Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7

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ABSTRACT

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Aims: To quantify the antibacterial properties of five essential oils (EO) on a non-toxigenic strain of *Escherichia coli* O157:H7 in the presence and absence of a stabilizer and an emulsifier and at three different temperatures.

Methods and Results: Five EOs known to exhibit antibacterial properties were screened by disc diffusion assay and the most active were selected for further study in microdilution colorimetric assays. Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*; light and red varieties) EO had the strongest bacteriostatic and bactericidal properties, followed by bay (*Pimenta racemosa*) and clove bud (*Eugenia caryophyllata* synonym: *Syzygium aromaticum*) EO. Oregano oil was colicidal at 625 μl l^{-1} at 10, 20 and 37°C. The addition of 0·05% (w/v) agar as stabilizer reinforced the antibacterial properties, particularly at 10°C, whereas 0·25% (w/v) lecithin reduced antibacterial activity. Scanning electron micrographs showed extensive morphological changes to treated cells.

Conclusions: Oregano and thyme EO possess significant *in vitro* colicidal and colistatic properties, which are exhibited in a broad temperature range and substantially improved by the addition of agar as stabilizer. Bay and clove bud EO are less active. Lecithin diminished antibacterial properties. The bactericidal concentration of oregano EO irreversibly damaged *E. coli* O157:H7 cells within 1 min.

Significance and Impact of the Study: Oregano and light thyme EO, particularly when enhanced by agar stabilizer, may be effective in reducing the number or preventing the growth of *E. coli* O157:H7 in foods.

Keywords: alamarBlue$^{\text{TM}}$, bactericidal, bacteriostatic, essential oil, *Escherichia coli* O157:H7, oregano, thyme.

INTRODUCTION

Escherichia coli O157:H7 is a concern to public health on a global scale (Mead and Griffin 1998) and is found in a wide variety of foodstuffs including meat and meat products, milk, yogurt, water, salad vegetables, fruits, fruit juices and cider (Buchanan and Doyle 1997; Mead and Griffin 1998). Pasteurization and cooking are adequate methods of ensuring that viable cells are eliminated, but heat treatment is not desirable for all foods and cross-contamination cannot always be prevented. Controlling the numbers and growth

of *E. coli* O157:H7 therefore remains an important objective for sectors of the food production industry.

It has long been acknowledged that some plant essential oils (EO) exhibit antimicrobial properties (Finnemore 1926; Koedam 1977). Recent studies have shown that EO of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), bay (*Pimenta racemosa*) and clove (*Eugenia caryophyllata* synonym: *Syzygium aromaticum*) are among the most active in this respect against strains of *E. coli* (Smith-Palmer *et al.* 1998; Hammer *et al.* 1999; Dorman and Deans 2000). Chemical analysis of these oils has shown the constituents to be principally carvacrol, thymol, citral, eugenol and their precursors (Salzer 1977; Lattaoui and Tantaoui-Elaraki 1994; Juliano *et al.* 2000; Demetzos and Perdetzoglou 2001), although the composition of EO from a particular species of plant can differ between harvesting seasons (Arras

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and Grella 1992; McGimpsey and Douglas 1994) and between geographical sources (Cosentino *et al.* 1999; Juliano *et al.* 2000). A number of constituents of EO exhibit significant antimicrobial properties when tested separately (Kim *et al.* 1995; Ultee *et al.* 1998; Lambert *et al.* 2001). However, there is evidence that EO are more strongly antimicrobial than is accounted for by the additive effect of their major antimicrobial components; minor components appear, therefore, to play a significant role (Lattaoui and Tantaoui-Elaraki 1994; Paster *et al.* 1995). For this reason, the present study was carried out using whole EO.

