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ANTIMICROBIAL AGENTS AND RESISTANCE

Inhibition of listeriolysin O and phosphatidylcholine-specific production in *Listeria monocytogenes* by subinhibitory concentrations of plant essential oils

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Successful infection by *Listeria monocytogenes* is dependent upon a range of bacterial extracellular proteins including a cytolysin termed listeriolysin O and phosphatidylcholine-specific phospholipase C. Five plant essential oils – bay, clove, cinnamon, nutmeg and thyme – significantly reduced the production of listeriolysin O by *L. monocytogenes*. The greatest change was observed after culture with oil of thyme, which reduced haemolysis to 52.1 haemolytic units (HU)/ml compared with 99.8 HU/ml observed with the control. Oil of clove was the only oil that also significantly reduced phosphatidylcholine-specific phospholipase C activity. These changes were observed despite the oils causing no change to the final bacterial concentration or total extracellular protein concentration.

Introduction

Listeria monocytogenes is the bacterium responsible for listeriosis, a disease with potentially serious complications including miscarriage, pre-term labour, meningitis and bacteraemia, and a mortality rate which can be as high as 30% [1]. Pregnancy is recognised as an important predisposing condition for *L. monocytogenes* infection. In non-pregnant adults, listeriosis occurs principally in those with a suppressed immune system. Predisposing conditions include lymphoproliferative disorders (leukaemia and lymphoma), other malignancies and immunosuppressive therapy associated with transplants [2]. The elderly are also at increased risk, as are patients with AIDS [3]. However, up to 30% of adults who develop listeriosis have no apparent immunocompromising condition [4].

L. monocytogenes is a gram-positive facultative intracellular pathogen capable of infecting a range of host cells including macrophages, fibroblasts and epithelial cells, by a process termed 'parasite-directed endocytosis' or 'induced phagocytosis'. InlA and InlB are two surface-bound proteins required for the invasion of many, but not all, non-phagocytic cell types [5]. A protein designated p60 is also involved in this process. Although predominately an extracellular protein, some p60 is also associated with the cell wall [6]. The subsequent survival and cell-to-cell spread of *L. monocytogenes* is dependent upon a range of extracellular proteins including catalase, superoxide dismutase, phosphatidylcholine-specific phospholipase C, phosphatidylinositol-specific phospholipase C [7–9] and listeriolysin O [10]. Listeriolysin O is a 60-kDa toxin belonging to the family of thiol-activated cytolysins. It disrupts the phagocytic membrane, allowing bacterial escape from the endosomal compartment. Following escape from the vacuole, *L. monocytogenes* induces host cell actin polymerisation which propels the bacteria through the host cell cytoplasm, ultimately leading to the formation of projections which are engulfed by neighbouring cells [11]. A number of the extracellular proteins are implicated in escape from the resultant double membrane vacuoles, especially the phospholipases.

The antimicrobial properties of herbs and spices have been recognised for many thousands of years. Recently

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there has been renewed interest in these products, in particular essential oils, in both the food and pharmaceutical industries [12–14]. As well as having antimicrobial properties, research has also shown that essential oils have the ability to alter bacterial pathogenicity and, in particular, toxin production. The work of González-Fandos *et al*. [15] demonstrated the influence of garlic on enterotoxin production by *Staphylococcus aureus*. Similarly, De Wit *et al*. [16] reported that garlic inhibited type A toxin production by *Clostridium botulinum*. However, data are unavailable regarding any changes in extracellular protein production by *L. monocytogenes*, despite considerable literature on the antilisterial properties of plant essential oils [13, 17]. This study investigated the ability of subinhibitory concentrations of selected plant essential oils to decrease the production of listeriolysin O and phosphatidylcholine-specific phospholipase C *in vitro*.

Materials and methods

Micro-organism

Listeria monocytogenes NCTC 11994 was maintained on Tryptone Soya Agar (Oxoid) at 4°C.

