Antibacterial activity of essential oils: potential applications in food

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Ph.D. thesis

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(met een samenvatting in het Nederlands)

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Contents

Chapter 1

Essential oils: their antibacterial properties and potential applications in foods - a review.

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ABSTRACT

In vitro studies have demonstrated antibacterial activity of essential oils against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus* at levels between 0.2-10 µl ml^{-1}. Gram-negative organisms are slightly less susceptible than Gram-positive bacteria. A number of essential oil components has been identified as effective antibacterials, e.g. carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid, having minimum inhibitory concentrations of 0.05-5 µl ml^{-1} in vitro. A higher concentration is needed to achieve the same effect in foods. Studies with fresh meat, meat products, fish, milk, dairy products, vegetables, fruit and cooked rice have shown that the concentration needed to achieve a significant antibacterial effect is around 0.5-20 µl g^{-1} in foods and about 0.1-10 µl ml^{-1} in solutions for washing fruit and vegetables. Essential oils comprise a large number of components and it is likely that their mode of action involves several targets in the cell. The hydrophobicity of essential oils enables them to partition in the lipids of the cell membrane, rendering them permeable and leading to leakage of cell contents. Physical conditions that improve the action of essential oils are low pH, low temperature and low oxygen levels. Synergism has been observed between carvacrol and its precursor *p*-cymene and between cinnamaldehyde and eugenol. Synergy between essential oil components and mild preservation methods has also been observed. Some components are legally registered flavourings in the EU and the USA. Undesirable organoleptic effects can be limited by careful selection of essential oils according to the type of food.

Keywords: Essential oils, antibacterial, preservatives, foodborne pathogens.

1. INTRODUCTION

In spite of modern improvements in slaughter hygiene and food production techniques, food safety is an increasingly important public health issue (191). It has been estimated that as many as 30% of people in industrialised countries suffer from a foodborne disease each year and in 2000 at least two million people died from diarrhoeal disease worldwide (191). There is therefore still a need for new methods of reducing or eliminating foodborne pathogens, possibly in combination with existing methods (the hurdle principle (97)). At the same time, Western society appears to be experiencing a trend of 'green' consumerism (163, 174), desiring fewer synthetic food additives and products with a smaller impact on the environment. Furthermore, the World Health Organization has recently called for a worldwide reduction in the consumption of salt in order to reduce the incidence of cardio-vascular disease (192). If the level of salt in processed foods is reduced, it is possible that other additives will be needed to maintain the safety of foods. There is therefore scope for methods of making food safe which have a natural or 'green' image. One such possibility is the use of essential oils as antibacterial additives.

Essential oils (EOs) (also called volatile or ethereal oils (61)) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). They can be obtained by expression, fermentation, enfleurage or extraction but the method of steam distillation is most commonly used for commercial production of EOs (183). The term *'essential oil'* is thought to derive from the name coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim; he named the effective component of a drug *Quinta essentia* (61). An estimated 3000 EOs are known, of which about 300 are commercially important – destined chiefly for the flavours and fragrances market (183). It has long been recognised that some EOs have antibacterial properties (17, 61) and these have been reviewed in the past (120, 152) as have the antibacterial properties of spices (152) but the relatively recent enhancement of interest in 'green' consumerism has lead to a renewal of scientific interest in these substances (120, 174). Besides antibacterial properties (21, 45, 116), EOs or their components have been shown to exhibit antiviral (14), antimycotic (1, 6, 74, 107), antitoxigenic (2, 76, 181), antiparasitic (126, 132), and insecticidal (82, 91) properties. These characteristics are possibly related to the function of these compounds in plants (61, 104).

The purpose of this paper is to provide an overview of the published data on the antibacterial activity of those EOs and their components that could be considered suitable for application in or on foods, and to describe their possible modes of action. The current knowledge on potential antagonists and synergists is presented; legal and safety aspects are discussed and areas for future research are

proposed. Although some data are presented on spoilage flora, this paper will focus chiefly on the antibacterial effect of EOs on foodborne pathogens and, in particular, those for which food animals are the major reservoir.

1.1. Historical use of essential oils

Although spices have been used for their perfume, flavour and preservative properties since antiquity (11), of the known EOs, only oil of turpentine was mentioned by Greek and Roman historians (61). Distillation as a method of producing EOs was first used in the East (Egypt, India and Persia) (61) more than 2000 years ago and was improved in the 9th century by the Arabs (11). The first authentic written account of distillation of essential oil is ascribed to Villanova (c. 1235-1311), a Catalan physician (61). By the 13th century EOs were being made by pharmacies and their pharmacological effects were described in pharmacopoeias (11) but their use does not appear to have been widespread in Europe until the 16th century, from which time they were traded in the City of London (39). Publishing separately in that century on the distillation and use of EOs, two Strassburg physicians, Brunschwig and Reiff, mention only a relatively small number of oils between them; turpentine, juniper wood, rosemary, spike (lavender), clove, mace, nutmeg, anise and cinnamon (61). According to the French physician, Du Chesne (Quercetanus), in the 17th century the preparation of EOs was well known and pharmacies generally stocked 15-20 different oils (61). The use of tea tree oil for medicinal purposes has been documented since the colonisation of Australia at the end of the 18th century, although it is likely to have been used by the native Australians before that (24). The first experimental measurement of the bactericidal properties of the vapours of EO is said to have been carried out by De la Croix in 1881 (17). However, in the course of the 19th and 20th centuries the use of EOs in medicine gradually became secondary to their use for flavour and aroma (61).

1.2 Current use of EOs

The greatest use of EOs in the European Union (EU) is in food (as flavourings), perfumes (fragrances and aftershaves) and pharmaceuticals (for their functional properties) (10, 183, 185). The well-known use of EO in aromatherapy constitutes little more than 2% of the total market (183). Individual components of EOs are also used as flavourings, either extracted from plant material or synthetically manufactured (122).

The antibacterial properties of essential oils and their components are exploited in such diverse commercial products as dental root canal sealers (105), antiseptics (10, 36) and feed supplements for lactating sows and weaned piglets (70, 184). A few food preservatives containing EOs are already commercially available. 'DMC Base Natural' is a food preservative produced by DOMCA S.A.

(Alhendín, Granada, Spain) and comprises 50% essential oils from rosemary, sage and citrus and 50% glycerol (114). 'Protecta One' and 'Protecta Two' are blended herb extracts produced by Bavaria Corp. (Apopka, FL, USA) and are classed as generally recognized as safe (GRAS) food additives in the U.S.A. Although the precise contents are not made known by the manufacturer, the extracts probably contain one or more EOs and are dispersed in solutions of sodium citrate and sodium chloride, respectively (40). Further physiological effects of EOs are made use of in widely differing products such as commercial potato sprout suppressants (67) and insect repellents (24).

2. COMPOSITION OF EOS

Steam distillation is the most commonly used method for producing EOs on a commercial basis. Extraction by means of liquid carbon dioxide under low temperature and high pressure produces a more natural organoleptic profile but is much more expensive (117).The difference in organoleptic profile indicates a difference in the composition of oils obtained by solvent extraction as opposed to distillation and this may also influence antimicrobial properties. This would appear to be confirmed by the fact that herb EOs extracted by hexane have been shown to exhibit greater antimicrobial activity than the corresponding steam distilled EOs (125). EOs are volatile and therefore need to be stored in airtight containers in the dark in order to prevent compositional changes.

Numerous publications have presented data on the composition of the various EOs. The major components of the economically interesting EOs are summarised by Bauer *et al*. (11). Detailed compositional analysis is achieved by gas chromatography and mass spectrometry of the EO or its headspace (42, 47, 75, 77, 148, 149, 193). EOs can comprise more than sixty individual components (146, 150). Major components can constitute up to 85 % of the EO whereas other components are present only as a trace (11, 150). The phenolic components are chiefly responsible for the antibacterial properties of EOs (35). The major components of a number of EOs with antibacterial properties are presented in Table 1 and the structural formulae of a number of antibacterial components are presented in Figure 1. These components have either been shown to have antibacterial activity or the data on their mode of action is discussed in this paper. There is some evidence that minor components have a critical part to play in antibacterial activity, possibly by producing a synergistic effect between other components. This has been found to be the case for sage (109), certain species of *Thymus* (95, 108, 129) and oregano (129).

Table 1. Major components of selected[a] EOs that exhibit antibacterial properties.

Common name of EO	Latin name of plant source	Major components	Approximate % composition[b]	References
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool E-2-decanal	26% 20%	(47)
Coriander	*Coriandrum sativum* (seeds)	Linalool E-2-decanal	70% -	(47)
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	65%	(99)
Oregano	*Origanum vulgare*	Carvacrol Thymol γ-Terpinene *p*-Cymene	Trace - 80% Trace - 64% 2 - 52% Trace - 52%	(28, 42, 48, 90, 96, 109, 137, 146, 158)
Rosemary	*Rosmarinus officinalis*	α-pinene Bornyl acetate Camphor 1,8-cineole	2-25 % 0-17% 2-14% 3-89%	(41, 42, 133)
Sage	*Salvia officinalis* L.	Camphor α-Pinene β-pinene 1,8-cineole α-tujone	6-15% 4-5% 2-10% 6-14% 20-42%	(109)
Clove (bud)	*Syzygium aromaticum*	Eugenol Eugenyl acetate	75 - 85 % 8 - 15 %	(11)
Thyme	*Thymus vulgaris*	Thymol Carvacrol γ-Terpinene *p*-Cymene	10 - 64 % 2 - 11 % 2 - 31 % 10 - 56 %	(35, 42, 77, 95, 96, 99, 108, 112, 150)

[a] EOs which have been shown to exert antibacterial properties in vitro or in food models and for which the composition could be found in the literature.
[b] Percentages of total volatiles rounded up to the nearest whole number.

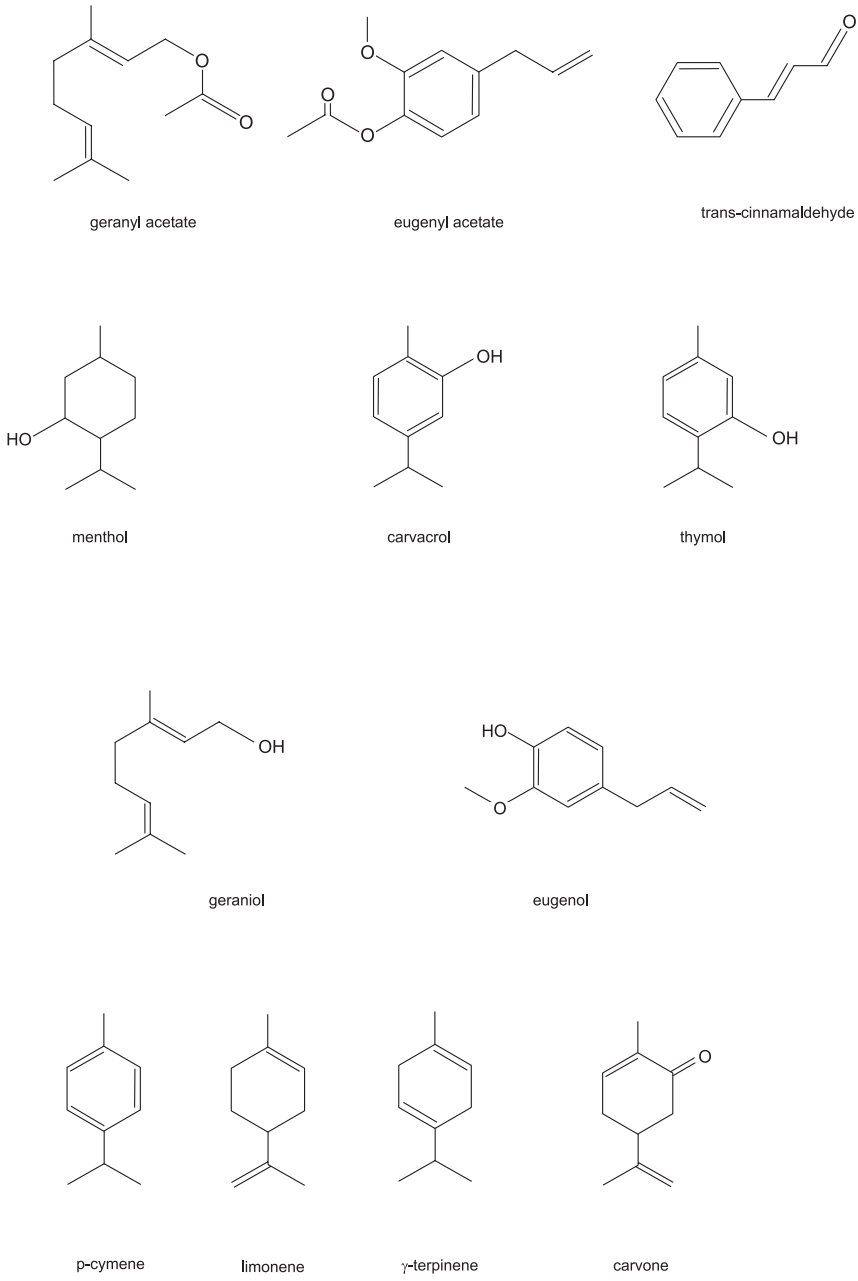

Figure 1. Structural formulae of selected components of EOs.

The composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources (4, 35, 54, 77, 108, 110, 112). This can be explained, at least in part, by the formation of antibacterial substances from their precursors. *p*-Cymene (1-methyl-4-(1-methylethyl)-benzene) and γ-terpinene (1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene) are the precursors of carvacrol (2-methyl-5-(1-methylethyl)phenol) and thymol (5-methyl-2-(1-methylethyl)phenol) in species of *Origanum* and *Thymus* (35, 75, 176). The sum of the amounts of these four compounds present in Greek oregano plants has been found to be almost equal in specimens derived from different geographical regions (90) and to remain stable in plants harvested during different seasons (75). The same is true of *T. vulgaris* from Italy (108). This indicates that the four compounds are biologically and functionally closely associated and supports the theory that thymol is formed via *p*-cymene from γ-terpinene in *Thymus vulgaris* (90). Generally, EOs produced from herbs harvested during or immediately after flowering possess the strongest antimicrobial activity (108, 112). Enantiomers of EO components have been shown to exhibit antimicrobial activity to different extents (101). The composition of EOs from different parts of the same plant can also differ widely. For example, EO obtained from the seeds of coriander (*Coriandrum sativum* L.) has a quite different composition to EO of cilantro, which is obtained from the immature leaves of the same plant (47).

3. IN VITRO TESTS OF ANTIBACTERIAL ACTIVITY

Tests of antimicrobial activity can be classified as diffusion, dilution or bioautographic methods (143). The principles and practice of these test methods are explained in the literature (7, 44, 69) but it appears that no standardised test has been developed for evaluating the antibacterial activity of possible preservatives against food-related microorganisms, although the need for such has been indicated (44). The NCCLS method for antibacterial susceptibility testing, which is principally aimed at the testing of antibiotics, has been modified for testing EOs (63, 118). Researchers adapt experimental methods to better represent possible future applications in their particular field. However, since the outcome of a test can be affected by factors such as the method used to extract the EO from plant material, the volume of inoculum, growth phase, culture medium used, pH of the media and incubation time and temperature (143), comparison of published data is complicated (57, 73). A number of reviewers has surveyed the methods used for antibacterial activity studies carried out with EOs (73, 87, 88, 120, 143, 152). In papers published since, the number of variations on culture medium, size of inoculum, choice of emulsifier and basic test method has further increased.

The minimum inhibitory concentration (MIC) is cited by most researchers as a measure of the antibacterial performance of EOs. The definition of the MIC differs between publications and this is another obstacle to comparison between studies. In some cases the minimum bactericidal concentration (MBC) or the bacteriostatic concentration is stated, both terms agreeing closely with the MIC. A list of the most frequently used terms in antibacterial activity testing of EOs is presented in Table 2. In addition, the term 'minimum cidal concentration' has been used but is not defined (63). The terms 'minimum lethal dilution (or concentration)' (72, 73) and ' minimum inhibitory dilution' (72) appear to have fallen out of use, at least in literature concerning EOs in food microbiology.

Table 2. Terms used in antibacterial activity testing

Term	Definition, with reference to concentration of EO	Reference
Minimum inhibitory concentration (MIC)	Lowest concentration resulting in maintenance or reduction of inoculum viability	(21)
	Lowest concentration required for complete inhibition of test organism up to 48 h incubation	(20, 187)
	Lowest concentration inhibiting visible growth of test organism	(47, 63, 79, 121)
	Lowest concentration resulting in a significant decrease in inoculum viability (>90%)	(35)
Minimum bactericidal concentration (MBC)	Concentration where 99.9% or more of the initial inoculum is killed	(20, 22, 35)
	Lowest concentration at which no growth is observed after subculturing into fresh broth	(121)
Bacteriostatic concentration	Lowest concentration at which bacteria fail to grow in broth, but are cultured when broth is plated onto agar	(164)
Bactericidal concentration	Lowest concentration at which bacteria fail to grow in broth, and are not cultured when broth is plated onto agar	(164)

A summary of the techniques used to test the antibacterial activity of EOs is presented in Table 3. Screening of EOs for antibacterial activity is often done by the disk diffusion method, in which a paper disk soaked with EO is laid on top of an inoculated agar plate. This is generally used as a preliminary check for antibacterial activity prior to more detailed studies. Factors such as the volume of EO placed on the paper disks, the thickness of the agar layer and whether a solvent is used vary considerably between studies. This means that this method is useful for selection between EOs but comparison of published data is not feasible. The

agar well test in which the EO is deposited into wells cut into the agar can be used as a screening method when large numbers of EOs and/or large numbers of bacterial isolates are to be screened (46, 51). In order to make bacterial growth easier to visualise, triphenyl tetrazolium chloride may be added to the growth medium (52, 116).

Table 3. Test methods used to measure the antibacterial activity of EOs and their constituents.

Purpose	Test method		References
Screening for antibacterial activity	Disk diffusion		(5, 19, 29, 52, 54-56, 83, 84, 125, 142, 151, 158, 159, 194)
	Agar wells		(45, 51, 145, 164, 187)
Determination of strength of antibacterial properties	Agar dilution method		(55, 63, 78, 79, 124, 127, 128, 133, 137, 167, 172) (194) (115, 119)
	Broth dilution	Visible growth	(9, 35, 47, 52, 116, 121)
		Optical density/turbidity	(27, 71, 83, 94, 136, 153, 157-159, 177, 181)
		Absorbance	(113, 164)
		Colorimetric	(19, 59)
		Conductance/conductivity/impedance	(108, 109, 168, 170, 187)
		Viable count	(13, 20, 57, 63, 89, 130, 131, 136, 153, 158, 160, 168)
Determination of rapidity and duration of antibacterial activity	Time-kill analysis/Survival curves		(5, 13, 19, 38, 113, 130, 133, 136, 153, 158, 159, 167, 168, 172, 177, 181, 187)
Observation of physical effects of antibacterial activity	Scanning electron microscopy		(19, 94, 159)

The strength of the antibacterial activity can be determined by dilution of EO in agar or broth. The published studies using dilution in agar have used different solvents to incorporate the EO in the medium (133, 137), different volumes of inoculum (1 – 100 µl) (78, 137), which is sometimes dotted (133) and sometimes streaked (55) onto the agar surface. Despite these variations, the MICs

of EOs determined by agar dilution generally appear to be in approximately the same order of magnitude (55, 133, 137). In broth dilution studies a number of different techniques exist for determining the end-point – the most used methods are that of optical density (OD) (turbidity) measurement and the enumeration of colonies by viable count. The former method has the advantage of being automated; the latter is labour intensive. The measurements of conductance/conductivity and end-point determination by visual monitoring have been less often used. A new microdilution method for determining the MIC of oil-based compounds uses the redox indicator resazurin as a visual indicator of the MIC. The results compare favourably with those obtained by viable count and OD measurement and the method is more sensitive than the agar dilution method (106). A patented colour indicator based on resazurin has been used to determine the MICs for methanolic extracts of plant materials (147) and EOs (19) and the method can be automated by measuring the end point by fluorescence instead of visual means (3). Triphenyl tetrazolium chloride has been used for visual end point determination in the study of tea tree oil in broth but, although it is an indicator of bacterial growth, the colour change did not fully correlate with the MIC (21).