When a portion of EO is shaken in broth, the oil phase separates from the water phase. Stabilization of the EO/broth mixture could improve the antimicrobial properties. A further advantage of the use of a stabilizer could be to lower the usage concentration of EO, thereby reducing the herbal aroma. Although the aroma of oregano oil was found to be pleasant when added at 0·05% (v/w) to cod fillets (Mejlholm and Dalgaard 2002) and acceptable up to 1% (v/w) in minced meat (Skandamis and Nychas 2001), it may be less acceptable in other food categories. A flavourless additive which improves the efficacy of EO, reducing the concentration required for an antimicrobial effect, could widen the perspectives for the use of EO as antimicrobials in food. The superiority of bacteriological agar compared with other substances (DMSO, ethanol, Tween- 20 and Tween-80) as dispersal agent or stabilizer in EO has been demonstrated (Remmal *et al.* 1993; Mann and Markham 1998). Lecithin, an emulsifier, was also chosen for testing as an aid to stabilization of EO. Both agar and lecithin are internationally approved as additives for certain foods (http://europa. eu.int/eur-lex/en/consleg/pdf/1995/en_1995L0002_do_001. pdf, http://www.cfsan.fda.gov/dms/eafus.html).

The purpose of this study was to select from bay, clove, oregano and thyme EO those with the most pronounced antibacterial properties, to determine at which concentration they were bacteriostatic and bactericidal to *E. coli* O157:H7, and to assess the effect of a stabilizer (agar), an emulsifier (lecithin) and temperature on their performance.

MATERIALS AND METHODS

Maintenance and preparation of cultures

Cultures of *E. coli* O157:H7 strain rr98089 phage type 34 isolated from bovine faeces were maintained on tryptone soya broth agar (TSBA) slants at 4°C. The agar slants were made by the addition of 1% (w/v) bacteriological agar Nr. 1 (Oxoid, Basingstoke, UK) to TSBA (Oxoid). Inocula were prepared by 16 h culture in Mueller–Hinton Broth (MHB; Oxoid) at 37°C. The strain harbours the *eae* and *ehly* genes and shows enterohaemolysis on enterohaemolysin agar, but does not carry genes for Shiga toxin production.

Essential oils

Essential oils from bay (*Pimenta racemosa*), clove bud (*Eugenia caryophyllata*, synonym: *Syzygium aromaticum*) and oregano (*Origanum vulgare*) and red and light thyme oils (both *Thymus vulgaris*) were obtained from C. Melchers Essential Oils Handels-GmbH, Bremen, Germany.

Disc diffusion assay

A 16-h culture was diluted with sterile physiological saline solution [PS; 0·85% (w/v) sodium chloride] with reference to the McFarland standard (bioMérieux, Marcy l'Etoile, France) to achieve an inoculum of approximately 10^6 CFU ml^{-1}. A 5-ml portion of this inoculum was placed onto the surface of pre-dried Mueller–Hinton agar (MHA; Oxoid) plates and allowed to remain in contact for 1 min. Excess inoculum was removed using a sterile syringe and the plates were allowed to dry for 20 min at room temperature. Sterile 6 mm filter paper discs (Schleicher and Schuell, Dassel, Germany) were placed on the plates and immediately 15-μl portions of the essential oils were added. Sterile PS was used as control. After allowing 1 h at room temperature for the essential oils to diffuse across the surface, the plates were incubated at 37°C for 24 h. The inhibition zone was measured in millimetre and the assay was carried out three times for each oil. To test for any additive effect between the three most inhibitive oils (oregano, light thyme, red thyme) they were mixed in the ratios of 1:1 and 1:1:1, and tested again.