Preparation of cultures and culture supernates

A 10-μl volume of an overnight culture of *L. monocytogenes* was used to inoculate 10 ml of tryptone soya broth (TSB) containing subinhibitory concentrations of plant essential oils of bay (*Pimenta racemosa*), cinnamon leaf (*Cinnamomum verum*), clove stem (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) at 0.01% v/v, and oil of nutmeg (*Myristica fragrans*) at 0.0025% v/v (kindly supplied by F.D. Copeland and Sons, London). Figs. 1–5 show the gas chromatography profiles of the plant essential oils and their major constituents. These five plant essential oils had previously been reported to have good antimicrobial activity against *L. monocytogenes* [13]. These subinhibitory concentrations were selected as they ensured that the bacterial concentration in the experimental samples after 24 h was the same as the control. Supernates from 24-h cultures were obtained by centrifugation (1600 *g*) for 10 min.

Determination of extracellular protein concentration

The determination of extracellular protein concentration was based on the method of Bradford [18] with Coomassie Brilliant Blue G dye (BioRad), diluted 1 in 5, and bovine serum albumin (Sigma-Aldrich) as the standard. Five ml of dye reagent were added to 1-ml samples of culture supernates and incubated at room temperature for 30 min. Absorbance was read in triplicate at 595 nm with a Dynatech MR 500 plate reader. Controls included TSB alone, the value for which was subtracted from the experimental value, to give the extracellular protein concentration.

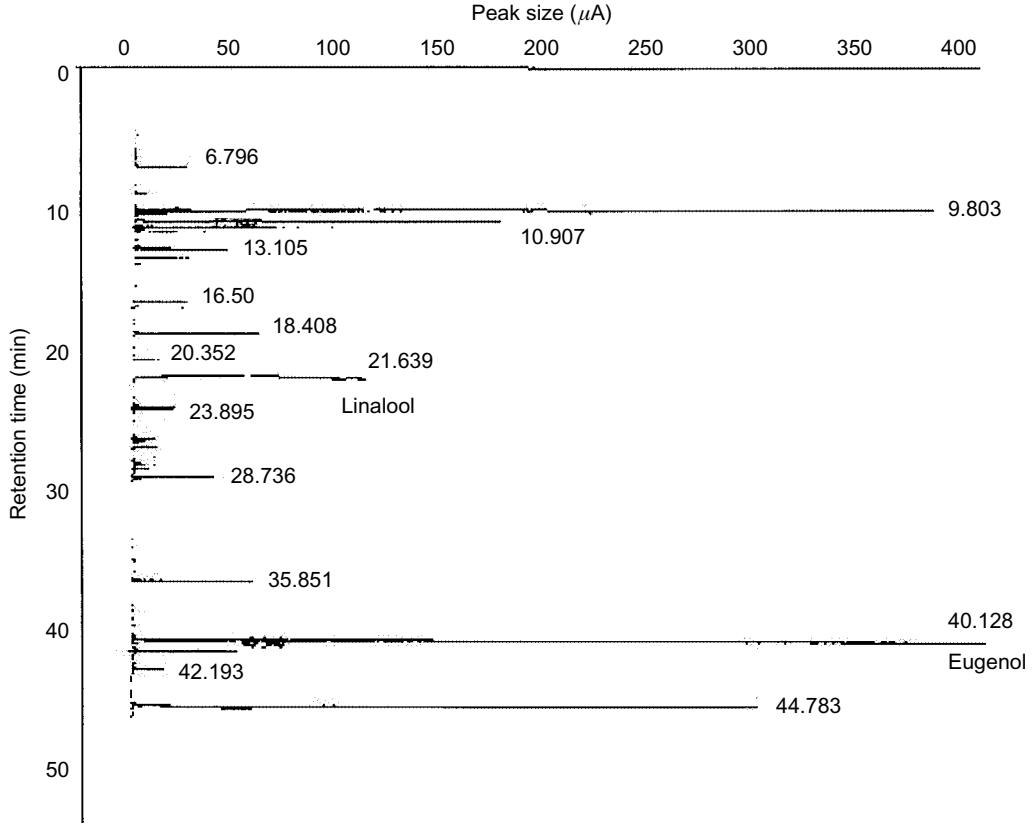

Fig. 1. Gas chromatography report for the composition of oil of bay (*Pimenta racemosa*).