The rapidity of a bactericidal effect or the duration of a bacteriostatic effect can be determined by time-kill analysis (survival curve plot) whereby the number of viable cells remaining in broth after the addition of EO is plotted against time. The most frequently used methods for this are measurement of OD and viable count after plating out onto agar. Damage to the bacterial cell wall and loss of cell contents can be studied by scanning electron microscopy (SEM) (19, 94, 159). Careful preparation of the samples for SEM is necessary to ensure that the observed difference between control and treated cells are due to the effect of the EO and not to the preparation method.

Several studies have used the measurement of OD or conductance to perform further calculations rather than directly stating the MIC. The OD of the test suspension and control may be used to calculate an inhibition index (27). Measurements of conductance can be used to calculate the period elapsing before growth can be detected, the detection time (DT), after treatment of cells with EO (108, 109, 170). Comparison of the maximum specific growth rate (μ_{max}) of bacteria based on data from viable counts or absorbance measurements has also been done in a number of studies (113, 160, 177). A new method of calculating the MIC from OD measurements has been found suitable for testing combinations of antibacterial substances (93, 94). In one study the percentage of EO resulting in a 50% decrease in the viable count was determined from plots of percentage kill against concentration (57). The diversity of ways of reporting the antibacterial activity of EOs limits comparison between studies and could lead to duplication of work.

One feature of test methods that varies considerably is whether or not an emulsifier or solvent is used to dissolve the EO or to stabilise it in water-based culture media. Several substances have been used for this purpose: ethanol (5, 13, 45, 78, 79, 95, 108, 109, 115, 124, 125, 127, 136, 157, 158), methanol (121), Tween-20 (63, 84, 106), Tween-80 (9, 25, 35, 78, 116, 128, 194), acetone in combination with Tween-80 (137), polyethylene glycol (133), propylene glycol (119), *n*-hexane (151), dimethyl sulfoxide (56) and agar (19, 47, 59, 106). However, a number of researchers found it unnecessary to use an additive (20, 29, 35, 51, 52, 94, 113, 142, 164, 170, 187). One study employed vigorous shaking in phosphate saline buffer to suspend EOs (57). The performance of the most frequently used solvents, ethanol and Tween-80, has been compared with that of agar for the stabilisation of oregano and clove oils. The use of agar (0.2%) was found to produce as homogenous a dispersion as a true solution in absolute ethanol (141). Furthermore, the MICs of oregano and clove EOs were significantly lower in the presence of agar than in the presence of Tween-80 or ethanol. It was concluded that solvents and detergents could decrease the antibacterial effect of EOs (140, 141). This is supported by the fact that Tween-80 has been recommended as a neutraliser of phenolic disinfectants (37) and this has been confirmed in work on the action of thyme oil against *Salmonella typhimurium* (78). Tween-80 has also been shown to protect *L. monocytogenes* from the antibacterial activity of an EO component during freeze-thaw cycles (38). A further disadvantage of the use of Tween-80 to dissolve EOs is the fact that the turbidity of the resulting dispersion can hamper visual observations and OD measurements (22).

A selection of MICs for EOs and EO components tested in vitro against foodborne pathogens is presented in Tables 4 and 5. Considering the diversity of test methods, bacterial isolates (clinical or reference) and origins of the EOs used, the range of MICs appears considerably narrow in most cases.

Table 4. Selected[a] MICs of essential oils tested in vitro against foodborne pathogens.

Plant from which EO is derived	Species of bacteria	MIC, approximate range (μl ml^{-1})[b]	Reference(s)
Rosemary	*Escherichia coli*	4.5->10	(55, 63, 133, 164)
	Salmonella typhimurium	>20	(63)
	Bacillus cereus	0.2	(27)
	Staphylococcus aureus	0.4-10	(55, 63, 133, 164)
	Listeria monocytogenes	0.2	(164)
Oregano	*E. coli*	0.5-1.2	(19, 63, 137)
	S. typhimurium	1.2	(63)
	Staph. aureus	0.5-1.2	(63, 137)
Lemongrass	*E. coli*	0.6	(63)
	S. typhimurium	2.5	(63)
	Staph. aureus	0.6	(63)
Sage	*E. coli*	3.5-5	(55, 63, 164)
	S. typhimurium	10-20	(63, 153)
	Staph. aureus	0.75-10	(55, 63, 153, 164)
	L. monocytogenes	0.2	(164)
Clove	*E. coli*	0.4-2.5	(55, 63, 164)
	S. typhimurium	>20	(63)
	Staph. aureus	0.4-2.5	(55, 63, 164)
	L. monocytogenes	0.3	(164)
Thyme	*E. coli*	0.45-1.25	(19, 35, 55, 63, 164)
	S. typhimurium	0.450->20	(35, 63)
	Staph. aureus	0.2-2.5	(35, 55, 63, 164)
	L. monocytogenes	0.156-0.45	(35, 56, 164)
Turmeric	*E. coli*	>0.2	(119)
	B. cereus	0.2	(119)
Tea bush (*Lippia* spp.)	*E. coli*	2.5->80	(9)
	Shigella dysenteria	5->80	(9)
	Staph. aureus	0.6-40	(9)
	B. cereus	5-10	(9)

[a] EOs derived from plants used as herbs, spices or infusions in cooking were selected and MICs for a selection of important foodborne pathogens cited.
[b] In the references MICs have been reported in the units ppm, mg ml^{-1}, % (v/v), μl l^{-1} and μg ml^{-1}. For ease of comparison these have been converted to μl ml^{-1}, whereby it was assumed that EOs have the same density as water. In some references the MIC was termed minimum bactericidal or bacteriostatic concentration (19, 35, 164).

Table 5. Selected[a] MICs of essential oil components tested in vitro against foodborne pathogens.

Essential oil component	Species of bacteria	MIC, approximate range (µl ml^{-1}) [b]	References
α-terpineol	*Escherichia coli*	0.450->0.9	(35)
	Salmonella typhimurium	0.225	(35)
	Staphylococcus aureus	0.9	(35)
	Listeria monocytogenes	>0.9	(35)
	Bacillus cereus	0.9	(35)
Carvacrol	*E. coli*	0.225-5	(35, 83)
	S. typhimurium	0.225-0.25	(35, 83)
	Staph. aureus	0.175-0.450	(35, 94)
	L. monocytogenes	0.375-5	(35, 83, 136)
	B. cereus	0.1875-0.9	(35, 136)
Citral	*E. coli*	0.5	(83, 121)
	S. typhimurium	0.5	(83)
	Staph. aureus	0.5	(121)
	L. monocytogenes	0.5	(83)
Eugenol	*E. coli*	1.0	(83)
	S. typhimurium	0.5	(83)
	L. monocytogenes	>1.0	(83)
Geraniol	*E. coli*	0.5	(83)
	S. typhimurium	0.5	(83)
	L. monocytogenes	1.0	(83)
Perillaldehyde	*E. coli*	0.5	(83)
	S. typhimurium	0.5	(83)
	L. monocytogenes	1.0	(83)
Thymol	*E. coli*	0.225-0.45	(35)
	S. typhimurium	0.056	(35)
	Staph. aureus	0.140-0.225	(35, 94)
	L. monocytogenes	0.450	(35)
	B. cereus	0.450	(35)

[a] EOs components present in plants used in cooking were selected and MICs for a selection of important foodborne pathogens cited.
[b] In the references MICs have been reported in the units mg ml^{-1}, % (v/v), µl l^{-1}, µg ml^{-1} and mmol l^{-1}. For ease of comparison these have been converted to µl ml^{-1}, whereby it was assumed that EOs have the same density as water. In one reference the MIC was termed minimum bactericidal concentration (35).

4. TESTS OF ANTIBACTERIAL ACTIVITY OF EOS IN FOOD SYSTEMS

Although, as mentioned previously, a small number of food preservatives containing EOs is commercially available, until the early 1990s very few studies of the activity of EOs in foods had been published (16). Since then a fair number of trials have been carried out with EOs in foods. An overview of the literature reporting studies on the antibacterial effect of EOs or their components in foods is presented in Table 6. Reports of studies using diluted foods or food slurries (135, 165) and studies using dried herbs or spices or their extracts (64, 65, 169) have not been included in the table.

However well EOs perform in antibacterial assays in vitro, it has generally been found that a greater concentration of EO is needed to achieve the same effect in foods (152, 163). The ratio has been recorded to be approximately twofold in semi-skimmed milk (81), 10-fold in pork liver sausage (127), 50-fold in soup (181) and 25 to 100-fold in soft cheese (114). An exception to this phenomenon is *Aeromonas hydrophila*; no greater proportion of EO was needed to inhibit this species on cooked pork and on lettuce in comparison to tests in vitro (167, 187). Several studies have recorded the effect of foodstuffs on microbial resistance to EOs but none appears to have quantified it or to have explained the mechanism, although suggestions have been made as to the possible causes. The greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster (59). Not only are the intrinsic properties of the food (fat/protein/water content, antioxidants, preservatives, pH, salt and other additives) relevant in this respect - the extrinsic determinants (temperature, packaging in vacuum/gas/air, characteristics of microorganisms) can also influence bacterial sensitivity (152, 168). Generally, the susceptibility of bacteria to the antimicrobial effect of EOs also appears to increase with a decrease in the pH of the food, the storage temperature and the amount of oxygen within the packaging (64). At low pH the hydrophobicity of an EO increases, enabling it to more easily dissolve in the lipids of the cell membrane of target bacteria (78).

It is generally supposed that the high levels of fat and/or protein in foodstuffs protect the bacteria from the action of the EO in some way (5, 127, 168). For example, if the EO dissolves in the lipid phase of the food there will be relatively less available to act on bacteria present in the aqueous phase (113). Another suggestion is that the lower water content of food compared to laboratory media may hamper the progress of antibacterial agents to the target site in the bacterial cell (165).

Table 6. Overview of studies testing the antibacterial activity of essential oils or their components in foods.

Food group	Food	EO or component	Concentration applied	Concentration applied (µl g^{-1} or µl ml^{-1})[a]	Bacterial species	Notes on experimental set-up[b]	Observations		References
							Extension of lag phase of growth	Reduction in final population[c]	
Meat	Minced mutton	Clove oil	0.5 – 1%	5-10	*L. monocytogenes*	Two storage temperatures	Yes	+	(186)
	Roast beef sirloin, sliced	Eugenol	0.1 ml spread over surface of 25 g slice		*L. monocytogenes*	Two levels of inoculum and two storage temperatures	Yes	++	(65)
					A. hydrophila	Two levels of inoculum and two storage temperatures	Yes	++	(65)
	Cooked chicken breast, pieces	Eugenol	0.1 ml spread over surface of 25 g piece of chicken		*L. monocytogenes*	Two levels of inoculum and two storage temperatures	Yes	++	(64)
					A. hydrophila	Two levels of inoculum and two storage temperatures	Yes	+++	(64)
	Pork liver sausage	Rosemary oil, encapsulated rosemary oil	1% and 5% respectively	10 and 50 respectively	*L. monocytogenes*		Yes	Rosemary oil: + Encapsulated oil: +++	(127)
	Chicken noodles, beef	Sage oil	200-500 ppm	0.2-0.5	*B. cereus, Staph. aureus, S. typhimurium*		No	Chicken noodles: 0 Beef: 0	(153)

	Minced beef	Oregano oil	0.05-1%	0.5-10	Natural flora	Three packaging regimes tested: air, carbon dioxide and modified atmosphere (40% CO_2, 30% N_2, 30% O_2)	n.d.[d]	+	(162)
	Cooked pork	Coriander oil	1250 µg/cm^2		*A. hydrophila*	Two storage temperatures and two inoculation levels tested.	Yes	++ to +++	(167)
		Clove oil	500 µg/cm^2		*A. hydrophila*	Two storage temperatures and two inoculation levels tested	Yes	++ to +++	(167)
	Beef fillets	Oregano oil	0.8% v/w	8	*L. monocytogenes* amongst natural flora	Modified atmosphere packaging (40% CO_2, 30% N_2, 30% O_2) and packaging in air	In MAP but not in air	MAP: +++ Air: +	(173)
	Pâté	Mint oil	0.5-2.0% v/w	5-20	*L. monocytogenes*	Two storage temperatures	No	0	(168)
					S. enteritidis	Two storage temperatures	No	0	(168)
	Minced pork	Thyme oil	0.02 ml mixed with 25g meat	0.8	*L. monocytogenes*		Yes	+	(5)
	Vacuum packed ham	Cilantro oil	0.1-6% v/v in coating	1-60	*L. monocytogenes*	Coating: gelatin gel or canola oil	No	0	(59)

	Vacuum packed minced pork product	Oregano oil	100-200 ppm	0.1-0.2	*C. botulinum* spores	Two levels of inoculation	No	0	(71)
	Cooked chicken sausage	Mustard oil	0.1% w/w	1	*E. coli*		Yes	0	(98)
Fish	Cooked shrimps	Thyme oil, Cinnamaldehyde	0.75-1.5% and 0.15-0.3% in coating respectively	7.5-15 and 1.5-3 in coating respectively	*Pseudomonas putida*		Very slight	++	(123)
	Red grouper fillet, cubed	Carvacrol, citral, geraniol	0.5-3.0% w/v in dipping solution	5-30 in dipping solution	*S. typhimurium*		Carvacrol at 30 µl ml^{-1} killed all cells	Carvacrol: ++ Citral and geraniol: +	(84)
	Asian sea bass, whole	Thyme oil or oregano oil	0.05% v/v (*sic*) sealed in packaging with whole fish	0.5	Natural flora		Both on surface and in flesh	Surface: ++ Flesh: +	(66)
	Cod fillets	Oregano oil	0.05% v/w	0.5	*Photobacterium phosphoreum*		Yes	+	(113)
	Salmon fillets	Oregano oil	0.05% v/w	0.5	*Photobacterium phosphoreum*		No	0	(113)
	Taramasalad (cod's roe salad)	Oregano oil	0.5-2.0% v/w	5-20	*S. enteritidis*	Various pHs and storage temperatures tested	Yes	+++	(92)
	Taramosalata (fish roe salad)	Mint oil	0.5-2.0% v/w	5-20	*S. enteritidis*	Two storage temperatures	No	0	(168)

					L. monocytogenes	Two storage temperatures	No	0	(168)
Dairy	Mozzarella cheese	Clove oil	0.5-1%	5-10	*L. monocytogenes*		Yes	+	(186)
	Semi skimmed milk	Carvacrol	2-3 mmol l^{-1}	0.3-0.45	*L. monocytogenes*	Two temperatures	n.d.	+	(81)
	Soft cheese	'DMC Base Natural' preservative comprising 50% EOs of rosemary, sage and citrus	250-2500 ppm	0.25-2.5	*L. monocytogenes pool* (10 strains)		From 1.0 µl g^{-1}	+	(114)
					E. coli O157:H7		No	0	(114)
	Yoghurt	Clove, cinnamon, cardamom, peppermint oils	0.005-0.5% in milk before fermentation	0.05-5	*Streptococcus thermophilus*		n.d.	Mint oil: + Cardamom, clove: ++ Cinnamon: +++	(12)
					Lactobacillus bulgaricus		n.d.	Mint oil: 0 Cardamom, clove: + Cinnamon: +++	(12)
	Tzatziki (yogurt and cucumber salad)	Mint oil	0.5-2.0% v/w	5-20	*S. enteritidis*	Two storage temperatures	From 1.5% v/w	++	(168)
					L. monocytogenes	Two storage temperatures	Increase in growth	_ _	(168)

Vegetables	Lettuce, Romaine	Thyme oil	0.1–10 ml l^{-1} in rinsing solution	0.1-10	*E. coli* O157:H7	EO added to washing water	n.d.	+	(156)
	Carrots	Thyme oil	0.1–10 ml l^{-1} in rinsing solution	0.1-10	*E. coli* O157:H7	EO added to washing water	n.d.	+ to ++	(156)
	Lettuce, Iceberg green	Basil methyl chavicol (BMC)	0.1–1.0 % v/v	1-10	Natural flora	BMC added to washing water	n.d.	++	(187)
	Eggplant salad	Oregano oil	0.7 – 2.1 % v/w	7-21	*E. coli* O157:H7	Four storage temperatures and three different pHs	Yes	++	(161)
	Alfalfa seeds	Cinnamaldehyde, thymol	200, 600 mg l^{-1} air		*Salmonella* spp., 6 serotypes	Fumigation at 50 or 70°C	n.d.	50°C: + 70°C: ++	(188)
Rice	Boiled rice	Carvacrol	0.15-0.75 mg g^{-1}	0.15-0.75	*B. cereus*		Yes – dose dependent effect	++	(180)
	Boiled rice	Sage oil	200-500 ppm	0.2-0.5	*B. cereus* *Staph. aureus* *S. typhimurium*		No	0	(153)
Fruit	Kiwifruit	Carvacrol	1 mM in dipping solution	0.15 µl ml^{-1} in dipping solution	Natural flora		n.d.	+++	(144)
		Cinnamic acid	1 mM in dipping solution	0.15 µl ml^{-1} in dipping solution	Natural flora	Two storage temperatures tested	n.d.	+++	(144)

	Honeydew melon	Carvacrol	1 mM in dipping solution	0.15 µl ml^{-1} in dipping solution	Natural flora		Yes	0	(144)
		Cinnamic acid	1 mM in dipping solution	0.15 µl ml^{-1} in dipping solution	Natural flora	Two storage temperatures tested	Yes	+	(144)

In papers where the combined effect of an EO or an EO component has been studied in combination with another preservation method, only the results for EO tested alone are cited.

[a] For ease of comparison, the concentration of EO or EO component applied has been converted into µl g^{-1} or µl ml^{-1} food, whereby it was assumed that EOs have the same density as water. Where no conversion could be calculated due to the experimental method this column is left blank.

[b] Products were mostly stored under refrigeration, but temperatures used range from 2 to 30°C.

[c] The following classification has been used:

+++ Large reduction compared to control (>3 log cfu g^{-1} or ml^{-1} fewer)

++ Medium reduction compared to control (1.5-3.0 log cfu g^{-1} or ml^{-1} fewer)

+ Slight reduction compared to control (up to 1.5 log cfu g^{-1} or ml^{-1} fewer)

0 Negligible effect compared to control

- Slight stimulation of growth compared to control (up to 1.5 log cfu g^{-1} or ml^{-1} more)

- - Medium stimulation of growth (>1.5 log cfu g^{-1} or ml^{-1} more).

These classifications apply to the end point of the experiment, which varies between references from 15 minutes to 33 days.

[d] n.d. = not done.

Mint oil in the high fat products pâté and fish roe salad (taramosalata) exhibited little antibacterial effect against *L. monocytogenes* and *S. enteritidis*, whereas in cucumber and yoghurt salad (tzatziki) (low fat) the same EO was much more effective (168). Although the improved effectiveness in cucumber and yoghurt salad may be partly attributed to the low pH (4.3 as opposed to pH 6.8 in pâté), fish roe salad also has a low pH (4.9). This would seem to indicate that fat percentage might exert a greater influence on the antibacterial effect of EOs than the pH.

A reaction between carvacrol, a phenolic component of various EOs, and proteins has been put forward as a limiting factor in the antibacterial activity against *Bacillus cereus* in milk (135). Protein content has also been put forward as a factor inhibiting the action of clove oil on *Salmonella enteritidis* in diluted low-fat cheese (165). Carbohydrates in foods do not appear to protect bacteria from the action of EOs as much as fat and protein do (153). A high water and/or salt level facilitates the action of EOs (153, 161, 168, 189).

