preservative should be attributed to its strong flavour negatively affecting the organoleptic properties of food. This problem may be overcome if valuable answers could be given to the following questions: (i) is the inhibitory effect of OEO attributable to only a few of its constituents? (ii) Does OEO provide a synergy of activity, which simple mixtures of components cannot deliver? (iii) What is the minimum inhibitory concentration of OEO or that of mixtures of its components that could enhance its use in food preservation?

Examining the inhibitory effect of preservative combinations by traditional methods is very time-consuming and resource-intensive. Through an advance on the method of Lambert and Pearson (2000), that examined the inhibition of single compounds, mixtures of preservatives could be analysed quickly and efficiently. Further, the method of analysis allows for the observation of preservative synergy or potentiation. With mixtures of thymol and carvacrol, neither

synergy nor antagonism was observed against either of the two organisms examined. The inhibition profiles observed were commensurate with those calculated on the basis of a purely additive effect of the two-component mixture. With respect to the destructive effects on membrane permeability, marked correlation was also evident between the effects of essential oil and its components for both micro-organisms.

When compared to OEO itself, eqn 5 suggests that the inhibitory effect due to the combination of thymol and carvacrol was enough to account for the inhibition observed with the oil itself. From the results, it would appear that against *Staph. aureus* the other components in the OEO may inhibit, to a small extent, the action of thymol and carvacrol. The result may be due to experimental errors such as inaccuracies in the measurement of inoculum size, which have been shown to have an effect on the MIC of OEO (Remmal *et al.* 1993b), and on the MIC of thymol (Lambert 2000).

From the results, therefore, the majority of the antimicrobial prowess of OEO can be attributed to two components acting independently. From the examination of proton, phosphate and potassium leakage, OEO creates membrane permeability problems for the organisms under investigation. Thus, *vide infra*, carvacrol and thymol are membrane permeabilizers.

Previously, the inhibition of ochratoxin A production by *Aspergillus achraceus* with 1000 mg l^{-1} of OEO was mentioned (Basilico and Basilico 1999). From this study, 1000 mg l^{-1} of OEO, would equate to the use of 50 mg l^{-1} of thymol and 250 mg l^{-1} of carvacrol. Although we cannot obtain actual MICs for thymol and carvacrol, the work presented here would allow MIC estimations of > 50 mg l^{-1} for thymol and > 250 mg l^{-1} for carvacrol. An approximate value of 200 mg l^{-1} for thymol and carvacrol against *Aspergillus niger* has been reported (Paulus 1993). It would be valuable to find out if inhibition, in this case, is also a simple additive effect of the thymol and carvacrol content of the OEO. The method would also allow for the replacement of one of the components with a more active inhibitor, but allow the formulation of the preservative mixture to be rationalized on the basis of the above calculations. Such advantages are of great importance considering the need for substitutes of OEO, being equally effective in combination with milder flavouring attributes.

Although the antimicrobial effects of essential oils are well established, the mechanism of action of such compounds is poorly understood. Furthermore, in contrast to the extended investigation on mode of action of bacteriocins and weak acids, there are too few studies that attempt such an insight for specific essential oils. Current research on the antimicrobial action of phenolic compounds focuses on their effects on cellular membranes (Davidson 1997; Ultee *et al.* 1999). Such an approach is expected for essential oils due to the presence, in their composition, of such compounds (Sivropoulou *et al.* 1996; Kokkini *et al.* 1997), which are known to cause structural and functional damage to plasma membranes (Sikkema *et al.* 1995). Indeed, the permeability of cell membranes is dependent on the hydrophobicity of the solutes that have to cross the membrane and the composition of the membrane (Sikkema *et al.* 1995). Given the latter, the partition coefficient of essential oils on cell membranes is a crucial determinant for their effectiveness. Thus, quantitative variations in the activity of essential oil are expected against different bacteria, especially when bacteria with different Gram staining response are examined. This was the case in our study, as far as the MIC is concerned. With respect to the mode of action, a marked correlation was evident between the effects of essential oil and its components for both microorganisms. Unlike many antibiotics, the hydrophobic constituents of essential oils are capable of gaining access to the periplasm of Gram-negative bacteria through the porin proteins of the outer membrane (Helander *et al.* 1998). Based on CLSM, it was clearly demonstrated that OEO, as well as thymol and carvacrol, disrupted the cell membrane, causing an increased permeabilization to the nuclear stain EB. Thymol and carvacrol were also found to disintegrate the outer membrane of *E. coli* and *S. typhimurium* at levels close to the MIC (Helander *et al.* 1998). It must also be noted, however, that the use of methods involving uptake of fluorescent probes for investigation of membrane integrity can cause misleading results, due to the ability of cells to extrude the probes or to be stained while retaining viability (Ueckert *et al.* 1995; Breeuwer and Abee 2000).

The hypothesized impairments in membranes are reflected mainly on the dissipation of the two components of the proton motive force, the pH gradient (ΔpH) and the electrical potential ($\Delta\psi$) (Sikkema *et al.* 1995; Davidson 1997). Further damage may be related to nutrient uptake, nucleic acid synthesis and ATPase activity, etc. (Denyer and Hugo 1991). Conner *et al.* (1984) demonstrated that most essential oils (at approx. 100 mg l^{-1}) impair the respiratory activity of *Saccharomyces cerevisiae*. Ultee *et al.* (1999) reported depletion of intracellular ATP pools and dissipation of proton motive force components of *Bacillus cereus* in the presence of 1 mM carvacrol. The pH gradient of bacteria examined in the present study was rapidly dissipated after the addition of OEO irrespective of glucose availability. Glucose plays a key role on pH homeostasis of cells, since it serves as energy source of proton-pumping H^+-ATPase (Holyoak *et al.* 1996). The latter is known to be involved in maintenance of pH_i homeostasis (Holyoak *et al.* 1996; Bracey *et al.* 1998; Coote *et al.* 1998). The detrimental effects of antimicrobial compounds on proton motive force are strongly correlated with leakage of specific ions (Kroll and Booth 1981; Bakker and Mangerich 1981). Indeed,

acting on the permeability barrier of cytoplasmic membrane various preservatives including essential oils, phenols and bacteriocins cause leakage of various substances, such as ions, ATP, nucleic acids and amino acids, e.g. glutamate, etc. (Tranter *et al*. 1993; Gonzalez *et al*. 1996; Tahara *et al*. 1996; Cox *et al*. 1998; Helander *et al*. 1998; Ultee *et al*. 1999; Tassou *et al*. 2000). It has been reported that essential oil from tea and mint, as well as carvacrol, can cause leakage of cellular material, e.g. material absorbing at 260 nm and K^+ (Cox *et al*. 1998; Gustafson *et al*. 1998; Ultee *et al*. 1999). This was also evident in our study with potassium and phosphate ions even at concentrations of OEO somewhat lower than the MIC.

Further study on the mode of action of certain combinations of essential oil mixtures at various levels against pathogenic and spoilage micro-organisms is needed in order to expand the knowledge on usage of such natural additives in industrial practice.

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