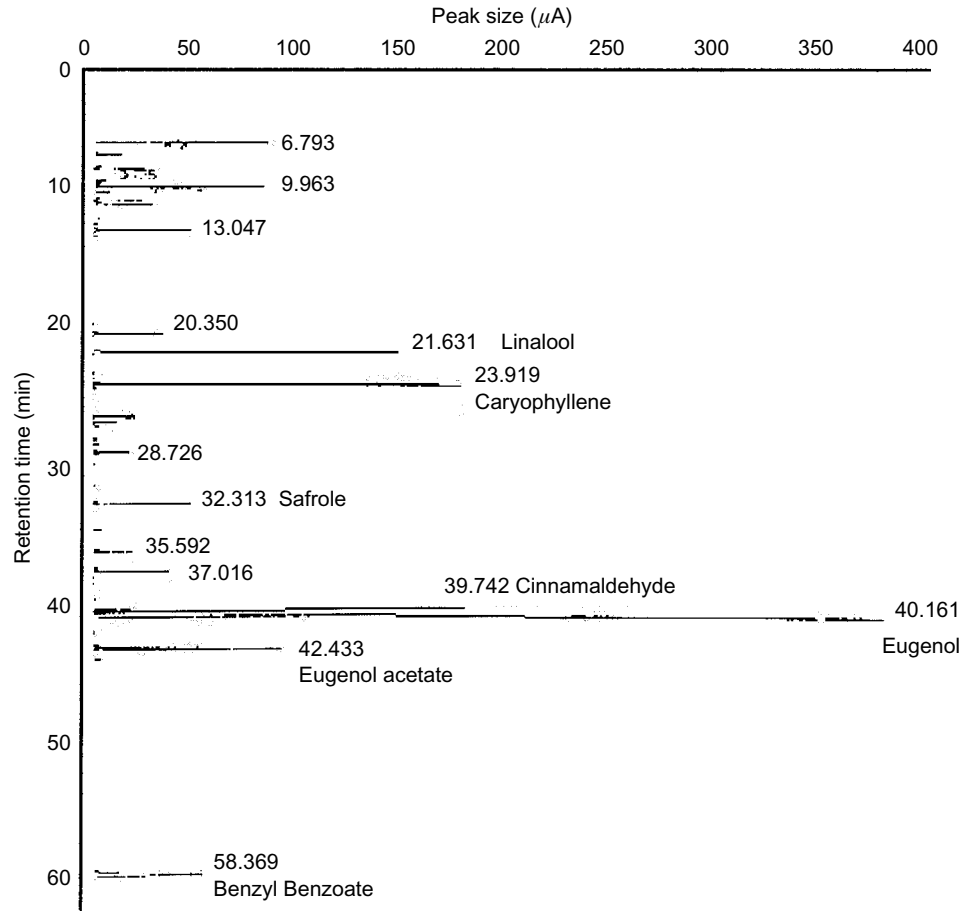

Fig. 2. Gas chromatography report for the composition of oil of cinnamon (*Cinnamomum verum*).