The physical structure of a food may limit the antibacterial activity of EO. A study of the relative performance of oregano oil against *S. typhimurium* in broth and in gelatin gel revealed that the gel matrix dramatically reduced the inhibitory effect of the oil. This was presumed to be due to the limitation of diffusion by the structure of the gel matrix (160). MICs for a particular EO on a particular bacterial isolate have been shown to be generally slightly lower in broth than in agar (63). Research into the growth characteristics of *Listeria monocytogenes* and *Yersinia enterocolitica* in oil-in-water emulsions has shown that, depending on the mean droplet size of the emulsion, the bacteria can grow in films, in colonies or as planktonic cells (18). It is known that colonial growth restricts diffusion of oxygen (195) and cells situated within a colony may be shielded to a certain extent by the outer cells from substrates in the emulsion. If the oil droplets in a food emulsion are of the appropriate size, it could be possible for bacteria growing within colonies to be protected from the action of EOs in this way.

4.1. Meat and meat products

With reference to Table 6, certain oils stand out as better antibacterials than others for meat applications. Eugenol and coriander, clove, oregano and thyme oils were found to be effective at levels of 5-20 μl g^{-1} in inhibiting *L. monocytogenes, Aeromonas hydrophila* and autochthonous spoilage flora in meat products, sometimes causing a marked initial reduction in the number of recoverable cells (5, 64, 65, 162, 167, 173) whilst mustard, cilantro, mint and sage oils were less effective or ineffective (59, 98, 153, 168). A high fat content appears to markedly reduce the action of EOs in meat products. For example, mint and cilantro EOs were not effective in products with a high level of fat, such as pâté (which generally contains 30-45% fat) and a coating for ham containing canola oil (59,

168). Immobilising cilantro EO in a gelatin gel, however, improved the antibacterial activity against *L. monocytogenes* in ham (59).

One study found that encapsulated rosemary oil was much more effective than standard rosemary EO against *L. monocytogenes* in pork liver sausage, although whether the effect was due to the encapsulation or the greater percentage level used was not further elucidated (127).

The activity of oregano EO against *Clostridium botulinum* spores has been studied in a vacuum packed and pasteurised minced (ground) pork product. Concentrations of up to 0.4 µl g^{-1} oregano EO were found not to significantly influence the number of spores or to delay growth. However, in the presence of low levels of sodium nitrite which delayed growth of bacteria and swelling of cans when applied alone, the same concentration of oregano EO enhanced the delay. The delay of growth was dependent on the number of inoculated spores; at 300 spores g^{-1} the reduction was greater than at 3000 spores g^{-1} (71).

4.2. Fish dishes

In fish, just as in meat products, a high fat content appears to reduce the effectiveness of antibacterial EOs. For example, oregano oil at 0.5 :l g^{-1} is more effective against the spoilage organism *Photobacterium phosphoreum* on cod fillets than on salmon, which is a fatty fish (113).

Oregano oil is more effective in/on fish than mint oil, even in fatty fish dishes; this was confirmed in two experiments with fish roe salad using the two EOs at the same concentration (5-20 µl g^{-1}) (92, 168). The spreading of EO on the surface of whole fish or using EO in a coating for shrimps appears effective in inhibiting the respective natural spoilage flora (66, 123).

4.3. Dairy products

Mint oil at 5-20 µl g^{-1} is effective against *S. enteritidis* in low fat yoghurt and cucumber salad (168). Mint oil inhibits the growth of yoghurt starter culture species at 0.05-5 µl g^{-1} but cinnamon, cardamom and clove oils are much more effective (12).

4.4. Vegetables

It appears that, in vegetable dishes just as for meat products, the antimicrobial activity of EOs is benefited by a decrease in storage temperature and/or a decrease in the pH of the food (161). Vegetables generally have a low fat content, which may contribute to the successful results obtained with EOs.

All EOs and their components that have been tested on vegetables appear effective against the natural spoilage flora and foodborne pathogens at levels of 0.1-10 µl g^{-1} in washing water (156, 187). Cinnamaldehyde and thymol are

effective against six *Salmonella* serotypes on alfalfa seeds when applied in hot air at 50°C as fumigation. Increasing the temperature to 70°C reduced the effectiveness of the treatment (188). This may be due to the volatility of the antibacterial compounds.

Oregano oil at 7-21 μl g^{-1} was effective at inhibiting *E. coli* O157:H7 and reducing final populations in eggplant salad compared to the untreated control. Although the salad recipe appears to have a high fat content, the percentage of fat was not stated (161).

4.5. Rice

Sage oil at 0.2-0.5 μl g^{-1} when used against *B. cereus* in rice was ineffective, whereas carvacrol at 0.15-0.75 μl g^{-1} was very effective at extending the lag phase and reducing the final population compared to a control (153, 180).

4.6. Fruit

Carvacrol and cinnamaldehyde were very effective at reducing the viable count of the natural flora on kiwifruit when used at 0.15 μl ml^{-1} in dipping solution, but less effective on honeydew melon. It is possible that this difference has to do with the difference in pH between the fruits; the pH of kiwifruit was 3.2-3.6 and of the melon 5.4-5.5 (144). As mentioned before, the lower the pH, the more effective EOs and their components generally are.

4.7. Food models

EOs of clove, cinnamon, bay and thyme were tested against *L. monocytogenes* and *S. enteritidis* in soft cheese diluted 1:10 in buffer. The former species was less easily inhibited in diluted full-fat cheese than in the low-fat version, indicating the protective action of fat. The level of fat in the cheese protected the bacterial cells to a different extent depending on which oil was used; clove oil was in fact more effective against *S. enteritidis* in full-fat than in low-fat cheese slurry (165).

In view of the published data on EOs in foods, the following approximate general ranking (in order of decreasing antibacterial activity) can be made: oregano/clove/coriander/cinnamon > thyme > mint > rosemary > mustard > cilantro/sage. An approximate general ranking of the EO components is as follows (in order of decreasing antibacterial activity): eugenol > carvacrol/cinnamic acid > basil methyl chavicol > cinnamaldehyde > citral/geraniol.

5. MODE OF ANTIBACTERIAL ACTION

Although the antimicrobial properties of essential oils and their components have been reviewed in the past (87, 88, 120, 152), the mechanism of action has not been studied in great detail (94). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (23, 159). The locations or mechanisms in the bacterial cell thought to be sites of action for EO components are indicated in Figure 2. Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted.

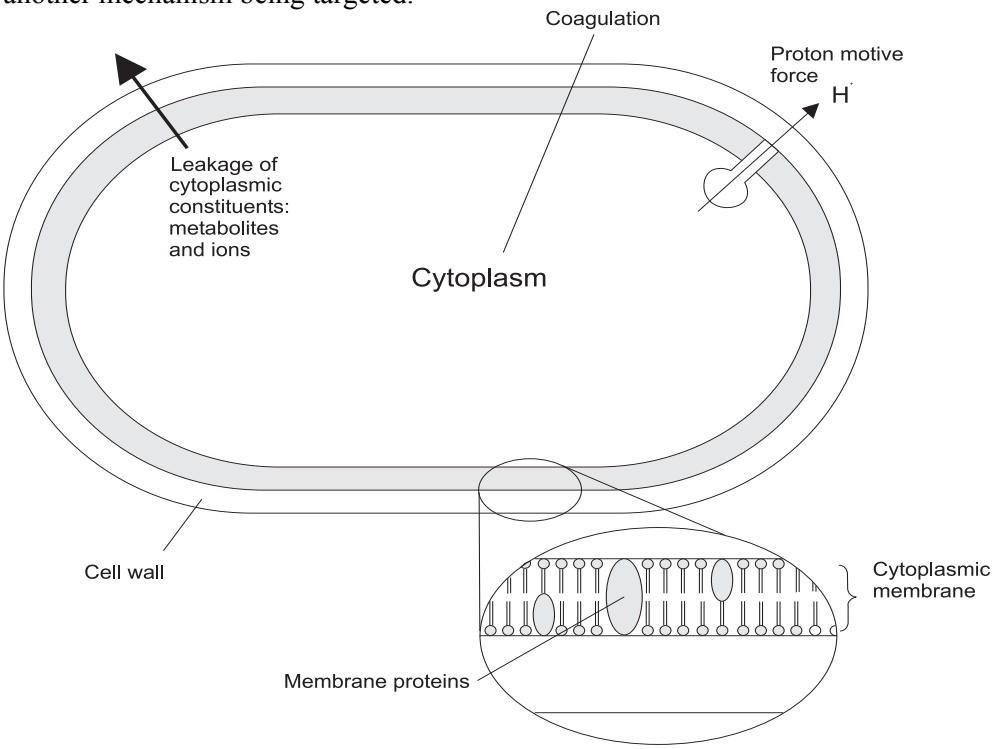

Figure 2. Locations and mechanisms in the bacterial cell thought to be sites of action for EO components: degradation of the cell wall (68, 171); damage to cytoplasmic membrane (85, 122, 154, 176, 178); damage to membrane proteins (78, 179); leakage of cell contents (36, 62, 68, 94, 122); coagulation of cytoplasm (62) and depletion of the proton motive force (179, 181).

An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable (86, 154). Leakage of ions and other cell contents can then occur (23, 36, 62, 68, 94, 122, 159, 176). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (49). There is some evidence from studies with tea tree oil and *E. coli* that cell death may occur before lysis (62).

Generally, the EOs possessing the strongest antibacterial properties against foodborne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl)phenol) and thymol (35, 51, 55, 77, 94, 171). It seems reasonable that their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (43, 50, 155).

The chemical structure of the individual EO components affects their precise mode of action and antibacterial activity (51). The importance of the presence of the hydroxyl group in phenolic compounds such as carvacrol and thymol has been confirmed (51, 86, 176). The relative position of the hydroxyl group on the phenolic ring does not appear strongly to influence the degree of antibacterial activity; the action of thymol against *B. cereus*, *Staph. aureus* and *Pseudomonas aeruginosa* appears to be comparable to that of carvacrol, for example (94, 176). However, in one study carvacrol and thymol were found to act differently against Gram-positive and Gram-negative species (51). The significance of the phenolic ring itself (destabilised electrons) is demonstrated by the lack of activity of menthol compared to carvacrol (176). In one study the addition of an acetate moiety to the molecule appeared to increase the antibacterial activity; geranyl acetate was more active against a range of Gram-positive and negative species than geraniol (51). As far as non-phenolic components of EOs are concerned, the type of alkyl group has been found to influence activity (alkenyl > alkyl). For example, limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) is more active than *p*-cymene (51).

Components of EO also appear to act on cell proteins embedded in the cytoplasmic membrane (85). Enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on these. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid-protein interaction; alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (78, 155) . Some EOs have been found to stimulate the growth of pseudomycelia (a series of cells

adhering end-to-end as a result of incomplete separation of newly formed cells) in certain yeasts. This could be an indication that EOs act on the enzymes involved in the energy regulation or synthesis of structural components (34). Cinnamon oil and its components have been shown to inhibit amino acid decarboxylases in *Enterobacter aerogenes*. The mechanism of action was thought to be the binding of proteins (190). Indications that EO components may act on proteins were also obtained from studies using milk containing different protein levels (135).

5.1. Carvacrol and thymol

The mode of action of carvacrol, one of the major components of oregano and thyme oils, appears to have received the most attention from researchers. Thymol is structurally very similar to carvacrol, having the hydroxyl group at a different location on the phenolic ring. Both substances appear to make the cell membrane permeable (94).

Carvacrol and thymol are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP. The presence of magnesium chloride has been shown to have no influence on this action, suggesting a mechanism other than chelation of cations in the outer membrane (68).

Studies with *B. cereus* have shown that carvacrol interacts with the cell membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains (178). This distortion of the physical structure would cause expansion and destabilisation of the membrane, increasing membrane fluidity, which in turn would increase passive permeability (176). Measurement of the average phase transition temperature of the bacterial lipids confirmed that membranes instantaneously became more fluid in the presence of carvacrol (178). The passage of *B. cereus* cell metabolites across the cell membrane on exposure to carvacrol has also been investigated. Intracellular and extracellular ATP measurements revealed that the level of ATP within the cell decreased whilst there was no proportional increase outside the cell. It is therefore presumed that the rate of ATP synthesis was reduced or that the rate of ATP hydrolysis was increased. Measurements of the membrane potential ($\Delta\psi$) of exponentially growing cells revealed a sharp decrease on the addition of carvacrol and indicated a weakening of the proton motive force. The pH gradient across the cell membrane was weakened by the presence of carvacrol and was completely dissipated in the presence of 1 mM or more. Furthermore, intracellular levels of potassium ions dropped whilst extracellular amounts increased proportionately, the total amount remaining constant (179). It was concluded that carvacrol forms channels through the membrane by pushing apart the fatty acid chains of the phospholipids, allowing ions to leave the cytoplasm (175). Oregano EO, which contains carvacrol as a

major component, causes leakage of phosphate ions from *Staph. aureus* and *P. aeruginosa* (94).

Aside from the inhibition of the growth of vegetative bacterial cells, the inhibition of toxin production is also of interest to food microbiologists. Carvacrol is able to inhibit the production of diarrhoeal toxin by *B. cereus* in broth and in soup. Two theories are offered for the mode of action of toxin limitation: If toxin excretion is an active process, there may be insufficient ATP or PMF to export it from the cell. Alternatively, the lower specific growth rate may mean that the cells use all the available energy to sustain viability, leaving little over for toxin production (181).

Juven *et al.* examined the working of thymol against *Salmonella typhimurium* and *Staph. aureus* and hypothesised that thymol binds to membrane proteins hydrophobically and by means of hydrogen bonding, thereby changing the permeability characteristics of the membrane. Thymol was found to be more inhibitive at pH 5.5 than 6.5. At low pH the thymol molecule would be undissociated and therefore more hydrophobic, and so may bind better to the hydrophobic areas of proteins and dissolve better in the lipid phase (78).

5.2. Eugenol

Eugenol is a major component (approximately 85%) of clove oil (55). Sub-lethal concentrations of eugenol have been found to inhibit production of amylase and proteases by *B. cereus*. Cell wall deterioration and a high degree of cell lysis were also noted (171). The hydroxyl group on eugenol is thought to bind to proteins, preventing enzyme action in *E. aerogenes* (190).

5.3. *p*-Cymene

The biological precursor of carvacrol, *p*-cymene is hydrophobic and causes swelling of the cytoplasmic membrane to a greater extent than does carvacrol (176). *p*-Cymene is not an effective antibacterial when used alone (51, 77, 78, 180), but when combined with carvacrol, synergism has been observed against *B. cereus* in vitro and in rice (180). The greater efficiency of *p*-cymene at being incorporated in the lipid bilayer of *B. cereus* very likely facilitates transport of carvacrol across the cytoplasmic membrane (176).

5.4. Carvone

When tested in a liposome model system at concentrations above the MIC, carvone (2-methyl-5-(1-methylethenyl)-2-cyclohexen-1-one) dissipated the pH gradient and membrane potential of cells. The specific growth rate of *E. coli, Streptococcus thermophilus* and *L. lactis* decreased with increasing concentrations of carvone, which suggests that it acts by disturbing the metabolic energy status of

cells (122). In contrast, another study found that carvone was ineffective on the outer membrane of *E. coli* and *S. typhimurium* and did not affect the intracellular ATP pool (68).

5.5. Cinnamaldehyde

Although cinnamaldehyde (3-phenyl-2-propenal) is known to be inhibitive to growth of *E. coli* O157:H7 and *S. typhimurium* at similar concentrations to carvacrol and thymol, it did not disintegrate the outer membrane or deplete the intracellular ATP pool (68). The carbonyl group is thought to bind to proteins, preventing the action of amino acid decarboxylases in *E. aerogenes* (190).

5.6. Terpinene

γ-Terpinene did not antagonize growth of *S. typhimurium* (78), whereas α-terpinene inhibited 11 of the 25 bacterial species screened (51).

6. SUSCEPTIBILITY OF GRAM-NEGATIVE AND GRAM-POSITIVE ORGANISMS

Most studies investigating the action of whole EOs against food spoilage organisms and foodborne pathogens agree that, generally, EOs are slightly more active against Gram-positive than Gram-negative bacteria (20, 29, 47, 48, 55, 66, 77, 94, 108, 109, 114, 119, 124, 133, 145, 151-153, 164). That Gram-negative organisms are less susceptible to the action of antibacterials is perhaps to be expected, since they possess an outer membrane surrounding the cell wall (138) which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (182). However, not all studies on EOs have concluded that Gram-positives are more susceptible (194). *A. hydrophila* (Gram-negative) appears in fact to be one of the most sensitive species (45, 64, 65, 167, 187). In one study mint (*Mentha piperita*) EO achieved a greater reduction in the viable count of *S. enteritidis* than for *L. monocytogenes* when added to the Greek appetisers taramosalata and tzatziki (168). In another study no obvious difference between Gram-positives and Gram-negatives was measured in the susceptibility after 24 h, but the inhibitory effect was more often extended to 48 h with Gram-negative than with Gram-positive organisms (124). A study testing 50 commercially available EOs against 25 genera found no evidence for a difference in sensitivity between Gram-negative and Gram-positive organisms (45). However, a later study using the same test method and the same bacterial isolates but apparently using freshly distilled EOs, revealed that Gram-positive bacteria were indeed more susceptible to two of the EOs tested and equally sensitive to four other EOs than were Gram-

negative species (51). It was postulated that individual components of EOs exhibit different degrees of activity against Gram-positives and Gram-negatives (51) and it is known that the chemical composition of EOs from a particular plant species can vary according to the geographical origin and harvesting period *(vide supra)*. It is therefore possible that variation in composition between batches of EOs is sufficient to cause variability in the degree of susceptibility of Gram-negative and Gram-positive bacteria.

Of the Gram-negative bacteria, Pseudomonads, and in particular *P. aeruginosa*, appear to be least sensitive to the action of EOs (35, 45, 51, 86, 101, 128, 133, 145, 151, 173, 194).

7. SYNERGISM AND ANTAGONISM BETWEEN COMPONENTS OF EOS

The inherent activity of an oil can be expected to relate to the chemical configuration of the components, the proportions in which they are present and to interactions between them (47, 51, 109). An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects (44). Some studies have concluded that whole EOs have a greater antibacterial activity than the major components mixed (59, 116), which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence.

The two structurally similar major components of oregano EO, carvacrol and thymol, were found to give an additive effect when tested against *Staph. aureus* and *P. aeruginosa* (94).

As discussed above, synergism between carvacrol and its biological precursor *p*-cymene has been noted when acting on *B. cereus* vegetative cells. It appears that *p*-cymene, a very weak antibacterial, swells bacterial cell membranes to a greater extent than carvacrol does. By this mechanism *p*-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are used together (180).

Fractions of cilantro, coriander, dill and eucalyptus EOs (each containing several components), when mixed in various combinations, resulted in additive, synergistic or antagonistic effects (47). A mixture of cinnamaldehyde and eugenol at 250 and 500 μg ml^{-1} respectively inhibited growth of *Staphylococcus* sp., *Micrococcus* sp. *Bacillus* sp. and *Enterobacter* sp. for more than 30 days

completely whereas the substrates applied individually did not inhibit growth (115).

8. SYNERGISM AND ANTAGONISM BETWEEN EO COMPONENTS AND FOOD PRESERVATIVES OR PRESERVATION METHODS

A number of potential synergists have been suggested for use with EOs: low pH, low water activity, chelators, low oxygen tension, mild heat and raised pressure, although not all of these have been researched in foodstuffs (60). This section will summarise studies on the combined effect of EOs or their components with the food additives sodium chloride, sodium nitrite and nisin and with preservation techniques of mild heat treatment, high hydrostatic pressure and anaerobic packaging.