Colorimetric determination of bacteriostatic and bactericidal concentrations

The EO which exhibited the greatest antibacterial effect in the disc diffusion assay (oregano, light thyme, red thyme) were further tested to determine the concentrations at which they were bacteriostatic and bactericidal using a colorimetric broth microdilution technique (Salvat *et al.* 2001). In order to test concentrations from 0·0078 to 1% (v/v) (78–10 000 μl l^{-1}) three sterile 96-well microplates with lids (Greiner) were set up as follows: in wells in row A were placed 200 μl portions of 2% essential oil in sterile MHB; wells in rows B to H received 100 μl of sterile MHB. Serial two fold dilutions were carried out from row A to row H and excess broth (100 μl) was discarded from row H. To each well was added 100 μl of inoculum and alamar-Blue^{TM} (Biosource International Inc., Camarillo, CA, USA) according to the method of Salvat *et al.* The inoculum was prepared using a 16-h culture adjusted by reference to the McFarland standard and further diluted with MHB to achieve approximately 10^6 CFU ml^{-1}. A positive control (containing inoculum but no EO) and negative control

(containing EO but no inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at 10°C for 96 h, at 20°C for 24 h or at 37°C for 24 h. A colour change from blue to pink or mauve was indicative of bacterial growth. In an extension to the method of Salvat *et al.*, aliquots of 5 μl from the wells remaining blue were plated onto MHA and incubated for 24 h at 37°C. Three replicates of each microassay were carried out and the experiment was carried out twice.

The bacteriostatic concentration was determined as the lowest concentration at which bacteria in at least five of the six replicates failed to grow in MHB but were cultured when plated onto MHA. The bactericidal concentration was the lowest concentration at which bacteria in at least five of the six replicates failed to grow in MHB and were not cultured after plating onto MHA. These definitions have been established by other workers (Smith-Palmer *et al.* 1998).

The experiment was repeated using MHB with the addition of bacteriological agar no. 1 (Oxoid) and MHB with the addition of soya lecithin (ICN Biomedicals Inc., Cleveland, OH, USA) with a view to stabilizing the EO in the broth and thereby improving the antibacterial properties. Prior experiments on the use of these additives determined the minimum concentrations which stabilized EO in MHB (i.e. no visible separation of the EO within 24 h at 37°C) (data not shown). The end concentrations in the microplate wells were 0·05% agar (w/v) and 0·25% (w/v) lecithin.

Survivor curves for *E. coli* O157:H7 in the presence of oregano EO

In order to assess the bactericidal effect of the EO with the lowest bactericidal and bacteriostatic concentrations (oregano) over time, survivor (time-kill) curves were plotted. A 16-h culture was harvested by centrifugation, washed twice with PS and resuspended in PS. The suspension was adjusted using the McFarland standard and was then further diluted in PS to achieve approximately 10^7 CFU ml^{-1}. Oregano EO was added to aliquots of 9 ml MHB in tubes in a waterbath at 37°C in amounts which would achieve concentrations of 0 (control), 78, 156, 312 and 625 μl l^{-1} after addition of the inoculum. Portions of 1 ml inoculum were then added to all tubes. Directly after addition of the inoculum and after incubation for 1, 5, 10 and 15 min, a 1 ml portion was removed from each tube for colony counting by decimal dilution in peptone physiological saline solution containing 0·85% (w/v) sodium chloride and 0·1% (w/v) peptone (Bacto^{TM} Peptone; Becton Dickinson, Sparks, MD, USA) and plating out on MHA. The experiment was carried out twice.

Scanning electron microscope observations

Scanning electron microscope (S.E.M.) observations were carried out on cells after 16 h incubation in MHB at 37°C. The suspension was divided into two portions and oregano EO was added to one portion so as to achieve a concentration of 625 μl l^{-1}. The other portion was left untreated as a control. After 1 min, the cells from both tubes were harvested by centrifugation, washed twice and resuspended in PS. A drop of each suspension was filtered through a polycarbonate membrane with 1-μm diameter pores (Costar, Cambridge, MA, USA) and the cells were frozen *in situ* using liquid nitrogen. They were observed with a field emission S.E.M. equipped with a cold stage and a cryo-preparation chamber.

RESULTS

Disc diffusion assay

The growth inhibition zones measured by disc diffusion method are presented in Table 1. Thyme oil, both light and red varieties, and oregano oil produced the strongest antibacterial effect; bay and clove bud EO were active to a lesser extent. The assay with mixtures of the three most inhibitive oils (oregano, light thyme and red thyme) in the ratios of 1:1 and 1:1:1 revealed no apparent additive effects (data not shown).