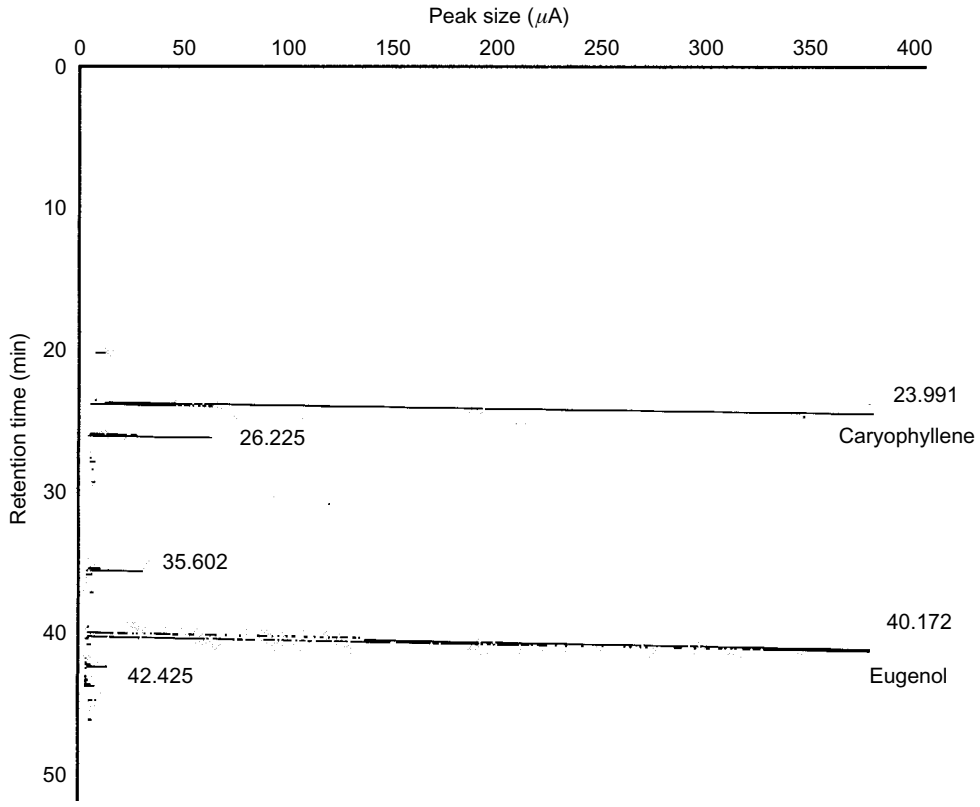

Fig. 3. Gas chromatography report for the composition of oil of clove (*Syzygium aromaticum*).

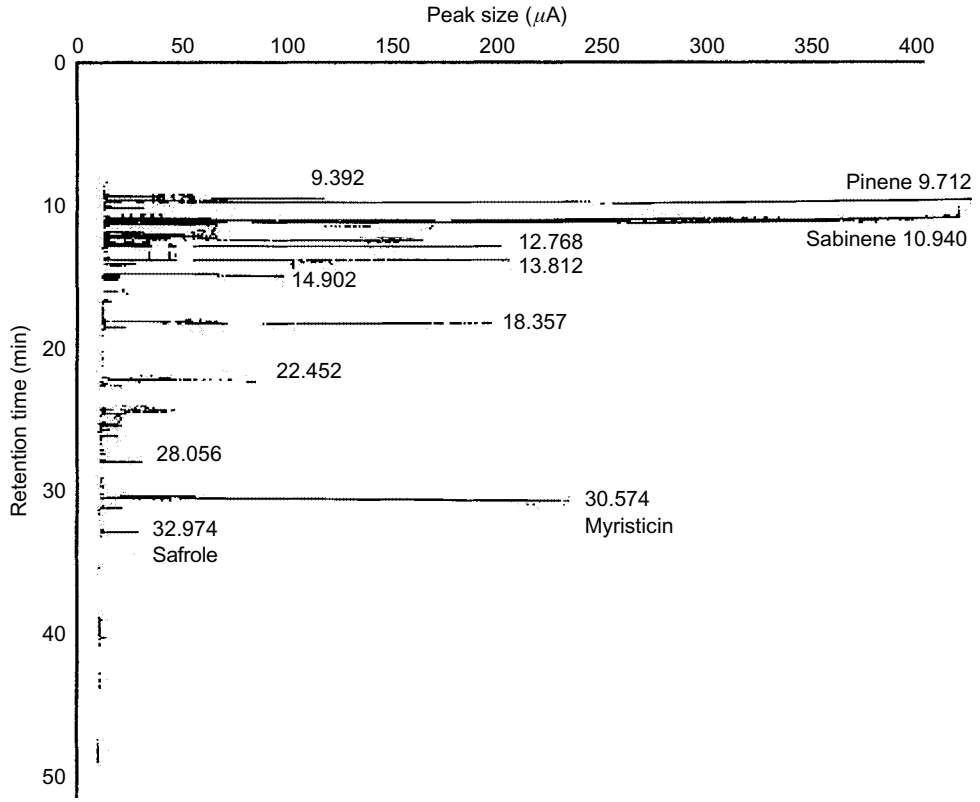

Fig. 4. Gas chromatography report for the composition of oil of nutmeg (*Myristica fragrans*).