Sodium chloride has been shown to work as a synergist and an antagonist under different circumstances with EOs and/or their components. Synergism between NaCl and mint oil against *S. enteritidis* and *L. monocytogenes* has been recorded in taramosalata (168). The combined use of 2-3% NaCl and 0.5% clove powder (containing eugenol and eugenyl acetate) in mackerel muscle extract has been found to totally prevent growth and histamine production by *E. aerogenes*. The suggested mechanism for this is that eugenol increases the permeability of the cells after which NaCl inhibits growth by its action on intracellular enzymes (189). Antagonistic effects of salt were found with carvacrol and *p*-cymene against *B. cereus* in rice: carvacrol and *p*-cymene worked synergistically, but this effect was reduced when salt was added (1.25 g l^{-1} rice) (180). In the same study, soy sauce was shown to exhibit synergy with carvacrol. However this synergy was also cancelled out by the presence of salt (180). Salt at 4% w/v in agar did not improve the antibacterial activity of cinnamaldehyde against Gram-positive and Gram-negative bacteria (115).

Combinations of oregano EO with sodium nitrite have been examined for their effect on growth and toxin production by *C. botulinum* (a combination of types A, B and E). Oregano oil acted synergistically with nitrite to inhibit growth in broth, whereas oregano oil applied alone at up to 400 ppm had no significant inhibitive effect on growth. The proposed mechanism of synergism depends on oregano EO reducing the number of spores that germinate and sodium nitrite inhibiting the outgrowth of spores. Both substances affect vegetative growth (71).

The simultaneous application of nisin (0.15 μg ml^{-1}) and carvacrol or thymol (0.3 mmol l^{-1} or 45 μg ml^{-1}) caused a larger decline in viable counts for strains of *B. cereus* than was observed when the antimicrobials were individually applied. The maximum reduction of viability was achieved in cells that had

experienced prior exposure to mild heat treatment at 45 °C (5 min for exponentially growing cells and 40 min for stationary phase cells) (131). Carvacrol was found not to increase the sensitivity of vegetative *B. cereus* cells to pulsed-electric-field (PEF) treatment nor did it sensitise spores to nisin or PEF (136). At pH 7 the synergistic action of nisin and carvacrol was significantly greater at 30°C than at 8°C, which would appear to indicate temperature-induced changes in the permeability of the cytoplasmic membrane (130). The mechanism of synergy is not known. Previously, it was hypothesised that carvacrol may increase the number, size or duration of existence of the pores created by nisin in the cell membrane (136). Later it became clear that this was not so – the mechanism may lie in the enhanced dissipation of the membrane potential and a reduction in the pH gradient and intracellular ATP (134).

The combined effect of carvone (5 mmol l^{-1}) and mild heat treatment (45°C, 30 min) on exponentially growing cells of *L. monocytogenes* grown at 8°C has been studied. Separately, the two treatments demonstrated no loss in viability but a decrease of 1.3 log units in viable cell numbers was recorded when they were combined. Cells grown at 35 or 45°C were not susceptible to the same combined treatment. The authors hypothesised that the phospholipid composition of the cytoplasmic membrane of cells grown at 8°C has a higher degree of unsaturation in order to maintain fluidity and function at low temperatures. This high degree of unsaturation causes the membranes of these cells to be more fluid at 45°C than the membranes of cells grown at that temperature. This increased fluidity would enable carvone to dissolve more easily into the lipid bilayer of cells grown at 8°C than into the bilayer of cells grown at 45°C. Membranes of cells grown at 45°C are less fluid because there is a 'normal' ratio of saturated to unsaturated fatty acids in their phospholipids and carvone is therefore less effective against them (80).

Thymol and carvacrol have been shown to have a synergistic effect with high hydrostatic pressure (HHP). The viable numbers of mid-exponential phase *L. monocytogenes* cells were reduced more by combined treatment with 300 MPa HHP and 3 mmol l^{-1} thymol or carvacrol than by the separate treatments. Since HHP is believed to cause damage to the cell membrane, it is suggested that this common target is the root of the observed synergism (81).

The antibacterial activity of EOs is influenced by the degree to which oxygen is available. This could be due to the fact that when little oxygen is present, fewer oxidative changes can take place in the EOs and/or that cells obtaining energy via anaerobic metabolism are more sensitive to the toxic action of EOs (128). The antibacterial activity of oregano and thyme EOs was greatly enhanced against *S. typhimurium* and *Staph. aureus* at low oxygen levels (128). The use of vacuum packing in combination with oregano EO may have a synergistic effect on the inhibition of *L. monocytogenes* and spoilage flora on beef fillets; 0.8% v/w

oregano EO achieved a 2-3 $\log_{10}$ initial reduction in the microbial flora but was found to be even more effective in samples packed under vacuum in low-permeability film when compared to aerobically stored samples and samples packaged under vacuum in highly permeable film (173). Similarly, the lethal effect of clove and coriander EOs on *A. hydrophila* on pork loin steak stored at 2°C and 10°C was more pronounced in vacuum packed pork than on samples stored in air (167). Oregano EO was found to delay microbial growth and to suppress final counts of spoilage microorganisms in minced beef under modified atmosphere packaging (MAP, 40% CO_2, 30% N_2 and 30% O_2) when, in contrast, no pronounced inhibition was evident in beef packed under air (162).

9. LEGAL ASPECTS OF THE USE OF EOS AND THEIR COMPONENTS IN FOODS

A number of EO components have been registered by the European Commission for use as flavourings in foodstuffs. The flavourings registered are considered to present no risk to the health of the consumer and include amongst others carvacrol, carvone, cinnamaldehyde, citral, *p*-cymene, eugenol, limonene, menthol and thymol. Estragole and methyl eugenol were deleted from the list in 2001 due to their being genotoxic (31). New flavourings may only be evaluated for registration after toxicological and metabolic studies have been carried out, which could entail a considerable financial outlay (30-33, 53).

The EU registered flavourings listed above also appear on the 'Everything Added to Food in the US' (EAFUS) list (http://www.cfsan.fda.gov/~dms/eafus.html, date consulted: 26 February 2003), which means that the United States Food and Drug Administration (FDA) has classified the substances as generally recognised as safe (GRAS) or as approved food additives. Estragole, specifically prohibited as flavouring in the EU, is also on the EAFUS list.

In other countries and if added to food for a purpose other than flavouring, these compounds may be treated as new food additives. Approval as a food additive would probably involve expensive safety and metabolic studies, the cost of which may be prohibitive. From a legislative point of view it would in those countries be economically more feasible to use a whole spice or herb or a whole EO as an ingredient than to use individual EO components (163).

10. SAFETY DATA

In spite of the fact that a considerable number of EO components are GRAS and/or approved food flavourings, some research data indicate irritation and toxicity. For example, eugenol, menthol and thymol, when applied in root canal treatments, have been known to cause irritation of mouth tissues. The results of a cytotoxicity study on these compounds suggest that gum irritation may be related to membrane lysis and surface activity and that tissue penetration may be related at least partly to membrane affinity and lipid solubility (105). Cinnamaldehyde, carvacrol, carvone and thymol appear to have no significant or marginal effects in vivo whilst in vitro they exhibit mild to moderate toxic effects at the cellular level. Genotoxicity data appear not to raise concern in view of the present levels of use (166).

Some EOs and their components have been known to cause allergic contact dermatitis in people who use them frequently. Preventive measures may be needed to ensure the well-being of workers if these substances were to be used on a larger scale (15, 26).

Some oils used in the fields of medicine, paramedicine and aromatherapy have been shown to exhibit spasmolytic or spasmogenic properties, although these are difficult to associate with a particular component (100, 103). Enantiomers of α-pinene have been shown to have very different spasmogenic effects (101).

It is recommended that more safety studies be carried out before EOs are more widely used or at greater concentrations in foods that at present.

11. ORGANOLEPTIC ASPECTS OF EOS IN FOODS

If EOs were to be more widely applied as antibacterials in foods, the organoleptic impact would be important. Foods generally associated with herbs, spices or seasonings would be the least affected by this phenomenon and information on the flavour impact of oregano EO in meat and fish supports this. The flavour of beef fillets treated with 0.8% v/w oregano oil was found to be acceptable after storage at 5°C and cooking (173). The flavour, odour and colour of minced beef containing 1% v/w oregano oil improved during storage under modified atmosphere packaging and vacuum at 5°C and was almost undetectable after cooking (162). Oregano oil (0.05% v/w) on cod fillets produced a 'distinctive but pleasant' flavour, which decreased gradually during storage at 2°C (113). Thyme and oregano oils spread on whole Asian sea bass at 0.05% (v/v) (*sic*) also imparted a herbal odour, which during storage up to 33 days at 0-2°C became more pronounced (66). The addition of thyme oil at up to 0.9% (v/w) in a coating for cooked shrimps had no ill effects on the flavour or appearance. However, 1.8% thyme oil in the coating

significantly decreased the acceptability of the shrimps (123). Individual EO components, many of them being approved food flavourings, also impart a certain flavour to foods. On fish, carvacrol is said to produce a 'warmly pungent' aroma; citral is 'lemon-like' and geraniol 'rose-like' (84). Treatment of fresh kiwifruit and honeydew melon with 1 mM carvacrol or cinnamic acid has been found to delay spoilage without causing adverse organoleptic changes (144).

12. FUTURE PERSPECTIVES

Arguably the most interesting area of application for EOs is the inhibition of growth and reduction in numbers of the more serious foodborne pathogens such as *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes*. The delay of spoilage and improvement of organoleptic qualities in vacuum packed meat or fish may also be interesting from a commercial point aspect. In view of their organoleptic properties, EOs could most readily be incorporated in manufactured foods that are traditionally associated with herbs (savoury dishes such as meat and fish dishes, cheese, vegetable dishes, soups and sauces) or with spices (drinks and desserts containing fruit and/or dairy products). It may be possible to use EOs in foods not previously associated with a herby or spicy flavour if the presence of one or more synergists can produce the desired antibacterial effect at a concentration which does not produce undesirable changes in the flavour or aroma.

The use of EOs in consumer goods is expected to increase in the future due to the rise of 'green consumerism', which stimulates the use and development of products derived from plants (174). This applies to the food and cosmetic sectors but also to medicinal products (8). If EOs were to be required in much greater volumes than at present, bioengineering of their synthesis in plants could provide greater yields (104, 111). International standardisation of the composition of commercially available EOs would be essential for reliable applications (26).

13. AREAS FOR FUTURE RESEARCH

The action of EO components on proteins embedded in the cytoplasmic membrane and on phospholipids in the membrane is not yet fully identified and is a focal area for future research. Further elucidation of these mechanisms would provide insights that may prove useful for technological applications.

The antibacterial activity against bacterial cells in the stationary phase is a particularly appropriate subject for study (139). The extent to which bacteria can adapt to the presence of EOs in foods is also important for further evaluation;

B. cereus has been shown to become less sensitive to carvacrol after being grown in the presence of non-lethal concentrations. The decrease in sensitivity was achieved by changing the fatty acid and phospholipid head-group composition in the membrane, which reduced fluidity and passive permeability of the cell membrane (178).

Interactions between EOs and their components and other food ingredients and food additives need to be investigated. Clove and oregano oils can acquire a dark pigmentation when in contact with iron (11); this may impose limitations on their application. Synergistic effects could be exploited so as to maximise the antibacterial activity of EOs and to minimise the concentrations required to achieve a particular antibacterial effect. Antagonism between EO and food ingredients is undesirable and research is needed so it can be avoided in practical applications.

The stability of EOs during food processing will also need to be studied. The heat stability of cinnamaldehyde has been investigated; it was found to decompose to benzaldehyde at temperatures approaching 60°C when heated alone. When combined with eugenol or cinnamon leaf oil, however, cinnamaldehyde was stable even after 30 min at 200°C (58).

Possible secondary or indirect consequences of the use of EOs would need to be explored: would the addition of EOs have any disadvantageous effects on the safety of the food, such as influencing the stress tolerance of pathogens? *L. monocytogenes* has been shown to become more tolerant of mild heat (56°C) after being stressed by the presence of ethanol, hydrogen peroxide or low pH (102). Could a similar phenomenon occur with EOs?

In the past there has been little standardisation of test methods for testing antibacterials for use in food. This is a field where a selection of standard methods would accelerate the study of promising antibacterial components and their synergistic or antagonistic action with each other and with food ingredients.

14. CONCLUSION

A number of EOS and several of their individual components exhibit antibacterial activity against foodborne pathogens in vitro and, to a lesser extent, in foods. The phenolic components are most active and appear to act principally as membrane permeabilisers. Gram-positive organisms are generally more sensitive to EOs than Gram-negative organisms. Undesirable organoleptic effects can be limited by careful selection of EO according to the type of food. Synergism and antagonism between components of EOs and food constituents require more study before these substances can reliably be used in commercial applications. If the active substances

are to be added to foods in greater concentrations than is currently normal practice for flavourings, further safety studies may be necessary.

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REFERENCES

1. **Akgül, A., and M. Kivanç**. 1988. Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. International Journal of Food Microbiology 6:263-268.
2. **Akgül, A., M. Kivanç, and S. Sert**. 1991. Effect of carvacrol on growth and toxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. Sciences des Aliments 11:361-370.
3. **Anonymous**. Alamar Blue™ product analysis sheet. Biosource International Inc., Camarillo, CA 93012, USA.
4. **Arras, G., and G. E. Grella**. 1992. Wild thyme, *Thymus capitatus*, essential oil seasonal changes and antimycotic activity. Journal of Horticultural Science 67:197-202.
5. **Aureli, P., A. Costantini, and S. Zolea**. 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. Journal of Food Protection 55:344-348.
6. **Azzouz, M. A., and L. B. Bullerman**. 1982. Comparative antimycotic effects of selected herbs, spices, plant components and commercial antifungal agents. Journal of Food Protection 45:1298-1301.
7. **Barry, A. L.** 1976. The antimicrobic susceptibility test: principles and practices. Lea and Febiger, Philadelphia.
8. **Bassett, I. B., D. L. Pannowitz, and R. S. C. Barnetson**. 1990. A comparitive study of tea-tree oil versus benzoylperoxide in the treatment of acne. Medical Journal of Australia 153:455-458.
9. **Bassole, I. H. N., A. S. Ouattara, R. Nebie, C. A. T. Ouattara, Z. I. Kabore, and S. A. Traore**. 2003. Chemical composition and antibacterial activities of the essential oils of *Lippia chevalieri* and *Lippia multiflora* from Burkina Faso. Phytochemistry 62:209-212.
10. **Bauer, K., and D. Garbe**. 1985. Common fragrance and flavor materials. Preparation, properties and uses. VCH Verlagsgesellschaft, Weinheim.
11. **Bauer, K., D. Garbe, and H. Surburg**. 2001. Common fragrance and flavor materials: Preparation, properties and uses., 4th ed. Wiley-VCH, Weinheim.
12. **Bayoumi, S.** 1992. Bacteriostatic effect of some spices and their utilization in the manufacture of yogurt. Chemie, Mikrobiologie und Technologie der Lebensmittel 14:21-26.
13. **Beuchat, L. R.** 1976. Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids. Journal of Food Science 41:899-902.

14. **Bishop, C. D.** 1995. Antiviral activity of the essential oil of *Melaleuca alternifolia* (Maiden and Betche) Cheel (tea tree) against tobacco mosaic virus. Journal of Essential Oil Research 7:641-644.
15. **Bleasel, N., B. Tate, and M. Rademaker**. 2002. Allergic contact dermatitis following exposure to essential oils. Australasian Journal of Dermatology 43:211-213.
16. **Board, R. G., and G. W. Gould**. 1991. Future prospects, p. 267-284. *In* N. J. R. a. G. W. Gould (ed.), Food preservatives. Blackie, Glasgow.
17. **Boyle, W.** 1955. Spices and essential oils as preservatives. The American Perfumer and Essential Oil Review 66:25-28.
18. **Brocklehurst, T. F., M. L. Parker, P. A. Gunning, H. P. Coleman, and M. M. Robins**. 1995. Growth of food-borne pathogenic bacteria in oil-in-water emulsions: II - Effect of emulsion structure on growth parameters and form of growth. Journal of Applied Bacteriology 78:609-615.
19. **Burt, S. A., and R. D. Reinders**. 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters in Applied Microbiology 36:162-167.
20. **Canillac, N., and A. Mourey**. 2001. Antibacterial activity of the essential oil of *Picea excelsa* on *Listeria*, *Staphylococcus aureus* and coliform bacteria. Food Microbiology 18:261-268.
21. **Carson, C. F., B. D. Cookson, H. D. Farrelly, and T. V. Riley**. 1995. Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia*. Journal of Antimicrobial Chemotherapy 35:421-424.
22. **Carson, C. F., K. A. Hammer, and T. V. Riley**. 1995. Broth micro-dilution method for determining the susceptibility of *Escherichia coli* and *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia* (tea tree oil). Microbios 82:181-185.
23. **Carson, C. F., B. J. Mee, and T. V. Riley**. 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. Antimicrobial Agents and Chemotherapy 46:1914-1920.
24. **Carson, C. F., and T. V. Riley**. 1993. Antimicrobial activity of the essential oil of *Melaleuca alternifolia*. Letters in Applied Microbiology 16:49-55.
25. **Carson, C. F., and T. V. Riley**. 1995. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. Journal of Applied Bacteriology 78:264-269.
26. **Carson, C. F., and T. V. Riley**. 2001. Safety, efficacy and provenance of tea tree (*Melaleuca alternifolia*) oil. Contact Dermatitis 45:65-67.
27. **Chaibi, A., L. H. Ababouch, K. Belasri, S. Boucetta, and F. F. Busta**. 1997. Inhibition of germination and vegetative growth of *Bacillus cereus* T and *Clostridium botulinum* 62A spores by essential oils. Food Microbiology 14:161-174.
28. **Charai, M., M. Mosaddak, and M. Faid**. 1996. Chemical composition and antimicrobial activities of two aromatic plants: *Origanum majorana* L. and *O. compactum* Benth. Journal of Essential Oil Research 8:657-664.
29. **Cimanga, K., K. Kambu, L. Tona, S. Apers, T. De Bruyne, N. Hermans, J. Totté, L. Pieters, and A. J. Vlietinck**. 2002. Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. Journal of Ethnopharmacology 79:213-220.
30. **Commission of the European Communities**. 1999. Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council on 28 October 1996. 1999/217/EC, Official Journal L084, 27/03/1999 p. 1-137. Commission of the European Communities,, Brussels, Belgium.