Colorimetric determination of bacteriostatic and bactericidal concentrations

The bacteriostatic and bactericidal concentrations of essential oils obtained by colorimetric assay followed by plating out on MHA are presented in Table 2. Without added stabilizers, oregano oil exhibited the strongest antibacterial effect of the three oils – light thyme was less active than oregano and red thyme was the least active. The inclusion of 0·05% agar in the broth substantially improved the antibacterial activity of all three EO. The differences in antibacterial properties between them became less pro-

Table 1 Antibacterial properties of essential oils against *E. coli* O157:H7 using the disc diffusion method. The diameter of the zone of inhibition includes the paper disc (6 mm)

Essential oil	Diameter of inhibition zone (mm) (Mean ± S.D.)
Bay	18·7 ± 1·5
Clove bud	15·7 ± 0·6
Oregano	24·3 ± 2·1
Thyme, light	25·7 ± 0·6
Thyme, red	24·0 ± 1·7

Table 2 Bacteriostatic and bactericidal concentrations for oregano and thyme EO against *E. coli* O157:H7 in MHB at three different temperatures and with the addition of stabilizers

Essential oil	T°C	MHB		MHB + 0·05% agar		MHB + 0·25% soy lecithin	
		Bacteriostatic concentration μl l^{-1}	Bactericidal concentration μl l^{-1}	Bacteriostatic concentration μl l^{-1}	Bactericidal concentration μl l^{-1}	Bacteriostatic concentration μl l^{-1}	Bactericidal concentration μl l^{-1}
Oregano oil	10	–*	625	78	156	1250	2500
	20	–	625	–	156	1250	2500
	37	–	625	–	156	–	1250
Thyme oil, light	10	625	1250	78	156	–	1250
	20	625	1250	78	156	–	1250
	37	625	1250	156	312	–	1250
Thyme oil, red	10	1250	2500	78	156	1250	2500
	20	1250	2500	156	312	–	2500
	37	–	1250	–	312	–	2500

*Where no bacteriostatic concentration is given, the lowest concentration which exhibited an antibacterial effect was bactericidal.

nounced but the relative activity was still in the order oregano > light thyme > red thyme. The inclusion of lecithin reduced the antibacterial activity of all three oils very markedly. A slight temperature effect was observed in broth stabilized by 0·05% agar and particularly for thyme – the bactericidal and bacteriostatic concentrations were lower at 10°C than at 37°C.

Survivor curves for *E. coli* O157:H7 in the presence of oregano EO

The survivor (time-kill) curves for *E. coli* O157:H7 in MHB at various concentrations of oregano oil are shown in Fig. 1. A concentration–effect relationship is apparent – 625 μl l^{-1} oregano EO being bactericidal (no viable cells detected)
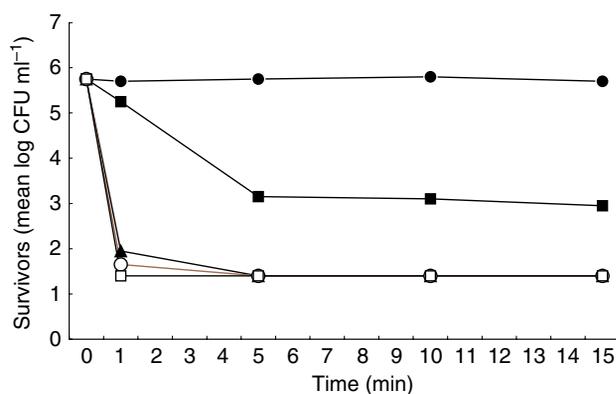

Fig. 1 Survivor curves for *E. coli* O157: H7 in Mueller-Hinton broth at different concentrations of oregano oil at 37°C: (●) 0 μl l^{-1} (control), (■) 78 μl l^{-1}, (▲) 156 μl l^{-1}, (○) 312 μl l^{-1}, (□) 625 μl l^{-1} oregano oil. The detection limit for viable cells was 1·4 log CFU ml^{-1}. Where no viable cells were recovered, the detection limit is indicated

within 1 min. Concentrations of 156 and 312 μl l^{-1} were bactericidal within 5 min. With 78 μl l^{-1} oregano EO the number of viable cells was reduced by more than 2 log factors within 5 min and thereafter did not change within the time span of the experiment (15 min).