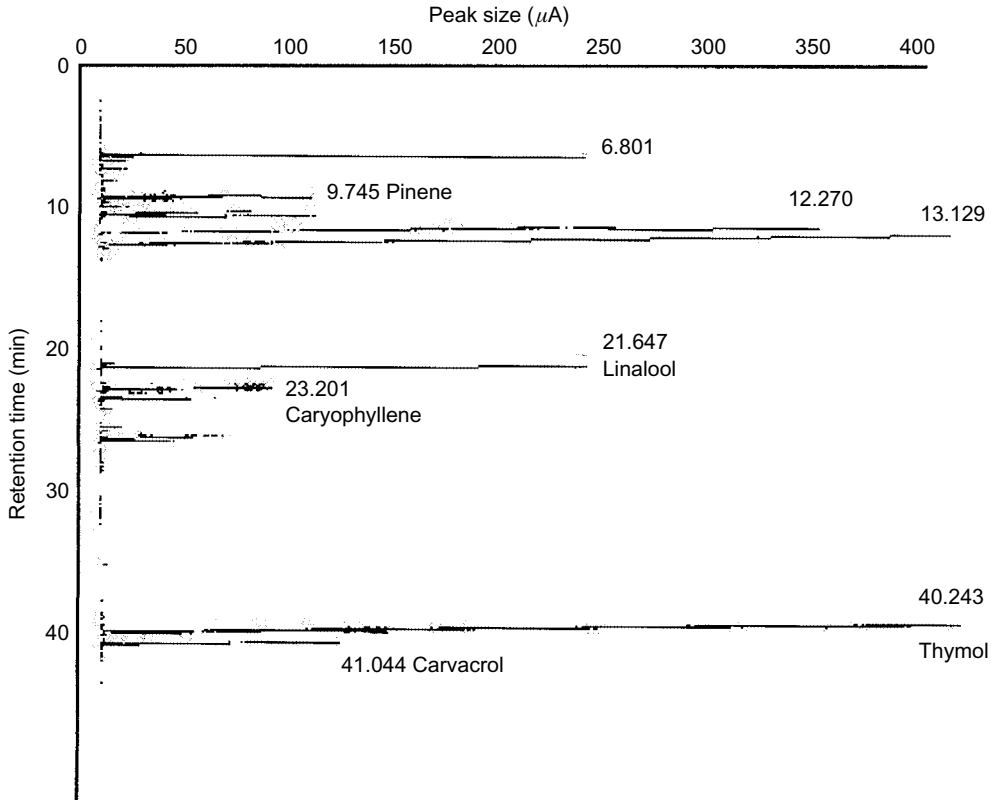

Fig. 5. Gas chromatography report for the composition of oil of thyme (*Thymus vulgaris*).

Listeriolysin O activity in the supernate after culture with subinhibitory concentrations of plant essential oils

Listeriolysin O activity was determined by a widely employed haemolysis assay [19]. Briefly, 1-ml samples of culture supernates were mixed with 485 μl of phosphate-buffered saline (PBS; Sigma-Aldrich) containing 20 mM cysteine (Sigma-Aldrich) and sheep erythrocytes (Oxoid) were added to achieve a final concentration of 1%. Tubes were gently mixed by inversion, incubated at 37°C for 30 min and then centrifuged (1000 *g*) for 5 min. The absorbance was read in triplicate at 550 nm with a Dynatech MR 500 plate reader. Experimental results were expressed as haemolytic units of activity. One haemolytic unit (HU) of activity was defined as the amount of activity that caused 50% haemolysis of the erythrocytes. To determine percentage haemolysis, controls were set up which contained 1% erythrocytes and de-ionised water, which was taken as 100% haemolysis. Other controls contained PBS, TSB or TSB with subinhibitory concentrations of plant essential oils. No significant haemolysis was detected in these preparations.