31. **Commission of the European Communities**. 2002. Decision of 23 January 2002 amending Commission Decision 1999/217/EC as regards the register of flavouring substances used in or on foodstuffs. 2002/113/EC, Official Journal L49 20/02/2002 p.1-160. Commission of the European Communities,, Brussels, Belgium.
32. **Commission of the European Communities**. 2000. Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council, Official Journal L180 19/07/2000 p. 8-16. Commission of the European Communities,, Brussels, Belgium.
33. **Commission of the European Communities**. 2002. Regulation (EC) No. 622/2002 of 11 April 2002 establishing deadlines for the submission of information of chemically defined flavouring substances used in or on foodstuffs, Official Journal L95 12/04/2002 p. 10-11. Commission of the European Communities,, Brussels, Belgium.
34. **Conner, D. E., and L. R. Beuchat**. 1984. Effects of essential oils from plants on growth of food spoilage yeasts. Journal of Food Science 49:429-434.
35. **Cosentino, S., C. I. G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi, and F. Palmas**. 1999. *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Letters in Applied Microbiology 29:130-135.
36. **Cox, S. D., C. M. Mann, J. L. Markham, H. C. Bell, J. E. Gustafson, J. R. Warmington, and S. G. Wyllie**. 2000. The mode of antimicrobial action of essential oil of *Melaleuca alternifola* (tea tree oil). Journal of Applied Microbiology 88:170-175.
37. **Cremieux, A., H. Guirard-Dauriac, and G. Dumenil**. 1981. Neutralisation des antiseptiques et désinfectants. Journal de Pharmacie de Belgique 36:223-226.
38. **Cressy, H. K., A. R. Jerrett, C. M. Osborne, and P. J. Bremer**. 2003. A novel method for the reduction of numbers of *Listeria monocytogenes* cells by freezing in combination with an essential oil in bacteriological media. Journal of Food Protection 66:390-395.
39. **Crosthwaite, D.** 1998. Presented at the International Federation of Essential Oils and Aroma Trades - 21st International Conference on Essential Oils and Aroma's, London, 8-12 November 1998.
40. **Cutter, C. N.** 2000. Antimicrobial effect of herb extracts against *Escherichia coli* O157:H7*, Listeria monocytogenes,* and *Salmonella typhimurium* associated with beef. Journal of Food Protection 63:601-607.
41. **Daferera, D. J., B. N. Ziogas, and M. G. Polissiou**. 2003. The effectiveness of plant essential oils in the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. Crop Protection 22:39-44.
42. **Daferera, D. J., B. N. Ziogas, and M. G. Polissiou**. 2000. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. Journal of Agricultural and Food Chemistry 48:2576-2581.
43. **Davidson, P. M.** 1997. Chemical preservatives and natural antimicrobial compounds, p. 520-556. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington.
44. **Davidson, P. M., and M. E. Parish**. 1989. Methods for testing the efficacy of food antimicrobials. Food Technology 43:148-155.
45. **Deans, S. G., and G. Ritchie**. 1987. Antibacterial properties of plant essential oils. International Journal of Food Microbiology 5:165-180.
46. **Deans, S. G., E. Simpson, R. C. Noble, A. MacPherson, and L. Penzes**. 1993. Natural antioxidants from *Thymus vulgaris* (thyme) volatile oil: the beneficial effects upon mammalian lipid metabolism. Acta Horticulturae 332:177-182.

47. **Delaquis, P. J., K. Stanich, B. Girard, and G. Mazza**. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. International Journal of Food Microbiology 74:101-109.
48. **Demetzos, C., and D. K. Perdetzoglou**. 2001. Composition and antimicrobial studies of the oils of *Origanum calcaratum* Juss. and *O. scabrum* Boiss. et Heldr. from Greece. Journal of Essential Oil Research 13:460-462.
49. **Denyer, S. P., and W. B. Hugo**. 1991. Biocide-induced damage to the bacterial cytoplasmic membrane, p. 171-188. *In* S. P. Denyer and W. B. Hugo (ed.), Mechanisms of action of chemical biocides. The Society for Applied Bacteriology, Technical Series No 27. Oxford Blackwell Scientific Publication, Oxford.
50. **Denyer, S. P., and W. B. Hugo**. 1991. Mechanisms of antibacterial action - A summary, p. 331-334. *In* S. P. Denyer and W. B. Hugo (ed.), Mechanisms of action of chemical biocides. Blackwell Scientific Publications, Oxford.
51. **Dorman, H. J. D., and S. G. Deans**. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88:308-316.
52. **Elgayyar, M., F. A. Draughon, D. A. Golden, and J. R. Mount**. 2001. Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. Journal of Food Protection 64:1019-1024.
53. **European Parliament**. 1996. Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996 laying down a Community procedure for flavouring substances used or intended for use in or on foodstuffs, Official Journal L299 23/11/1996 p.1-4.
54. **Faleiro, M. L., M. G. Miguel, F. Ladeiro, F. Venancio, R. Tavares, J. C. Brito, A. C. Figueiredo, J. G. Barroso, and L. G. Pedro**. 2002. Antimicrobial activity of essential oils isolated from Portuguese endemic species if *Thymus*. Letters in Applied Microbiology 36:35-40.
55. **Farag, R. S., Z. Y. Daw, F. M. Hewedi, and G. S. A. El-Baroty**. 1989. Antimicrobial activity of some Egyptian spice essential oils. Journal of Food Protection 52:665-667.
56. **Firouzi, R., M. Azadbakht, and A. Nabinedjad**. 1998. Anti-listerial activity of essential oils of some plants. Journal of Applied Animal Research 14:75-80.
57. **Friedman, M., P. R. Henika, and R. E. Mandrell**. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes* and *Salmonella enterica*. Journal of Food Protection 65:1545-1560.
58. **Friedman, M., N. Kozuekue, and L. A. Harden**. 2000. Cinnamaldehyde content in foods determined by gas chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 48:5702-5709.
59. **Gill, A. O., P. Delaquis, P. Russo, and R. A. Holley**. 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. International Journal of Food Microbiology 73:83-92.
60. **Gould, G. W.** 1996. Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. Journal of Food Protection 1996 supplement:82-86.
61. **Guenther, E.** 1948. The essential oils, vol. 1. D. Van Nostrand Company Inc., New York.
62. **Gustafson, J. E., Y. C. Liew, S. Chew, J. L. Markham, H. C. Bell, S. G. Wyllie, and J. R. Warmington**. 1998. Effects of tea tree oil on *Escherichia coli*. Letters in Applied Microbiology 26:194-198.
63. **Hammer, K. A., C. F. Carson, and T. V. Riley**. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86:985-990.

64. **Hao, Y. Y., R. E. Brackett, and M. P. Doyle**. 1998. Efficacy of plant extracts in inhibiting Aeromonas hydrophila and *Listeria monocytogenes* in refrigerated cooked poultry. Food Microbiology 15:367-378.
65. **Hao, Y. Y., R. E. Brackett, and M. P. Doyle**. 1998. Inhibition of *Listeria monocytogenes* and *Aeromonas hydrophila* by plant extracts in refrigerated cooked beef. Journal of Food Protection 61:307-312.
66. **Harpaz, S., L. Glatman, V. Drabkin, and A. Gelman**. 2003. Effects of herbal essential oils used to extend the shelf life of freshwater-reared Asian sea bass fish (*Lates calcarifer*). Journal of Food Protection 66:410-417.
67. **Hartmans, K. J., P. Diepenhorst, W. Bakker, and L. G. M. Gorris**. 1995. The use of carvone in agriculture: sprout suppression of potatoes and antifungal activity against potato tuber and other plant diseases. Industrial Crops and Products 4:3-13.
68. **Helander, I. M., H.-L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. Von Wright** 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.
69. **Hodges, N. A., and G. W. Hanlon**. 1991. Detection and measurement of combined biocide action, p. 297-310. *In* S. P. Denyer and W. B. Hugo (ed.), Mechanisms of action of chemical biocides. Blackwell Scentific Publications, Oxford.
70. **Ilsley, S., H. Miller, H. Greathead, and C. Kamel**. 2002. Herbal sow diets boost pre-weaning growth. Pig Progress 18:8-10.
71. **Ismaiel, A. A., and M. D. Pierson**. 1990. Effect of sodium nitrite and origanum oil on growth and toxin production of *Clostridium botulinum* in TYG broth and ground pork. Journal of Food Protection 53:958-960.
72. **Janssen, A. M.** 1989. Antimicrobial activities of essential oils - a pharmacological study. Ph.D. University of Leiden, Leiden
73. **Janssen, A. M., J. J. C. Scheffer, and A. Baerheim Svendsen**. 1987. Antimicrobial activity of essential oils: A 1976-1986 literature review. Aspects of the test methods. Planta Medica 53:396-398.
74. **Jayashree, T., and C. Subramanyam**. 1999. Antiaflatoxigenic activity of eugenol is due to inhibition of lipid peroxidation. Letters in Applied Microbiology 28:179-183.
75. **Jerkovic, I., J. Mastelic, and M. Milos**. 2001. The impact of both the season of collection and drying on the volatile constituents of *Origanum vulgare* L. ssp. *hirtum* grown wild in Croatia. International Journal of Food Science and Technology 36:649-654.
76. **Juglal, S., R. Govinden, and B. Odhav**. 2002. Spice oils for the control of co-occurring mycotoxin-producing fungi. Journal of Food Protection 65:683-687.
77. **Juliano, C., A. Mattana, and M. Usai**. 2000. Composition and *in vitro* antimicrobial activity of the essential oil of *Thymus herba-barona* Loisel growing wild in Sardinia. Journal of Essential Oil Research 12:516-522.
78. **Juven, B. J., J. Kanner, F. Schved, and H. Weisslowicz**. 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. Journal of Applied Bacteriology 76:626-631.
79. **Karapinar, M., and S. E. Aktug**. 1987. Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole. International Journal of Food Microbiology 4:161-166.
80. **Karatzas, A. K., M. H. J. Bennik, E. J. Smid, and E. P. W. Kets**. 2000. Combined action of S-carvone and mild heat treatment on *Listeria monocytogenes* Scott A. Journal of Applied Microbiology 89:296-301.

81. **Karatzas, A. K., E. P. W. Kets, E. J. Smid, and M. H. J. Bennik**. 2001. The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. Journal of Applied Microbiology 90:463-469.
82. **Karpouhtsis, I., E. Pardali, E. Feggou, S. Kokkini, Z. G. Scouras, and P. Mavragani-Tsipidou**. 1998. Insecticidal and genotoxic activities of oregano essential oils. Journal of Agricultural and Food Chemistry 46:1111-1115.
83. **Kim, J., M. R. Marshall, and C. I. Wei**. 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. Journal of Agricultural and Food Chemistry 43:2839-2845.
84. **Kim, J. M., M. R. Marshall, J. A. Cornell, J. F. Preston, and C. I. Wei**. 1995. Antibacterial activity of carvacrol, citral, and geraniol against Salmonella typhimurium on culture medium and on fish cubes. Journal of Food Science 60:1364-1374.
85. **Knobloch, K., A. Pauli, B. Iberl, H. Weigand, and N. Weis**. 1989. Antibacterial and antifungal properties of essential oil components. Journal of Essential Oil Research 1:119-128.
86. **Knobloch, K., H. Weigand, N. Weis, H.-M. Schwarm, and H. Vigenschow**. 1986. Action of terpenoids on energy metabolism, p. 429-445. *In* E. J. Brunke (ed.), Progress in Essential Oil Research: 16th International Symposium on Essential Oils. De Gruyter, Berlin.
87. **Koedam, A.** 1977. Antimikrobielle Wirksamkeit ätherischer Öle: Eine Literaturarbeit 1960-1976 - Fortsetzung und Schluss. Riechstoffe, Aromen, Kosmetika 27:36-41.
88. **Koedam, A.** 1977. Antimikrobielle Wirksamkeit ätherischer Öle: Eine Literaturarbeit 1960-1976 - Teil I. Riechstoffe, Aromen, Kosmetika 27:6,8-11.
89. **Koidis, P., E. Iossifidou, A. Abrahim, and I. Ambrosiadis**. 2000. The effectiveness of different spices as inhibitors for *Escherichia coli* O157:H7 in nutrient broth stored at 4°C or 12°C. Archiv fuer Lebensmittelhygiene 51:129-152.
90. **Kokkini, S., R. Karousou, A. Dardioti, N. Krigas, and T. Lanaras**. 1997. Autumn essential oils of Greek oregano. Phytochemistry 44:883-886.
91. **Konstantopoulou, I., L. Vassilopoulou, P. Mavragani-Tsipidou, and Z. G. Scouras**. 1992. Insecticidal effects of essential oils. A study of the effects of essential oils extracted from eleven Greek aromatic plants on *Drosophila auraria*. Experientia 48:616-619.
92. **Koutsoumanis, K., K. Lambropoulou, and G.-J. E. Nychas**. 1999. A predictive model for the non-thermal inactivation of *Salmonella enteritidis* in a food model system supplemented with a natural antimicrobial. International Journal of Food Microbiology 49:63-74.
93. **Lambert, R. J. W., and J. Pearson**. 2000. Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. Journal of Applied Microbiology 88:784-790.
94. **Lambert, R. J. W., P. N. Skandamis, P. Coote, and G.-J. E. Nychas**. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91:453-462.
95. **Lattaoui, N., and A. Tantaoui-Elaraki**. 1994. Individual and combined antibacterial activity of the main components of three thyme essential oils. Rivista Italiana EPPOS 13:13-19.
96. **Lawrence, B. M.** 1984. The botanical and chemical aspects of oregano. Perfumer and Flavorist 9:41-51.
97. **Leistner, L.** 1978. Hurdle effect and energy saving, p. 553. *In* W. K. Downey (ed.), Food quality and nutrition. Applied Science Publishers, London.

98. **Lemay, M.-J., J. Choquette, P. J. Delaquis, C. Gariépy, N. Rodrigue, and L. Saucier**. 2002. Antimicrobial effect of natural preservatives in a cooked and acidified chicken meat model. International Journal of Food Microbiology 78:217-226.
99. **Lens-Lisbonne, C., A. Cremieux, C. Maillard, and G. Balansard**. 1987. Methodes d'evaluation de l'activité antibacterienne des huiles essentielles: Application aux essences de thym et de cannelle. Journal de Pharmacie de Belgique 42:297-302.
100. **Lis-Balchin, M., S. Hart, S. G. Deans, and E. Eaglesham**. 1996. Comparison of the pharmacological and antimicrobial action of commercial plant essential oils. Journal of Herbs, Spices and Medicinal Plants 4:69-86.
101. **Lis-Balchin, M., R. J. Ochoka, S. G. Deans, M. Asztemborska, and S. Hart**. 1999. Differences in bioactivity between the enantiomers of α-pinene. Journal of Essential Oil Research 11:393-397.
102. **Lou, Y., and A. E. Yousef**. 1996. Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. Journal of Food Protection 59:465-471.
103. **Madeira, S. V. F., F. J. A. Matos, J. H. Leal-Cardoso, and D. N. Criddle**. 2002. Relaxant effects of the essential oil of *Ocimum gratissimum* on isolated ileum of the guinea pig. Journal of Ethnopharmacology 81:1-4.
104. **Mahmoud, S. S., and R. B. Croteau**. 2002. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends in Plant Science 7:366-373.
105. **Manabe, A., S. Nakayama, and K. Sakamoto**. 1987. Effects of essential oils on erythrocytes and hepatocytes from rats and dipalitoyl phophatidylcholine-liposomes. Japanese Journal of Pharmacology 44:77-84.
106. **Mann, C. M., and J. L. Markham**. 1998. A new method for determining the minimum inhibitory concentration of essential oils. Journal of Applied Microbiology 84:538-544.
107. **Mari, M., P. Bertolini, and G. C. Pratella**. 2003. Non-conventional methods for the control of post-harvest pear diseases. Journal of Applied Microbiology 94:761-766.
108. **Marino, M., C. Bersani, and G. Comi**. 1999. Antimicrobial activity of the essential oils of *Thymus vulgaris* L. measured using a bioimpedometric method. Journal of Food Protection 62:1017-1023.
109. **Marino, M., C. Bersani, and G. Comi**. 2001. Impedance measurements to study the antimicrobial activity of essential oils from *Lamiacea* and *Compositae*. International Journal of Food Microbiology 67:187-195.
110. **Marotti, M., R. Piccaglia, and E. Giovanelli**. 1994. Effects of planting time and mineral fertilization on peppermint (*Mentha piperita* L.) essential oil composition and its biological activity. Flavour and Fragrance Journal 9:125-129.
111. **McCaskill, D., and R. Croteau**. 1999. Strategies for bioengineering the development and metabolism of glandular tissues in plants. Nature Biotechnology 17:31-36.
112. **McGimpsey, J. A., M. H. Douglas, J. L. Van Klink, D. A. Beauregard, and N. B. Perry**. 1994. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris* L. in New Zealand. Flavour and Fragrance Journal 9:347-352.
113. **Mejlholm, O., and P. Dalgaard**. 2002. Antimicrobial effect of essential oils on the seafood spoilage micro-organism *Photobacterium phosphoreum* in liquid media and fish products. Letters in Applied Microbiology 34:27-31.
114. **Mendoza-Yepes, M. J., L. E. Sanchez-Hidalgo, G. Maertens, and F. Marin-Iniesta**. 1997. Inhibition of *Listeria monocytogenes* and other bacteria by a plant essential oil (DMC) en Spanish soft cheese. Journal of Food Safety 17:47-55.
115. **Moleyar, V., and P. Narasimham**. 1992. Antibacterial activity of essential oil components. International Journal of Food Microbiology 16:337-342.

116. **Mourey, A., and N. Canillac**. 2002. Anti-*Listeria monocytogenes* activity of essential oils components of conifers. Food Control 13:289-292.
117. **Moyler, D.** 1998. Presented at the International Federation of Essential Oils and Aroma Trades - 21st International Conference on Essential Oils and Aroma's., London, 8-12 November 1998.
118. **NCCLS**. 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard - Fifth edition. NCCLS document M7-A5. [ISBN 1-56238-394-9] NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.
119. **Negi, P. S., G. K. Jayaprakasha, L. Jagan Mohan Rao, and K. K. Sakariah**. 1999. Antibacterial activity of turmeric oil: a byproduct from curcumin. Journal of Agricultural and Food Chemistry 47:4297-4300.
120. **Nychas, G. J. E.** 1995. Natural antimicrobials from plants, p. 58-89. *In* G. W. Gould (ed.), New methods of food preservation, 1st ed. Blackie Academic & Professional, London.
121. **Onawunmi, G. O.** 1989. Evaluation of the antimicrobial activity of citral. Letters in Applied Microbiology 9:105-108.
122. **Oosterhaven, K., B. Poolman, and E. J. Smid**. 1995. S-carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound. Industrial Crops and Products 4:23-31.
123. **Ouattara, B., S. F. Sabato, and M. Lacroix**. 2001. Combined effect of antimicrobial coating and gamma irradiation on shelf life extension of pre-cooked shrimp (*Penaeus* spp.). International Journal of Food Microbiology 68:1-9.
124. **Ouattara, B., R. E. Simard, R. A. Holley, G. J.-P. Piette, and A. Bégin**. 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. International Journal of Food Microbiology 37:155-162.
125. **Packiyasothy, E. V., and S. Kyle**. 2002. Antimicrobial properties of some herb essential oils. Food Australia 54:384-387.
126. **Pandey, R., A. Kalra, S. Tandon, N. Mehrotra, H. N. Singh, and S. Kumar**. 2000. Essential oil compounds as potent source of nematicidal compounds. Journal of Phytopathology 148:501-502.
127. **Pandit, V. A., and L. A. Shelef**. 1994. Sensitivity of *Listeria monocytogenes* to rosemary (*Rosmarinus officinalis* L.). Food Microbiology 11:57-63.
128. **Paster, N., B. J. Juven, E. Shaaya, M. Menasherov, R. Nitzan, H. Weisslowicz, and U. Ravid**. 1990. Inhibitory effect of oregano and thyme essential oils on moulds and foodborne bacteria. Letters in Applied Microbiology 11:33-37.
129. **Paster, N., M. Menasherov, U. Ravid, and B. Juven**. 1995. Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. Journal of Food Protection 58:81-85.
130. **Periago, P. M., and R. Moezelaar**. 2001. Combined effect of nisin and carvacrol at different pH and temperature levels on the variability of different strains of *Bacillus cereus*. International Journal of Food Microbiology 68:141-148.
131. **Periago, P. M., A. Palop, and P. S. Fernandez**. 2001. Combined effect of nisin, carvacrol and thymol on the viability of *Bacillus cereus* heat-treated vegetative cells. Food Science and Technology International 7:487-492.
132. **Pessoa, L. M., S. M. Morais, C. M. L. Bevilaqua, and J. H. S. Luciano**. 2002. Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus*. Veterinary Parasitology 109:59-63.
133. **Pintore, G., M. Usai, P. Bradesi, C. Juliano, G. Boatto, F. Tomi, M. Chessa, R. Cerri, and J. Casanova**. 2002. Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. oils from Sardinia and Corsica. Flavour and Fragrance Journal 17:15-19.