S.E.M. observations

Cells treated with 625 μl l^{-1} oregano oil underwent considerable morphological alterations in comparison with the control when observed by a S.E.M. (Fig. 2). The cell structures appeared to be empty of contents and the remains were flaccid. Control cells were whole. Although the samples were not prepared in a quantitative manner, it was apparent from observations at low magnification that the number of cells retained on the membrane was significantly greater in the control than in the suspension treated with oregano EO.

DISCUSSION

Oregano and thyme EO were found to exhibit stronger antimicrobial properties than clove and bay in the disc diffusion assay. Studies testing EO from the same species against *E. coli* using the agar dilution method (Hammer *et al.* 1999) and well test (Dorman and Deans 2000) support this ranking. The concentrations at which oil of oregano, in particular, exerts a colistatic and colicidal effect at 10, 20 and 37°C indicate that there may be possibilities for its use as an additive to foodstuffs where a reduction in the number or prevention of growth of *E. coli* O157:H7 is desired.

The substantial improvement in these properties in the presence of agar as stabilizer is assumed to be attributable to the slowing of the separation of EO from the water phase, which would enable the more effective inhibition of bacterial
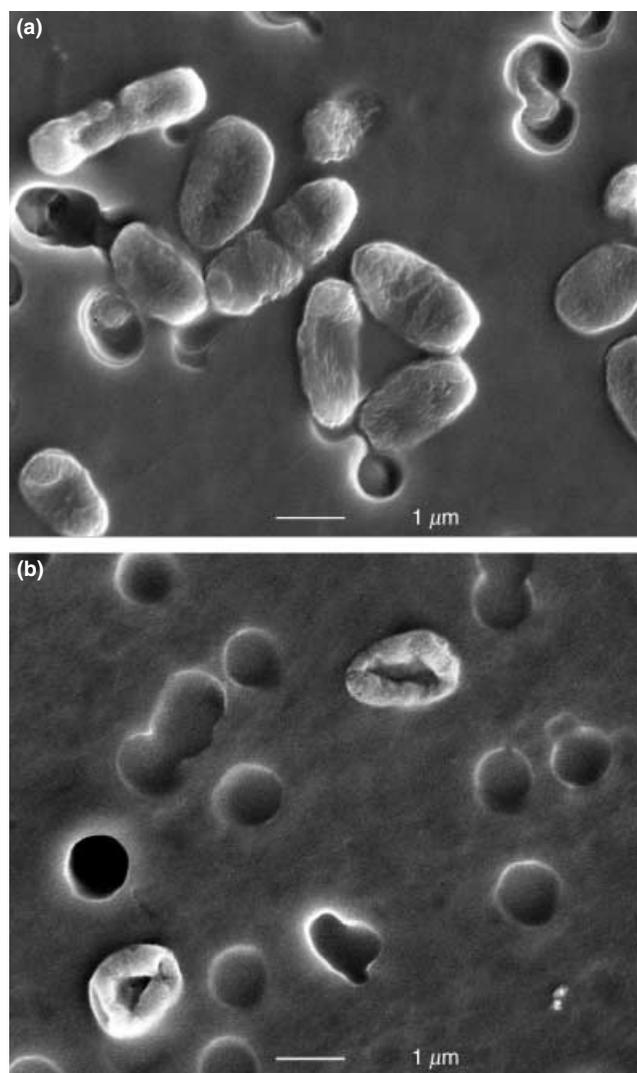
Fig. 2 Scanning electron microscope images of *E. coli* O157:H7 cellsafter treatment with oregano essential oil (12 000 ×). (a) Untreated cells (control). (b) Cells damaged after treatment with 625 μl l^{-1} oregano oil