Experiments were conducted to determine whether the reducing agent dithiothreitol (DTT; Sigma-Aldrich) could restore haemolysis lost as a result of culture with plant essential oils. The culture supernates were obtained as before and DTT was added to achieve a final concentration of 1 mM. The samples were mixed for 1 h at 37°C and the haemolytic activity was measured as before.

Direct effect of plant essential oils on listeriolysin O activity

Experiments were conducted to determine whether plant essential oils directly inhibited the activity of listeriolysin O. Samples of the plant essential oils (5 μl) were added to supernates from *L. monocytogenes* cultured in TSB only (therefore known to contain high levels of listeriolysin O) to achieve concentrations of each oil greater than the subinhibitory concentrations. All tubes were incubated at 37°C for 1 h with constant mixing, and then the samples were left to settle for 15 min, enabling the plant essential oils to separate out. Then 1-ml samples were removed and haemolytic activity was measured as before.

Determination of intracellular listeriolysin O activity after culture with subinhibitory concentrations of plant essential oils

Experiments were conducted to determine whether culture with plant essential oils prevented listeriolysin O synthesised in the cell being exported into the culture supernate. *L. monocytogenes* was cultured with subinhibitory concentrations of plant essential oils for 24 h, the supernate was discarded, the cells were washed twice in PBS and resuspended in 10 ml of PBS. Cells were lysed on ice by sonication for 5 min and examined by light microscopy to ensure that all cells were lysed. The lysate was centrifuged (1600 *g*) for 10 min to remove cell debris and the supernate was examined for haemolytic activity as above.

Influence of plant essential oils on the phosphatidylcholine-specific phospholipase C activity of L. monocytogenes

Phosphatidylcholine-specific phospholipase C activity was determined by the method of Coffey *et al.* [20]. One ml of culture supernate was incubated with 2 ml of a 10% solution of egg-yolk lecithin (Merck) and 7 ml of 0.15 M NaCl (Sigma-Aldrich) at 37°C for 1 h and the amount of H^+ liberated was determined by titration and changes in pH. Controls of TSB and TSB with subinhibitory concentrations of plant essential oils showed no significant liberation of H^+.

Reproducibility and statistics

All measurements were conducted in triplicate within each experiment, and each experiment was performed on at least three separate occasions. Two-tailed Student's *t* tests were used to analyse the data. Differences were judged to be statistically significant when $p \leqslant 0.05$.

Results

To make a valid comparison of the influence of subinhibitory concentrations of plant essential oils on listeriolysin O, it was necessary to ensure that the same cell numbers and quantity of extracellular protein were produced when cultured with plant essential oils as the control. At the concentrations used there was no reduction in bacterial growth (data not shown) nor in the amount of extracellular protein produced (Table 1). In contrast, listeriolysin O activity in the culture supernate was significantly reduced after culture with all five oils (Table 2). The greatest changes were observed after culture with the oils of clove and thyme with 55.9 and 52.1 HU/ml, respectively, compared with 99.8 HU/ml observed with the control. Cells cultured with the oils of bay and cinnamon showed very similar

Table 1. The quantity of extracellular protein produced by *L. monocytogenes* after culture for 24 h with subinhibitory concentrations of plant essential oils

Culture conditions	Mean (SEM) extracellular protein concentration (μg/ml)
Control	34.66 (0.54)
Oil of bay	33.80 (2.59)
Oil of cinnamon	34.00 (2.03)
Oil of clove	33.66 (1.36)
Oil of nutmeg	34.20 (1.11)
Oil of thyme	34.62 (0.54)

Table 2. Listeriolysin O activity in supernates from *L. monocytogenes* cultured with subinhibitory concentrations of plant essential oils

	Mean (SEM) listeriolysin O activity (HU/ml)	
Culture conditions	without DTT	with DTT*
Control	99.8 (5.6)	95.3 (4.1)
Oil of bay	63.1 (3.1)†	61.3 (2.1)†
Oil of cinnamon	63.7 (4.6)†	63.1 (5.4)†
Oil of clove	55.9 (5.6)†	56.9 (5.5)†
Oil of nutmeg	80.1 (5.7)†	83.5 (4.6)†
Oil of thyme	52.1 (1.5)†	57.3 (4.1)†