134. **Pol, I. E., J. Krommer, and E. J. Smid**. 2002. Bioenergetic consequences of nisin combined with carvacrol towards *Bacillus cereus*. Innovative Food Science and Emerging Technologies 3:55-61.
135. **Pol, I. E., H. C. Mastwijk, R. A. Slump, M. E. Popa, and E. J. Smid**. 2001. Influence of food matrix on inactivation of *Bacillus cereus* by combinations of nisin, pulsed electric field treatment and carvacrol. Journal of Food Protection 64:1012-1018.
136. **Pol, I. E., and E. J. Smid**. 1999. Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*. Letters in Applied Microbiology 29:166-170.
137. **Prudent, D., F. Perineau, J. M. Bessiere, G. M. Michel, and J. C. Baccou**. 1995. Analysis of the essential oil of wild oregano from Martinique (*Coleus aromaticus* Benth.) - evaluation of its bacterioatatic and fungistatic properties. Journal of Essential Oil Research 7:165-173.
138. **Ratledge, C., and S. G. Wilkinson**. 1988. An overview of microbial lipids, p. 3-22. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press Limited, London.
139. **Rees, C. E. D., C. E. R. Dodd, P. T. Gibson, I. R. Booth, and G. S. A. B. Stewart**. 1995. The significance of bacteria in stationary phase to food microbiology. International Journal of Food Microbiology 28:263-275.
140. **Remmal, A., T. Bouchikhi, K. Rhayour, and M. Ettayebi**. 1993. Improved method for the determination of antimicrobial activity of essential oils in agar medium. Journal of Essential Oil Research 5:179-184.
141. **Remmal, A., T. Bouchikhi, A. Tantaoui-Elaraki, and M. Ettayebi**. 1993. Inhibition of antibacterial activity of essential oils bij Tween 80 and ethanol in liquid medium. Journal de Pharmacie de Belgique 48:352-356.
142. **Renzini, G., F. Scazzocchio, M. Lu, G. Mazzanti, and G. Salvatore**. 1999. Antibacterial and cytotoxic activity of *Hyssopus officinalis* L. oils. Journal of Essential Oil Research 11:649-654.
143. **Rios, J. L., M. C. Recio, and A. Villar**. 1988. Screening methods for natural antimicrobial products with antimicrobial activity: a review of the literature. Journal of Ethnopharmacology 23:127-149.
144. **Roller, S., and P. Seedhar**. 2002. Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwifruit at 4°C and 8°C. Letters in Applied Microbiology 35:390-394.
145. **Ruberto, G., M. T. Baratta, S. G. Deans, and H. J. D. Dorman**. 2000. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. Planta Medica 66:687-693.
146. **Russo, M., G. C. Galletti, P. Bocchini, and A. Carnacini**. 1998. Essential oil chemical composition of wild populations of Italian oregano spice (*Origanum vulgare* ssp. *hirtum* (Link) Ietswaart): A preliminary evaluation of their use in chemotaxonomy by cluster analysis. 1. Inflorescences. Journal of Agricultural and Food Chemistry 46:3741-3746.
147. **Salvat, A., L. Antonnacci, R. H. Fortunato, E. Y. Suarez, and H. M. Godoy**. 2001. Screening of some plants fron Northern Argentina for their antimicrobial activity. Letters in Applied Microbiology 32:293-297.
148. **Salzer, U.-J.** 1977. The analysis of essential oils and extracts (oleoresins) from seasonings - a critical review. Critical Reviews in Food Science and Nutrition 9:345-373.
149. **Scheffer, J. J. C., and A. Baerheim Svendsen**. 1981. Zur Analytik ätherische Öle: Zehn Jahre Ätherische-Öle-Forschung 1969 bis 1979. Riechstoffe, Aromen, Kosmetika 31:39-41.
150. **Senatore, F.** 1996. Influence of harvesting time on yield and composition of the essential oil of a thyme (*Thymus pulegioides* L.) growing wild in Campania (Southern Italy). Journal of Agricultural and Food Chemistry 44:1327-1332.

151. **Senatore, F., F. Napolitano, and M. Ozcan**. 2000. Composition and antibacterial activity of the essential oil from *Crithmum maritimum* L. (Apiaceae) growing wild in Turkey. Flavour and Fragrance Journal 15:186-189.
152. **Shelef, L. A.** 1983. Antimicrobial effects of spices. Journal of Food Safety 6:29-44.
153. **Shelef, L. A., E. K. Jyothi, and M. A. Bulgarelli**. 1984. Growth of enteropathogenic and spoilage bacteria in sage-containing broth and foods. Journal of Food Science 49:737-740, 809.
154. **Sikkema, J., J. A. M. De Bont, and B. Poolman**. 1994. Interactions of cyclic hydrocarbons with biological membranes. Journal of Biological Chemistry 269:8022-8028.
155. **Sikkema, J., J. A. M. De Bont, and B. Poolman**. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews 59:201-222.
156. **Singh, N., R. K. Singh, A. K. Bhunia, and R. L. Stroshine**. 2002. Efficacy of chlorine dioxide, ozone and thyme essential oil or a sequential washing in killing *Escherichia coli* O157:H7 on lettuce and baby carrots. Lebensmittelnwissenschaft und -Technologie 35:720-729.
157. **Sivropoulou, A., S. Kokkini, T. Lanaras, and M. Arsenakis**. 1995. Antimicrobial activity of mint essential oils. Journal of Agricultural and Food Chemistry 43:2384-2388.
158. **Sivropoulou, A., E. Papanikolaou, C. Nikolaou, S. Kokkini, T. Lanaras, and M. Arsenakis**. 1996. Antimicrobial and cytotoxic activities of *Origanum* essential oils. Journal of Agricultural and Food Chemistry 44:1202-1205.
159. **Skandamis, P., K. Koutsoumanis, K. Fasseas, and G.-J. E. Nychas**. 2001. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. Italian Journal of Food Science 13:65-75.
160. **Skandamis, P., E. Tsigarida, and G.-J. E. Nychas**. 2000. Ecophysiological attributes of *Salmonella typhimurium* in liquid culture and within a gelatin gel with or without the addition of oregano essential oil. World Journal of Microbiology and Biotechnology 16:31-35.
161. **Skandamis, P. N., and G.-J. E. Nychas**. 2000. Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs and oregano essential oil concentrations. Applied and Environmental Microbiology 66:1646-1653.
162. **Skandamis, P. N., and G.-J. E. Nychas**. 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. Journal of Applied Microbiology 91:1011-1022.
163. **Smid, E. J., and L. G. M. Gorris**. 1999. Natural antimicrobials for food preservation, p. 285-308. *In* M. S. Rahman (ed.), Handbook of food preservation. Marcel Dekker Inc., New York.
164. **Smith-Palmer, A., J. Stewart, and L. Fyfe**. 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Food Microbiology 26:118-122.
165. **Smith-Palmer, A., J. Stewart, and L. Fyfe**. 2001. The potential application of plant essential oils as natural food preservatives in soft cheese. Food Microbiology 18:463-470.
166. **Stammati, A., P. Bonsi, F. Zucco, R. Moezelaar, H.-l. Alakomi, and A. Von Wright**. 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food and Chemical Toxicology 37:813-823.
167. **Stecchini, M. L., I. Sarais, and P. Giavedoni**. 1993. Effect of essential oils on A*eromonas hydrophila* in a culture medium and in cooked pork. Journal of Food Protection 56:406-409.

168. **Tassou, C., E. H. Drosinos, and G.-J. E. Nychas**. 1995. Effects of essential oil from mint (*Mentha piperita*) on *Salmonella enteritidis* and *Listeria monocytogenes* in model food systems at 4°C and 10°C. Journal of Applied Bacteriology 78:593-600.
169. **Tassou, C., E. H. Drosinos, and G.-J. E. Nychas**. 1996. Inhibition of resident microbial flora and pathogen inocula on cold fresh fish fillets in olive oil, oregano, and lemon juice under modified atmosphere or air. Journal of Food Protection 59:31-34.
170. **Tassou, C., K. Koutsoumanis, and G.-J. E. Nychas**. 2000. Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil. Food Research International 33:273-280.
171. **Thoroski, J., G. Blank, and C. Biliaderis**. 1989. Eugenol induced inhibition of extracellular enzyme production by *Bacillus cereus*. Journal of Food Protection 52:399-403.
172. **Ting, W. T. E., and K. E. Deibel**. 1991. Sensitivity of *Listeria monocytogenes* to spices at two temperatures. Journal of Food Safety 12:129-137.
173. **Tsigarida, E., P. Skandamis, and G.-J. E. Nychas**. 2000. Behaviour of *Listeria monocytogenes* and autochthonous flora on meat stored under aerobic, vacuum and modified atmosphere packaging conditions with or without the presence of oregano essential oil at 5°C. Journal of Applied Microbiology 89:901-909.
174. **Tuley de Silva, K.** (ed.). 1996. A manual on the essential oil industry. United Nations Industrial Development Organization, Vienna.
175. **Ultee, A.** 2000. Bactericidal action of carvacrol towards the food pathogen *Bacillus cereus.* A case study of a novel approach to mild food preservation. Ph.D. thesis. Wageningen University, Wageningen, The Netherlands. ISBN 90-5808-219-9.
176. **Ultee, A., M. H. J. Bennink, and R. Moezelaar**. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 68:1561-1568.
177. **Ultee, A., L. G. Gorris, and E. J. Smit**. 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. Journal of Applied Microbiology 85:211-218.
178. **Ultee, A., E. P. W. Kets, M. Alberda, F. A. Hoekstra, and E. J. Smid**. 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Archives of Microbiology 174:233-238.
179. **Ultee, A., E. P. W. Kets, and E. J. Smid**. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65:4606-4610.
180. **Ultee, A., R. A. Slump, G. Steging, and E. J. Smid**. 2000. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. Journal of Food Protection 63:620-624.
181. **Ultee, A., and E. J. Smid**. 2001. Influence of carvacrol on growth and toxin production by *Bacillus cereus*. International Journal of Food Microbiology 64:373-378.
182. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiological Reviews 56:395-411.
183. **Van de Braak, S. A. A. J., and G. C. J. J. Leijten**. 1999. Essential oils and oleoresins: a survey in the Netherlands and other major markets in the European Union. CBI, Centre for the Promotion of Imports from Developing Countries, Rotterdam.
184. **Van Krimpen, M. M., and G. P. Binnendijk**. 2001. Ropadiar® as alternative for antimicrobial growth promoter in diets of weanling pigs. Report nr. 205. Praktijkonderzoek Veehouderij Lelystad, The Netherlands.
185. **Van Welie, R. T. H.** 1997. Alle cosmetica ingrediënten en hun functies. Nederlandse Cosmetica Vereniging, Nieuwegein.
186. **Vrinda Menon, K., and S. R. Garg**. 2001. Inhibitory effect of clove oil on *Listeria monocytogenes* in meat and cheese. Food Microbiology 18:647-650.

187. **Wan, J., A. Wilcock, and M. J. Coventry**. 1998. The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. Journal of Applied Microbiology 84:152-158.
188. **Weissinger, W. R., K. H. McWatters, and L. R. Beuchat**. 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. Journal of Food Protection 64:442-450.
189. **Wendakoon, C. N., and M. Sakaguchi**. 1993. Combined effect of sodium chloride and clove on growth and biogenic amine formation of *Enterobacter aerogenes* in mackerel muscle extract. Journal of Food Protection 56:410-413.
190. **Wendakoon, C. N., and M. Sakaguchi**. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. Journal of Food Protection 58:280-283.
191. **WHO**. 2002. Food safety and foodborne illness, World Health Organization Fact sheet 237, revised January 2002, Geneva.
192. **WHO**. 2002. World health report 2002: Reducing risks, promoting healthy life. Report nr. World Health Organization Geneva.
193. **Wilkins, C., and J. O. Madsen**. 1991. Oregano headspace constituents. Zeitschrift für Lebensmittel-Untersuchung und -Forschung 192:214-219.
194. **Wilkinson, J. M., M. Hipwell, T. Ryan, and H. M. A. Cavanagh**. 2003. Bioactivity of *Backhousia citriodora*: Antibacterial and antifungal activity. Journal of Agricultural and Food Chemistry 51:76-81.
195. **Wimpenny, J. W. T., and M. W. A. Lewis**. 1977. The growth and respiration of bacterial colonies. Journal of General Microbiology 103:9-18.

Chapter 2

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

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ABSTRACT

The aim of this study was to quantify the antibacterial properties of five essential oils 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. Five essential oils 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) essential oils had the strongest bacteriostatic and bactericidal properties, followed by bay (Pimenta racemosa) and clove bud (*Eugenia caryophyllata* synonym: *Syzygium aromaticum*) essential oils. 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. Oregano and thyme essential oils 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 essential oils are less active. Lecithin diminished antibacterial properties. The bactericidal concentration of oregano oil irreversibly damaged *E. coli* O157:H7 cells within 1 min. Oregano and light thyme essential oils, 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™, 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 (18) and is found in a wide variety of foodstuffs including meat and meat products, milk, yogurt, water, salad vegetables, fruits, fruit juices and cider (2, 18). 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 exhibit antimicrobial properties (6, 12, 13). Recent studies have shown that essential oils 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* (5, 7, 26). Chemical analysis of these oils has shown the constituents to be principally carvacrol, thymol, citral, eugenol and their precursors (4, 9, 15, 23), although the composition of essential oils from a particular species of plant can differ between harvesting seasons (1, 17) and between geographical sources (3, 9). A number of constituents of essential oils exhibit significant antimicrobial properties when tested separately (11, 14, 28). However, there is evidence that essential oils 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 (15, 20). For this reason, the present study was carried out using whole essential oil.

When a portion of essential oil is shaken in broth, the oil phase separates from the water phase. Stabilization of the essential oil/broth mixture could improve the antimicrobial properties. A further advantage of the use of a stabilizer could be to lower the usage concentration of essential oils, 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 (19) and acceptable up to 1% (v/w) in minced meat (25), it may be less acceptable in other food categories. A flavourless additive that improves the efficacy of essential oils, reducing the concentration required for an antimicrobial effect, could widen the perspectives for the use of essential oils 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 essential oils has been demonstrated (16, 21). Lecithin, an emulsifier, was also chosen for testing as an aid to stabilization of essential oil. 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 essential oils 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 essential oils that 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 (22). In order to test concentrations from 0.0078 to 1% (v/v) (78–10 000 µl) 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™ (Biosource International Inc., Camarillo, CA, USA) according to the method of Salvat et al (22). 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 $CFUml^{-1}$. A positive control (containing inoculum but no essential oil) and negative control (containing essential oil 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 assay were made 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 (26).

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 essential oil in the broth and thereby improving the antibacterial properties. Prior experiments on the use of these additives determined the minimum concentrations that stabilized essential oil in MHB (i.e. no visible separation of the essential oil 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 essential oil

In order to assess the bactericidal effect of the essential oil 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 oil was added to aliquots of 9 ml MHB in tubes in a waterbath at 37°C in amounts that 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™ 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 oil 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 oils 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).

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 ± SD)
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

Colorimetric determination of 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.

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

		MHB		MHB + 0.05% agar		MHB + 0.25% soy lecithin	
Essential oil	T (°C)	MIC	MBC	MIC	MBC	Bacteriostatic concentration	Bactericidal concentration
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 stated, the value is the same as the bactericidal concentration.

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 essential oils. The differences in antibacterial properties between them became less pronounced 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 essential oil

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 oil being bactericidal (no viable cells detected) within 1 min. Concentrations of 156 and 312 μl l^{-1}were bactericidal within 5 min. With 78 μl l^{-1} oregano oil 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).

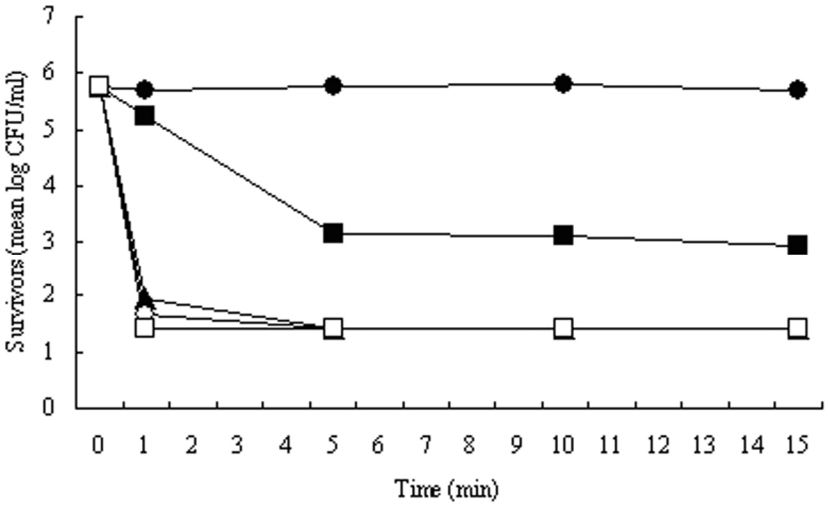

Figure 1 Survivor curves for *E. coli* O157: H7 in Mueller-Hinton broth at different concentrations of oregano oil at 37°C: (●) 0 μl l^{-1} (control), (■) 78 μl l^{-1}, (▲) 156 μl l^{-1}, (○) 312 μl l^{-1}, (□) 625 μl l^{-1} oregano oil. The detection limit for viable cells was 1.4 log CFU ml^{-1}. Where no viable cells were recovered, the detection limit is indicated.

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 essential oil.

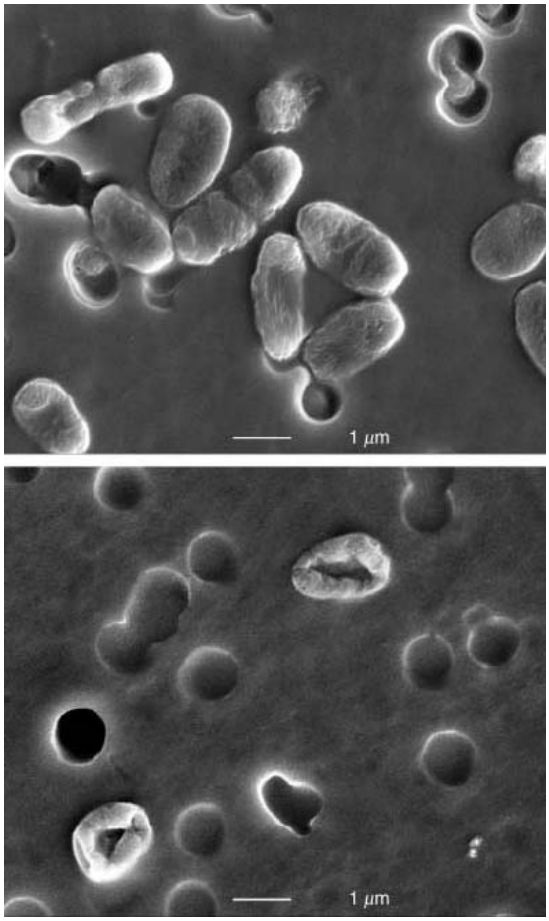

Figure 2 Scanning electron microscope images of *E. coli* O157:H7 cells after treatment with oregano essential oil (12000 x). (a) Untreated cells (control). (b) Cells damaged after treatment with 625 μl l^{-1} oregano oil.

DISCUSSION

Oregano and thyme essential oils were found to exhibit stronger antimicrobial properties than clove and bay in the disc diffusion assay. Studies testing essential oils from the same species against *E. coli* using the agar dilution method (7) and well test (5) 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 essential oil from the water phase, which would enable the more effective inhibition of bacterial 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 essential oil and the bacterial cells. Alternatively, it is possible that essential oils exert their antibacterial effect on phospholipids in the outer layer of the bacterial cell membrane and are 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 essential oils 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 (29). In another study it was shown that *E. coli* K-12 increased the saturation level of its lipid 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 (10). This observation has implications for the possible application of essential oils in foods and may partly account for the observed reductions in antibacterial activity of some essential oils in food systems compared with in vitro performance (8).