cells. The reduction in activity in the presence of lecithin was marked. A possible explanation for this observation is that lecithin, in orienting itself between the oil micelles and the water phase, may have physically hindered the interaction between the EO and the bacterial cells. Alternatively, it is possible that EO exert their antibacterial effect on phospholipids in the outer layer of the bacterial cell membrane and were therefore effectively partly neutralized by the presence of lecithin, an additional source of phospholipids. The latter hypothesis is supported by work on carvacrol, a phenolic constituent of EO such as oregano and thyme, which was found to cause changes in the fatty acid and head-group composition of the phospholipid

bilayer of *Bacillus cereus*. Cells grown in sublethal concentrations of carvacrol were found to synthesize two additional phospholipids and to lack one of the original phospholipids (Ultee *et al.* 2000). In another study it was shown that *E. coli* K-12 increased the saturation level of its lipids when grown in sublethal concentrations of phenol and related compounds. Supplementation of the growth media with saturated fatty acids or lecithin reduced the inhibitive effect of the phenols. It is supposed that free fatty acid impurities in lecithin may be available to *E. coli* for uptake in the cell membrane, thereby providing a level of protection from phenolics (Keweloh *et al.* 1991). This observation has implications for the possible application of EO in foods and may partly account for the observed reductions in antibacterial activity of some EO in food systems compared with *in vitro* performance (Ismaiel and Pierson 1990).

The lack of a temperature effect between 10 and 37°C on the antibacterial properties of EO in broth without stabilizer is an encouraging observation, considering the broad application of low temperature storage in food preservation. That thyme oil retains its bacteriostatic/bactericidal effect at low temperatures was also demonstrated in studies with *Listeria monocytogenes* (Ting and Deibel 1991; Smith-Palmer *et al.* 1998). With agar as stabilizer a slight temperature effect was observed, particularly for thyme EO, whereby the agar-induced improvement in performance was greater at 10°C than at 37°C. The agar matrix is presumably firmer at low temperatures, which further retards the separation of the oil and water phases, thereby improving the antibacterial properties.

The oregano oil used in the present study appears particularly effective with respect to the time needed to exert its bactericidal effect. The bactericidal concentration of oregano EO without stabilizer (625 μl l^{-1}) was successful in killing >10^4 CFU ml^{-1} within 1 min (no viable cells recovered). Other researchers found oregano EO bactericidal to a different strain of *E. coli* O157:H7 at concentrations greater than 0·05% (v/v) (500 μl l^{-1}). The time-kill performance was, however, less substantial. When a similar number of CFU per ml^{-1} were treated with 0·05% oregano EO in broth, the number of viable cells decreased by approximately 2 log factors over a period of days at 37°C but a total kill was apparently not achieved (Skandamis *et al.* 2001).

From the s.e.m. observations it appears that, after loss of contents, *E. coli* O157:H7 cells treated with oregano EO collapsed, which enabled them to pass more easily through the pores of the membrane than the untreated control cells. Lambert *et al.* (2001) confirms that carvacrol and thymol (major constituents of oregano EO) render bacterial cell membranes permeable.

As mentioned before, the composition of EO from the same species of plant can vary with harvesting season and geographical location. Studies carried out with a particular batch of EO should, however, exhibit good reproducibility.

In summary, this study shows that oregano and thyme EO possess significant *in vitro* colicidal and colistatic properties, which are exhibited in a broad temperature range and are amplified by the addition of agar as stabilizer, and could be further researched for application in the food sector to improve food safety by the partial or total elimination of *E. coli* O157:H7. Particularly interactions with other food ingredients necessitate more thorough investigation.

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