*DTT was added to each supernate to achieve a final concentration of 1 mM, haemolytic activity was measured after incubation for 1 h at 37°C.
†$p \leq 0.05$ compared to corresponding control.

reductions in listeriolysin O activity with 63 HU/ml. Oil of nutmeg proved to be the least influential with 80.1 HU/ml. Table 2 also shows that the addition of DTT did not significantly alter the haemolytic activity in supernates from cells cultured with plant essential oils compared to samples without DTT. The plant essential oils were shown not to directly inhibit haemolytic activity present in supernates from cells cultured in TSB only and thus known to contain high levels of listeriolysin O (Table 3). Similarly the plant essential oils did not inhibit the export of listeriolysin O from the cell, as ≤4 HU/ml was detected in all the samples of cell lysate (Table 4).

Fig. 6 shows H^+ production as a result of lecithin degradation by phosphatidylcholine-specific phospholipase C; 65 μmole H^+/h were produced by the control and no significant differences were detected for samples cultured with the oils of bay, cinnamon, nutmeg or thyme. A significant reduction of 17 μmole H^+/h was observed for cells cultured with oil of clove.

Table 3. Direct effect of plant essential oils on listeriolysin O activity

Plant essential oil	Mean (SEM) listeriolysin O activity (HU/ml)
Control	102.4 (5.6)
Oil of bay	102.9 (7.9)
Oil of cinnamon	98.9 (5.2)
Oil of clove	103.1 (5.1)
Oil of nutmeg	100.2 (5.3)
Oil of thyme	102.4 (2.4)

The haemolytic activity observed after 5 μl of plant essential oil was added to 10 ml of culture supernate from *L. monocytogenes* cultured without plant essential oils and incubated at 37°C for 1 h, before the haemolytic activity was determined.

Table 4. Listeriolysin O activity in the cell lysate from *L. monocytogenes* cultured with subinhibitory concentrations of plant essential oils

Culture conditions	Mean (SEM) listeriolysin O activity (HU/ml)
Control	3.3 (0.1)
Oil of bay	2.5 (0.1)
Oil of cinnamon	3.2 (0.2)
Oil of clove	3.5 (0.2)
Oil of nutmeg	3.6 (0.1)
Oil of thyme	4.0 (0.3)

The haemolytic activity in the cell lysate obtained after *L. monocytogenes* was cultured for 24 h with subinhibitory concentrations of plant essential oils.

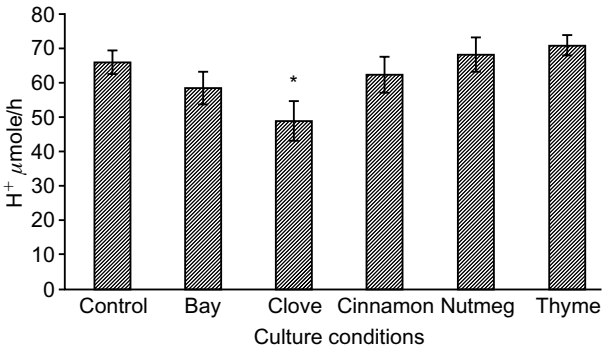

Fig. 6. Production of H^+ from egg yolk lecithin in 60 min (μmole H^+/h) by phosphatidylcholine-specific phospholipase C from *L. monocytogenes* cultured with subinhibitory concentrations of plant essential oils. *$p \leq 0.05$ significant difference in μmole H^+/h compared with control.

Discussion

The data presented here show the ability of all five plant essential oils to significantly reduce the production of listeriolysin O at subinhibitory concentrations. The production of phosphatidylcholine-specific phospholipase C was inhibited by oil of clove. None of the oils used had a significant effect on the overall quantity of extracellular protein produced.