The lack of a temperature effect between 10 and 37°C on the antibacterial properties of essential oil 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* (26, 27). With agar as stabilizer a slight temperature effect was observed, particularly for thyme essential

oil, 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 oil 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 oil 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 was treated with 0.05% oregano essential oil in broth, the number of viable cells decreased by approximately 2 log cycles over a period of days at 37°C but a total kill was apparently not achieved (24).

From the S.E.M. observations it appears that, after loss of contents, *E. coli* O157:H7 cells treated with oregano oil collapsed, which enabled them to pass more easily through the pores of the membrane than the untreated control cells. Lambert et al. (14) confirms that carvacrol and thymol (major constituents of oregano essential oil) render bacterial cell membranes permeable.
As mentioned before, the composition of essential oils from the same species of plant can vary with harvesting season and geographical location. Studies carried out with a particular batch of oil should, however, exhibit good reproducibility.

In summary, this study shows that oregano and thyme essential oils 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. These properties 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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REFERENCES

1. **Arras, G., and G. E. Grella**. 1992. Wild thyme, *Thymus capitatus*, essential oil seasonal changes and antimycotic activity. Journal of Horticultural Science 67:197-202.
2. **Buchanan, R. L., and M. P. Doyle**. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli.* Food Technology 51:69-76.
3. **Cosentino, S., C. I. G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi, and F. Palmas**. 1999. *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Letters in Applied Microbiology 29:130-135.
4. **Demetzos, C., and D. K. Perdetzoglou**. 2001. Composition and antimicrobial studies of the oils of *Origanum calcaratum* Juss. and *O. scabrum* Boiss. et Heldr. from Greece. Journal of Essential Oil Research 13:460-462.
5. **Dorman, H. J. D., and S. G. Deans**. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88:308-316.
6. **Finnemore, H.** 1926. The essential oils. Ernest Benn Limited, London.
7. **Hammer, K. A., C. F. Carson, and T. V. Riley**. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86:985-990.
8. **Ismaiel, A. A., and M. D. Pierson**. 1990. Effect of sodium nitrite and origanum oil on growth and toxin production of *Clostridium botulinum* in TYG broth and ground pork. Journal of Food Protection 53:958-960.
9. **Juliano, C., A. Mattana, and M. Usai**. 2000. Composition and *in vitro* antimicrobial activity of the essential oil of *Thymus herba-barona* Loisel growing wild in Sardinia. Journal of Essential Oil Research 12:516-522.
10. **Keweloh, H., R. Diefenbach, and H.-J. Rehm**. 1991. Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. Archives of Microbiology 157:49-53.
11. **Kim, J., M. R. Marshall, and C. I. Wei**. 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. Journal of Agricultural and Food Chemistry 43:2839-2845.
12. **Koedam, A.** 1977. Antimikrobielle Wirksamkeit ätherischer Öle: Eine Literaturarbeit 1960-1976 - Fortsetzung und Schluss. Riechstoffe, Aromen, Kosmetika 27:36-41.
13. **Koedam, A.** 1977. Antimikrobielle Wirksamkeit ätherischer Öle: Eine Literaturarbeit 1960-1976 - Teil I. Riechstoffe, Aromen, Kosmetika 27:6,8-11.
14. **Lambert, R. J. W., P. N. Skandamis, P. Coote, and G.-J. E. Nychas**. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91:453-462.
15. **Lattaoui, N., and A. Tantaoui-Elaraki**. 1994. Individual and combined antibacterial activity of the main components of three thyme essential oils. Rivista Italiana EPPOS 13:13-19.
16. **Mann, C. M., and J. L. Markham**. 1998. A new method for determining the minimum inhibitory concentration of essential oils. Journal of Applied Microbiology 84:538-544.
17. **McGimpsey, J. A., M. H. Douglas, J. L. Van Klink, D. A. Beauregard, and N. B. Perry**. 1994. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris* L. in New Zealand. Flavour and Fragrance Journal 9:347-352.
18. **Mead, P. S., and P. M. Griffin**. 1998. *Escherichia coli* O157:H7. Lancet 352:1207-12.
19. **Mejlholm, O., and P. Dalgaard**. 2002. Antimicrobial effect of essential oils on the seafood spoilage micro-organism *Photobacterium phosphoreum* in liquid media and fish products. Letters in Applied Microbiology 34:27-31.

20. **Paster, N., M. Menasherov, U. Ravid, and B. Juven**. 1995. Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. Journal of Food Protection 58:81-85.
21. **Remmal, A., T. Bouchikhi, A. Tantaoui-Elaraki, and M. Ettayebi**. 1993. Inhibition of antibacterial activity of essential oils bij Tween 80 and ethanol in liquid medium. Journal de Pharmacie de Belgique 48:352-356.
22. **Salvat, A., L. Antonnacci, R. H. Fortunato, E. Y. Suarez, and H. M. Godoy**. 2001. Screening of some plants fron Northern Argentina for their antimicrobial activity. Letters in Applied Microbiology 32:293-297.
23. **Salzer, U.-J.** 1977. The analysis of essential oils and extracts (oleoresins) from seasonings - a critical review. Critical Reviews in Food Science and Nutrition 9:345-373.
24. **Skandamis, P., K. Koutsoumanis, K. Fasseas, and G.-J. E. Nychas**. 2001. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. Italian Journal of Food Science 13:65-75.
25. **Skandamis, P. N., and G.-J. E. Nychas**. 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. Journal of Applied Microbiology 91:1011-1022.
26. **Smith-Palmer, A., J. Stewart, and L. Fyfe**. 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Food Microbiology 26:118-122.
27. **Ting, W. T. E., and K. E. Deibel**. 1991. Sensitivity of *Listeria monocytogenes* to spices at two temperatures. Journal of Food Safety 12:129-137.
28. **Ultee, A., L. G. Gorris, and E. J. Smit**. 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. Journal of Applied Microbiology 85:211-218.
29. **Ultee, A., E. P. W. Kets, M. Alberda, F. A. Hoekstra, and E. J. Smid**. 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Archives of Microbiology 174:233-238.

Chapter 3

Increased activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers.

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ABSTRACT

The major components of oregano and thyme essential oils that had previously been shown to inhibit *Escherichia coli* O157:H7 were determined by high-performance liquid chromatography with UV detection and liquid chromatographic tandem mass spectrometry. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of carvacrol, thymol, *p*-cymene and γ-terpinene against a strain of *E. coli* O157:H7 phage type 34 isolated from bovine faeces were determined by microdilution assay. The constituents were then tested in checkerboard assays to detect possible interactions. Carvacrol and thymol displayed bacteriostatic and bactericidal properties with MICs of 1.2 mmol/l and were additive in combination. *p*-Cymene and γ-terpinene displayed no measurable antibacterial activity up to 50 mmol/l and neither influenced the activity of carvacrol or thymol. Growth curves in the presence of non-lethal concentrations of carvacrol with the addition of agar (0.05% w/v) or carrageenan (0.125% w/v) as stabilizer were produced by optical density measurement. The stabilizers agar and carrageenan both significantly improved the effectiveness of carvacrol in broth, possibly because of a delay in the separation of the hydrophobic substrate from the aqueous phase of the medium. When carvacrol was dissolved in ethanol before addition to broth, stabilizers were not needed. Carvacrol and thymol, particularly when used in combination with a stabilizer or in an ethanol solution, may be effective in reducing the number or preventing growth of *E. coli* O157:H7 in liquid foods.

Keywords: carvacrol, thymol, oregano, essential oil, *Escherichia coli* O157:H7, agar, carrageenan.

INTRODUCTION

Controlling the numbers and growth of *Escherichia coli* O157:H7 remains an important objective for the food industry because this pathogen is found in a wide variety of foodstuffs and causes serious outbreaks of foodborne disease (2, 28). Bovine faeces are the chief source of contamination, which means that insufficiently heated ground beef, raw dairy products and leafy vegetables that have been treated with bovine manure are the foods most often associated with human cases of infection (6). More than 5,000 cases of enterohaemorrhagic *E. coli* infection per year are recorded worldwide, with the highest incidence in young children. Cases can be severe and are sometimes fatal. The infection can therefore be classed amongst the most serious of foodborne infections (46).

Recent studies have revealed that the essential oils of oregano and thyme are active against strains of *E. coli* (3, 4, 12, 16, 40). This activity could lead to opportunities for the use of these essential oils or their components in improving the safety of certain foods. Chemical analysis of these oils has identified the major components as carvacrol, thymol, *p*-cymene and γ-terpinene (22, 26, 27), although the composition of the essential oil from a particular species of plant can differ between harvesting seasons (1, 27) and geographical sources (7, 19). A number of constituents of essential oil exhibit significant antimicrobial properties when tested separately (21, 23, 43). However, there is some evidence that essential oils are more strongly antimicrobial than is accounted for by the additive effect of their major antimicrobial components, which may indicate that minor components also play a role and/or that there may be synergy between components (23, 24, 33, 34, 41).

Essential oils and their components are hydrophobic whereas many foods have an aqueous phase. This could restrict their potential application in foods. However, it may be possible to delay separation of essential oil components from the water phase by the addition of food stabilizers, which would prevent coalescence of hydrophobic droplets by increasing the viscosity of the aqueous medium.

The purpose of this study was to compare the composition of two oregano essential oils and two thyme essential oils and to determine to what extent their activity against *E. coli* O157:H7 is related to the content of carvacrol, thymol, *p*-cymene and γ-terpinene. Possible interactions among these constituents were also evaluated. The influence of two permitted food stabilizers, agar and carrageenan, on the activity of carvacrol also was investigated.

MATERIALS AND METHODS

Maintenance and preparation of cultures.
Cultures of *E. coli* O157:H7 strain rr98089 phage type 34 isolated by our laboratory from bovine faeces were maintained on tryptone soy agar slants (Oxoid, Basingstoke, UK) at 4°C. Inocula were prepared by 16 h culture in Mueller Hinton broth (MHB; Oxoid) at 37 °C. This *E. coli* strain harbours the *eae* and *ehly*-genes and shows enterohaemolysis on enterohaemolysin agar, but does not carry genes for Shiga toxin production. The strain was chosen as a surrogate for Shiga toxin-producing *E. coli*. Before use, the optical density (OD) of the suspension was measured using a Pharmacia Ultrospec III spectrophotometer at 620 nm and the suspension was diluted in MHB to the appropriate concentration.

Essential oils and components.
Essential oils of oregano (*Origanum vulgare*) were obtained from Jacob Hooy (Limmen, The Netherlands) and C. Melchers GmbH (Bremen, Germany). Two thyme (*Thymus vulgaris*) essential oils were obtained from C. Melchers GmbH. Carvacrol (98%) and γ-terpinene (97%) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); thymol (98%) was from Merck KGaA (Darmstadt, Germany) and *p*-cymene (>99.5%) was from Fluka Chemie GmbH (Buchs, Switzerland).

HPLC-UV.
All solvents were from J.T. Baker (Deventer, The Netherlands) and were degassed before use. Aliquots of 20 µl of 0.5% essential oil diluted in 40% (v/v) acetonitrile were injected into a high-performance liquid chromatography (HPLC) system consisting of a quaternary gradient HPLC pump (type L-7100, Hitachi, Tokyo, Japan), a UV detector (type 785A, Applied Biosystems, Foster City, CA, USA) and an autosampler (type Triathlon, Spark-Holland, Emmen, The Netherlands). The HPLC column was a Phenomenex Luna C18 (150 x 4.6 mm, Phenomenex, Torrance, CA, USA). Elution was performed at a flow of 0.8 ml/min using a mixture of water (A) and acetonitrile (B) with the following elution characteristics. For the first two minutes there was an isocratic flow with 40% B, from 2 to 17 min B increased to 60%, and then the column was washed with 90% B for 12 minutes. After 6 min of equilibration, the next sample was injected. The effluent was monitored at 254 nm (for detection of carvacrol, *p*-cymene and thymol) or 210 nm (for detection of γ-terpinene). Chromatograms were recorded and processed by Chromquest software package (version 2.51, Thermoquest, San Jose, CA, USA).

For calibration, standard solutions of 200 to 1000 µg/ml pure essential oil components were injected.

LC-MS-MS.
For liquid chromatographic tandem mass spectrometry (LC-MS-MS) analysis, 10 µl aliquots of diluted essential oil solution were injected into an HPLC system consisting of two HPLC pumps (PE200 series, Applied Biosystems, Foster City, CA, USA), an autosampler (PE200 series, Applied Biosystems) and an MDS SCIEX API-365 MS detector (Applied Biosystems) equipped with an APCI interface. The LC-MS-MS was controlled by Analyst software (version 1.1, Applied Biosystems). The HPLC column was a Phenomenex Luna C18 (150 x 4.6 mm, Phenomenex) and elution was performed as described. The APCI of the MS operated at an ionization current of 2 µA and a source temperature of 350°C. The entrance, declustering and focusing potentials were set at 10, –26 and –90V, respectively. Tandem MS analysis was performed in negative multireaction monitoring mode. The collision energy was set at –35V. The following traces were monitored to detect carvacrol and thymol: *m/z* 149.0 → *m/z* 133.0, *m/z* 149.0 → *m/z* 106.0, *m/z* 149.0 → *m/z* 91.0. Ionization and consequently detection of *p*-cymene and γ-terpinene was not possible because of the lack of a hydroxyl group on these molecules.

Determination of the MICs and MBCs.
The MICs and MBCs of the essential oils and components were determined using a colourimetric broth microdilution technique as previously described (4), with small adaptations. Serial dilutions of the substances to be tested were made up in sterile MHB in sterile 96-well microplates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). To each well was added 80 µl of inoculum and 20 µl alamarBlue (Biosource International Inc., Camarillo, CA, USA). The inoculum was prepared using a 16 h culture adjusted by reference to the OD at 620 nm and further diluted with MHB to achieve approximately 1.25 x 10^6 cfu/ml, which produced a total bacterial load of approximately 10^5 cfu/well. A positive control (containing inoculum but no essential oil) and negative control (containing essential oil but no inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at 37°C for 24 h. A colour change from blue to pink or mauve was indicative of bacterial growth. Aliquots of 5 µl from the wells that remained blue were placed on Mueller Hinton Agar (MHA; Oxoid) and incubated for 24 h at 37°C. Three replicates of each assay were carried out and the experiment was carried out twice; averages were calculated for MICs and MBCs. The MIC was the lowest concentration at which bacteria failed to grow in MHB

but were cultured when plated onto MHA. The MBC was the lowest concentration at which bacteria failed to grow in MHB and were not culturable after plating onto MHA. These definitions have been established by other workers (40).

Checkerboard assay for synergism/antagonism.
To detect any synergism or antagonism between the essential oil components, a checkerboard assay was carried out whereby increasing concentrations of one component were dispensed in the rows and increasing concentrations of the other component were dispensed in the columns of a microplate. Inocula and colour indicator were added as for the colourimetric assay. Carvacrol was tested against thymol; carvacrol and thymol were both tested against *p*-cymene and γ-terpinene. MICs were determined for each component in the presence of the second component and fractional inhibitory concentrations (FICs) were calculated from these as follows: $FIC_{(A)} = MIC_{(A \text{ in the presence of B})} / MIC_{(A \text{ alone})}$ and $FIC_{(B)} = MIC_{(B \text{ in the presence of A})} / MIC_{(B \text{ alone})}$. The FIC index was obtained by adding the individual FICs. The results were interpreted as synergistic when the FIC index was ≤0.5, as additive when between 0.5 and 1.0, as indifferent when between 1.0 and 2.0 and as antagonistic when the index was ≥2.0 (13).

Effect of stabilizers on the antimicrobial activity of carvacrol.
The effect of carvacrol on the growth of the test strain was verified in the presence and absence of the stabilizers with an automated OD reader (software version 2.28, Bioscreen, Oy Growth Curves AB Ltd., Helsinki, Finland). Stock preparations of 1 mol/l carvacrol were made up in 96% ethanol or by shaking in sterile distilled water. The appropriate test concentrations of carvacrol were made up in MHB, MHB containing 0.1% bacteriological agar no. 1 (Oxoid) or MHB containing 0.25% carrageenan type II (Sigma) (predominantly iota carrageenan) and 100 µl portions were placed in wells in a sterile Bioscreen microplate with a lid. At these concentrations the stabilizers increased the viscosity of the medium without forming a firm gel (5, 8). Aliquots of 100 µl of bacterial suspension containing approximately 10^6 cfu/ml were added to all wells with mixing, and during the 18 h incubation with continuous shaking the OD measurements were automatically plotted in an Excel file. Each experiment was carried out on two different days with three to five replicates each time. A positive growth control containing no essential oil component was run on every occasion. The maximum specific growth rate, μ_{max}, and time to reach μ_{max} were calculated automatically by the software.

Determination of final bacterial population after incubation with carvacrol.
Viable counts were carried out by serial 10-fold dilutions in sterile physiological salt solution (0.85% sodium chloride in distilled water) and plating out of

appropriate dilutions in duplicate on MHA. Plates were incubated at 37°C for 24 h.

Statistical analysis.
For the experiments using stabilizers, the μ_{max} and time to reach μ_{max} were compared in an analysis of variance using SPSS software (version 10.0, SPSS, Chigao, IL, USA).

RESULTS

The composition of the four essential oils was determined using HPLC-UV and LC-MS-MS, and the results are presented in Table 1 with the MICs and MBCs of the whole essential oils. The oil with the highest MIC and therefore the least antibacterial activity had the lowest percentage content of carvacrol plus thymol.

Table 1. Percentage composition of the four major components of essential oils (EO) of oregano and thyme and their MICs and MBCs, determined by HPLC-UV and LC-MS-MS.

EO	Composition (%, v/v)						MIC	MBC	Calculated
	Carvacrol	Thymol	*p*-Cymene	γ-Terpinene	Carvacrol + thymol	Other components	(%, v/v)	(%, v/v)	MIC[a] (%, v/v)
Oregano 1	14.6	27.7	6.1	3.7	42.3	47.9	0.06	0.08	0.04
Oregano 2	12.5	14.6	8.3	6.4	27.1	58.2	0.14	0.14	0.07
Thyme 1	21.2	22.3	5.2	3.8	43.5	47.5	0.05	0.07	0.04
Thyme 2	-[b]	33.0	6.0	11.5	33.0	49.5	0.08	0.08	0.05

[a] MIC calculated on the basis of the carvacrol plus thymol content and on MICs of carvacrol and thymol (see Table 2).
[b] Percent composition too low to be determined.

The MICs and MBCs of the four major components of the essential oils are shown in Table 2. Carvacrol and thymol were active against *E. coli* O157:H7 but no antibacterial activity was detected for *p*-cymene or γ-terpinene. The MBC for carvacrol was slightly higher than the MIC; for thymol, the MBC was the same as the MIC.

Table 2. MIC, MBC and fractional inhibitory concentration (FIC) index for the four major components of oregano and thyme essential oils.

	MIC (mmol/l)	MBC (mmol/l)	FIC-index
Carvacrol	1.2	1.4	1.1
Thymol	1.2	1.2	
p-Cymene	>50	>50	
γ-Terpinene	>50	>50	

The checkerboard assay revealed that the effects of carvacrol and thymol were additive (FIC = 1.1). Further tests with combinations of the four components revealed no antagonism or synergism (data not shown). Since neither *p*-cymene nor γ-terpinene had antibacterial activity no MICs or FIC indices could be calculated for them.