All the haemolytic activity detected in the culture supernates was assumed to be the result of listeriolysin O. Although phosphatidylcholine-specific phospholipase C has some haemolytic activity, it is not active against sheep erythrocytes, probably because they contain very little phosphatidylcholine [9]. Culture of *L. monocytogenes* with subinhibitory concentrations of all five plant essential oils, particularly oil of thyme, significantly reduced haemolysis indicative of listeriolysin O. The results indicate that the inhibition was probably at the level of listeriolysin O production, rather than the oils interfering directly with listeriolysin O activity. Firstly, activity was not restored by the addition of DTT, known to reverse activity lost as a result of oxidation or mercurials [19]. Secondly, the addition of plant essential oils to the supernate from *L. monocytogenes* cultured in TSB alone did not significantly reduce haemolysis (Table 3). This is the first account of listeriolysin O production being inhibited by plant essential oils. However, it is widely recognised that production is influenced by other factors including glucose concentration [21, 22], NaCl [23, 24], cellobiose [25] and β-lactam antibiotics [26]. Kouassi and Shelef reported that sorbate suppressed listeriolysin O secretion and its activation by cysteine [27]. These findings led them to suggest that the addition of sorbate

to foods could reduce listeriolysin O production. Similar conclusions could be drawn from the data presented here regarding the addition of plant essential oils.

A significant reduction in H^+ production resulting from the breakdown of lecithin by phosphatidylcholine-specific phospholipase C was detected only with cells previously cultured with oil of clove. It has been reported previously that pH, temperature, salt concentration and nutrient availability all have a significant impact on the production of phosphatidylcholine-specific phospholipase C [20]. Hence, it is not surprising that production can also be influenced by the presence of antimicrobial agents such as plant essential oils. It was possible that the inhibition observed did not result from oil of clove directly inhibiting the production or activity of the phospholipase *per se,* but rather the metalloprotease. The metalloprotease activates the precursor of the phospholipase secreted by the cells to the active enzyme [28]. The reduction in phosphatidylcholine-specific phospholipase C activity by oil of clove has the potential to limit the cell-to-cell spread of *L. monocytogenes*, especially when coupled with the significant reduction in listeriolysin O.

It is known that a number of virulence genes in *L. monocytogenes* – including *hly* encoding listeriolysin O, *plcB* encoding phosphatidylcholine-specific phospholipase C, *mpl* encoding metalloprotease and *actA* which encodes a protein necessary for actin polymerisation – are positively regulated by PrfA, a transcriptional activator [29, 30]. Because oil of clove was the only oil to inhibit the production of both toxins, it was possibly the only one to act at the level of genetic regulation. However, the remaining oils may have directly or indirectly inhibited *hly* specifically rather than the regulation of this gene. Similarly, cellobiose has been reported to repress *hly* [25] but not phosphatidylcholine-specific phospholipase C [20].

Another possible explanation for the inhibition of toxin production was disruption at the ribosomal level. It is believed that extracellular proteins are generally synthesised on membrane-associated ribosomes, whereas intracellular proteins are synthesised on cytoplasmic ribosomes [31]. Thus, toxin production is sensitive to changes in the membrane caused by plant essential oils, and the membrane is recognised as an important target site for many plant essential oils [32, 33]. Furthermore, components of plant essential oils actually penetrating the membrane would more readily interact with membrane-bound ribosomes than cytoplasmic ribosomes. Any changes to the cell membrane caused by the plant essential oils did not interfere with the cell's ability to export extracellular proteins, as no change in intracellular haemolytic activity was detected (Table 4) and, furthermore, there was no change in total extracellular protein concentration (Table 1).

The work presented here shows the potential of selected plant essential oils at subinhibitory concentrations to reduce the pathogenicity of *L. monocytogenes* by reducing the production of two key factors, listeriolysin O and phosphatidylcholine-specific phospholipase C. Reductions in pathogenicity could have important implications when considering potential applications in the food and pharmaceutical industries, especially at a time when there is already considerable interest in plant essential oils because of their antimicrobial action [12, 13] and antioxidant properties.

We acknowledge F.D. Copeland and Sons, Colanol House, 5 Westfield Street, London for the generous supply of plant essential oils.

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