The effect of the addition of stabilizers on the activity of carvacrol is represented in Figure 1. When a stock preparation of carvacrol shaken in distilled water was mixed with broth to achieve the desired test concentration (rather than first being dissolved in ethanol as in the MIC determination), little effect was achieved up to and including 6 mmol/l carvacrol. The use of broth containing stabilizer significantly improved the action of carvacrol so that an extension of the lag phase and a reduction in the final population density were achieved compared to the control. Standard deviations for the mean ODs were large (not presented in Figure 1 to preserve legibility); therefore the mean μ_{max} and time to μ_{max} were compared by statistical methods and are reported in Table 3. The use of agar or carrageenan as stabilizer resulted in significantly better performance of carvacrol compared with no stabilizer, and agar performed significantly better than carrageenan in the reduction of μ_{max}. The stabilizers were not significantly different from each other in respect to the period of time to reach μ_{max} ($p < 0.05$). The use of 4 or 6 mmol/l carvacrol produced a significant reduction in μ_{max} in the presence of both stabilizers compared with the controls, but 6 mmol/l carvacrol was not significantly more effective than 4 mmol/l ($p < 0.05$) (Table 3). The use of carrageenan at 0.05% (w/v) had no apparent effect on the activity of carvacrol (data not shown).

When the stock preparation of carvacrol was made by solution in 96% ethanol instead of by shaking in distilled water, the antibacterial activity was improved much more than by the action of stabilizers. A concentration-dependent effect was seen (Figure 2) whereby carvacrol extended the lag phase and reduced both the μ_{max} and the maximum population density in comparison with the untreated control.

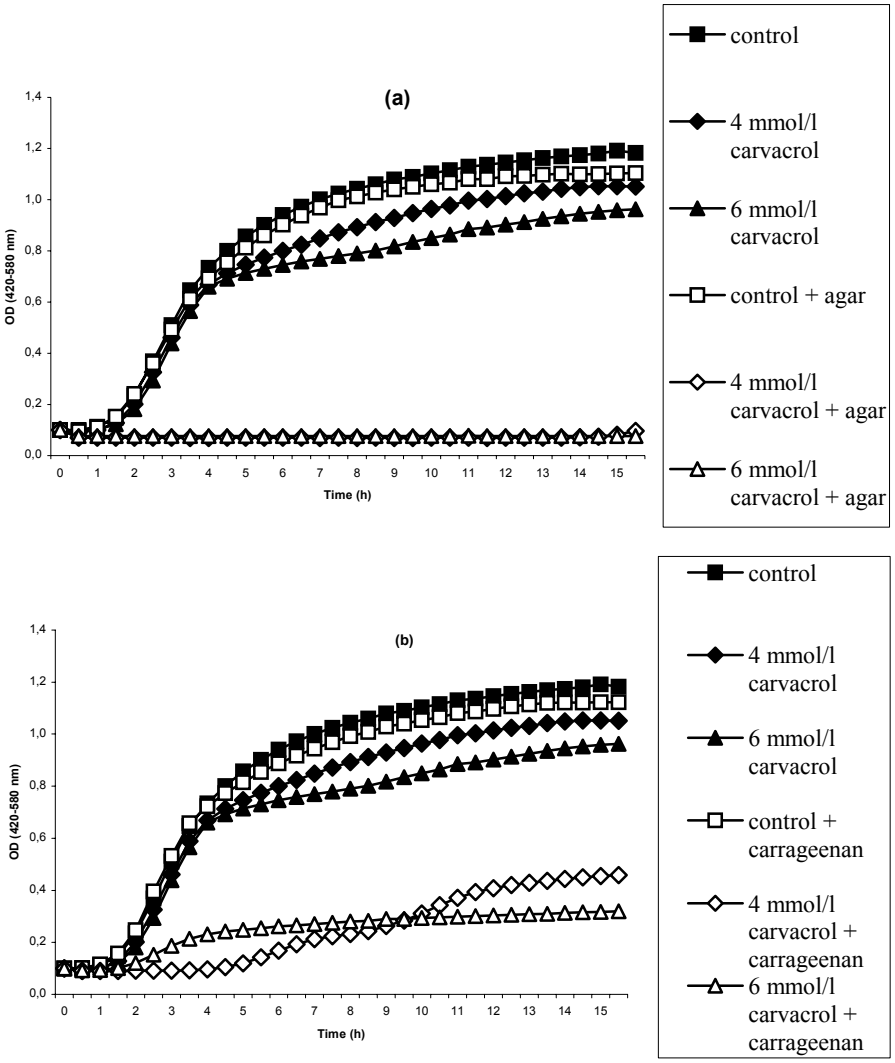

Figure 1. Effect of (a) agar (0 or 0.05% w/v) and (b) carrageenan (0 or 0.125% w/v) as stabilizers on the activity of carvacrol (0, 4 or 6 mmol/l) against *E. coli* O157:H7 in Mueller Hinton broth at 37°C. The mean ODs are plotted for the outcomes of three experiments with three replicates each.

Table 3. Maximum specific growth rate (μ_{max}), time to reach μ_{max} and final population for *E. coli* incubated in broth containing carvacrol and the stabilizers agar or carrageenan.

Concentration of carvacrol (mmol/l)	Concentration of stabilizer (% w/v)	μ_{max} (h^{-1}) (mean ± SD)	Time to μ_{max} (h) (mean ± SD)	Mean population density at 16 h (log_{10} cfu/ml)
0 (control)	0 (control)	0.97 ± 0.09 [a]	2.10 ± 0.21 [a]	8.6
4	0	0.97 ± 0.03 [a]	2.33 ± 0.25 [a]	ND[d]
4	0.05% agar	0.07 ± 0.04 [b]	8.67 ± 5.47 [b]	ND
4	0.125% carrageenan	0.42 ± 0.44 [c]	9.87 ± 4.05 [b]	2.3
6	0	0.98 ± 0.07 [a]	2.50 ± 0.00 [a]	8.3
6	0.05% agar	0.06 ± 0.02 [b]	11.33 ± 5.31 [b]	--[e]
6	0.125% carrageenan	0.25 ± 0.37 [c]	7.07 ± 4.05 [b]	--

[a,b,c] Means within a column with different superscript letters are significantly different ($p < 0.05$).

[d] Not done.

[e] No colonies incubated.

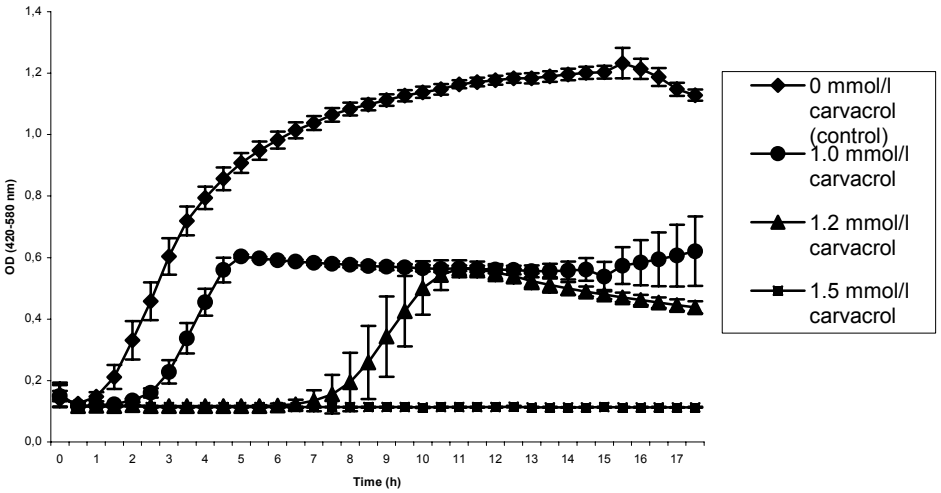

Figure 2. Effect of 0, 1.0, 1.2 and 1.5 mmol/l carvacrol on the growth of *E. coli* O157:H7 in Mueller Hinton broth at 37°C after dissolution of the carvacrol in 96% ethanol. Data points represent the mean and standard deviation for two experiments with five replicates each.

The mean population densities after 18 h incubation were 8.6 log cfu/ml for controls, 8.3 log cfu/ml for 1.2 mmol/l carvacrol, and 2.7 log cfu/ml for 1.5 mmol/l carvacrol. Population densities for 1.0 mmol/l carvacrol were not measured. The use of broth containing agar or carrageenan did not further increase the antibacterial activity when carvacrol was dissolved in ethanol (data not shown).

DISCUSSION

The composition of essential oils is dependent on many things, including geographical location of the plant, harvesting season, extraction method and the part of the plant used (1, 10, 18, 27, 29). This variability is reflected in the range of composition of essential oils noted by other workers. Oregano oils have been reported as containing carvacrol, thymol, *p*-cymene and *γ*-terpinene in the ranges: from trace up to 80, 64, 52 and 52% respectively (3, 9, 30, 38). Thyme oils have been reported as containing the same components in the ranges of 0-11, 10-64, 5-56 and 2-31% respectively. The proportions of the four components in the oregano oils tested in this study fall within these ranges. The thymol, *p*-cymene and *γ*-terpinene contents of the two thyme oils also were in agreement with reported ranges. Thyme oil 1 contained more carvacrol than found in previous studies, which is unusual. Thyme oil 2 lacked carvacrol altogether; this is not common but has been described before (3, 9, 19, 30, 38).

The MICs of the essential oils reported here agree very closely with those found by other researchers working with *E. coli* strains in liquid media. MICs of 0.03- 0.125% (v/v equivalent) have been determined for oils of *O. vulgare* (16, 31), and MICs of 0.03-0.08% (v/v equivalent) for oils derived from *T. vulgaris* (14, 16, 37, 40).

The MIC for carvacrol is very close to that reported by other workers using strains of *E. coli* in liquid media. When converted from the cited units into mmol/l, the range is equivalent to 1.5-3.3 mmol/l (7, 17, 19, 21, 32). The MIC for thymol obtained in the present study agrees with MICs of 1.2-3.0 mmol/l reported previously (7, 17, 32). In another study the MIC for thymol was between 0.66 and 3.3 mmol/l (45). The range of values in the literature reflects the differences in media composition, methodology and strains of bacteria used.

To get an impression of the proportion of the antibacterial activity that could be attributed to the carvacrol and thymol content, theoretical MICs for the essential oils were calculated based on the carvacrol and thymol content (Table 1). The measured MICs are all slightly higher than the calculated MICs except for oregano oil 2, which has a much higher MIC (is less antibacterial) than would be

expected even based on its low carvacrol plus thymol content. The measured MICs for essential oils are slightly higher than the calculated MICs for oregano oil 1 and both thyme oils because the essential oils were shaken in broth before MIC determination and were therefore in suspension, whereas carvacrol and thymol had been dissolved in ethanol and remained in solution when added to the test broth. Droplets of oil in suspension gradually coalesce and separate from the aqueous phase (broth), which limits the activity of the oil. Individual components in solution cannot coalesce and separate and would presumably therefore be in closer contact with the bacteria in the aqueous phase. In oregano oil 2 the proportion of minor components (i.e. those other than carvacrol, thymol, *p*-cymene and γ-terpinene) were higher than in the other three oils (58.2% versus 47.5 to 49.5%). In this essential oil, other minor components may have had an antagonistic effect on carvacrol and/or thymol. This phenomenon has been suggested to occur in certain varieties of thyme and oregano (24, 26, 33).

No antagonism or synergism was detected between the four major components. The lack of synergy between carvacrol and *p*-cymene in this study is in contrast to the findings of a study carried out with Gram positive organisms in which a particular combination of carvacrol and cymene was synergistic when tested against *Bacillus cereus* in buffer and on rice (44). Carvacrol and *p*-cymene also exhibited a synergistic effect on the viability of *Listeria monocytogenes* (34). Ultee et al. postulated that the synergy between carvacrol and *p*-cymene when acting on *B. cereus* depends on cymene expanding the cell membrane, resulting in membrane destabilization that allows carvacrol to enter the cell and exert an antibacterial effect (42, 44). *E. coli* and other Gram negative organisms possess an additional outer cell membrane (35) that may inhibit the action of *p*-cymene. In most studies, essential oils and their components have been more effective against Gram positive than against Gram negative organisms, and cymene does not appear to have an effect on Gram negative bacteria (3, 11, 19, 20). The lack of synergy between *p*-cymene and carvacrol or thymol in this study could therefore be due to physiological differences between Gram positive and Gram negative bacteria, which could give an indication about their mode of action.

Carvacrol and thymol were additive in their activity against this strain of *E. coli*, as has been reported for *Staphylococcus aureus* and *Pseudomonas aeruginosa* (23). These two components have a very similar chemical structure consisting of a system of delocalized electrons and a hydroxyl group, which makes it likely that they have a similar mechanism of antimicrobial activity (42).

To our knowledge, there have been no other studies on the effect of carrageenan on the antibacterial activity of essential oils or their components, although the effect of agar on whole essential oils has been studied. Agar at 0.2% (w/v) was sufficient to achieve a stable dispersion for oregano and clove essential

oils in MIC determinations with *E. coli*, *B. megaterium*, *Salmonella* Hadar and *S. aureus* and performed better than Tween 80 or ethanol (36). A concentration of 0.15% (w/v) agar produced a more stable emulsion with tea tree oil than was obtained with Tween 20, Tween 80, ethanol or dimethyl sulfoxide (25) and 0.05% (w/v) agar markedly improved the action of oregano and thyme oils (4). In the present study, stabilizers significantly reduced the μ_{max} and time to reach μ_{max} for carvacrol. We hypothesized that the flexible three-dimensional matrix formed by the polysaccharide molecules entraps droplets of the hydrophobic essential oil or the active component and forms a physical barrier that retards their separation from the aqueous phase. This action would extend the period of time that the oil is in contact with bacterial cells, thereby improving the antibacterial activity. This hypothesis is supported by work carried out with gelatin gels and essential oils. Surface treatment of cooked ham with up to 6% (v/v) cilantro oil was only significantly effective against *L. monocytogenes* when the essential oil was applied in a 7% (w/v) gelatin gel coating. The authors supposed that the immobilization of the cilantro oil in the gel held the oil in contact with the ham and thereby increased the effectiveness of the treatment (15). Limitation of diffusion of oregano essential oil by structural effects caused by gelatin was also thought to have been significant in a study with *Salmonella* Typhimurium. However, in that study the essential oil was less effective in the gelatin gel than in broth. This reduction in the antibacterial effect was attributed to the limitation of diffusion because of the high concentration of gelatine (10%, w/v) *(39)*. Perhaps there is an optimum level of structural effects on essential oils, i.e., restriction of coalescence in liquid media is useful to increase the antibacterial activity but too much restriction of diffusion, e.g., in solid gels, may be counter-productive.

The large standard deviations in μ_{max} for carrageenan and time to reach μ_{max} for agar and carrageenan (Table 3) may have been caused by the fact that solution in wells had to be mixed by hand using a pipette to achieve an adequate suspension of carvacrol in the growth medium. Hand mixing is difficult to standardize, and because essential oils exert their antibacterial effect within 1 – 5 min (4), slight variations in mixing can have a large influence on the parameters, therefore increasing the standard deviation.

When carvacrol was dissolved in ethanol before use, the presence of a stabilizer did not further improve the antibacterial activity. The concentration-dependent antibacterial effect of carvacrol when dissolved in ethanol has also been established for *L. monocytogenes* and *B. cereus* and with thymol for *Ps. aeruginosa* (34, 44, 45).

In conclusion, the results of this study confirm that carvacrol and thymol are the major antibacterial components of oregano and thyme essential oils and that they have an additive antibacterial effect on *E. coli* O157:H7 strain rr98089 phage

type 34. The activity of the carvacrol and thymol in the oils appears to be related to the antibacterial activity of the whole oils and is not influenced by the presence of the other two major components, *p*-cymene and γ-terpinene, which have no apparent antibacterial effect on this strain. However, other minor components can have an antagonistic effect on the action of carvacrol and thymol.

The addition of agar or carrageenan stabilizers to an aqueous medium markedly improved the antibacterial action of carvacrol by significantly reducing μ_{max} , extending the time to μ_{max} , and reducing the final population density. The effect is presumed to be due to the delay in separation of the hydrophobic carvacrol from the aqueous broth medium and thereby the prolongation of contact between carvacrol and bacterial cells. Dissolution of carvacrol in ethanol before use improved the antibacterial activity even more than the use of stabilizers, presumably because the carvacrol could come into closer association with the bacterial cells in the aqueous phase than was possible in the presence of stabilizers. Oregano and thyme essential oils, carvacrol and thymol have possible uses as food preservatives. The addition of stabilizers could improve the performance of carvacrol or thymol in liquid foods when the use of ethanol as solvent is not feasible and where lower concentrations are desirable for organoleptic reasons.

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REFERENCES

1. **Arras, G., and G. E. Grella**. 1992. Wild thyme, *Thymus capitatus*, essential oil seasonal changes and antimycotic activity. Journal of Horticultural Science 67:197-202.
2. **Buchanan, R. L., and M. P. Doyle**. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli.* Food Technology 51:69-76.
3. **Burt, S.** 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology 94:223-253.
4. **Burt, S. A., and R. D. Reinders**. 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters in Applied Microbiology 36:162-167.
5. **C. P. Kelco**, posting date. GENU carrageenan. [Online.]
6. **Codex Alimentarius Commission**. 2002. Risk profile for enterohemorragic *E. coli* including the identification of the commodities of concern, including sprouts, ground beef and pork Report nr. CX/FH 03/5_Add. 4. FAO and WHO Orlando, USA.

7. **Cosentino, S., C. I. G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi, and F. Palmas**. 1999. *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Letters in Applied Microbiology 29:130-135.
8. **CyberColloids** 2004, posting date. Introduction to agar. [Online.]
9. **Daferera, D. J., B. N. Ziogas, and M. G. Polissiou**. 2000. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. Journal of Agricultural and Food Chemistry 48:2576-2581.
10. **Delaquis, P. J., K. Stanich, B. Girard, and G. Mazza**. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. International Journal of Food Microbiology 74:101-109.
11. **Dorman, H. J. D., and S. G. Deans**. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88:308-316.
12. **Dorman, H. J. D., Deans, S.G.** 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88:308-316.
13. **EUCAST**. 2000. Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (EUCAST) definitive document E.Def 1.2, 503-508, CMI, vol. 6.
14. **Farag, R. S., Z. Y. Daw, F. M. Hewedi, and G. S. A. El-Baroty**. 1989. Antimicrobial activity of some Egyptian spice essential oils. Journal of Food Protection 52:665-667.
15. **Gill, A. O., P. Delaquis, P. Russo, and R. A. Holley**. 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. International Journal of Food Microbiology 73:83-92.
16. **Hammer, K. A., C. F. Carson, and T. V. Riley**. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86:985-990.
17. **Helander, I. M., H.-L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. Von Wright** 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.
18. **Hudaib, M., E. Speroni, A. M. Di Pietra, and V. Cavrini**. 2002. GC/MS evaluation of thyme (*Thymus vulgaris* L.) oil composition and variations during the vegetative cycle. Journal of Pharmaceutical and Biomedical Analysis 29:691-700.
19. **Juliano, C., A. Mattana, and M. Usai**. 2000. Composition and *in vitro* antimicrobial activity of the essential oil of *Thymus herba-barona* Loisel growing wild in Sardinia. Journal of Essential Oil Research 12:516-522.
20. **Juven, B. J., J. Kanner, F. Schved, and H. Weisslowicz**. 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. Journal of Applied Bacteriology 76:626-631.
21. **Kim, J., M. R. Marshall, and C. I. Wei**. 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. Journal of Agricultural and Food Chemistry 43:2839-2845.
22. **Kokkini, S., R. Karousou, A. Dardioti, N. Krigas, and T. Lanaras**. 1997. Autumn essential oils of Greek oregano. Phytochemistry 44:883-886.
23. **Lambert, R. J. W., P. N. Skandamis, P. Coote, and G.-J. E. Nychas**. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91:453-462.
24. **Lattaoui, N., and A. Tantaoui-Elaraki**. 1994. Individual and combined antibacterial activity of the main components of three thyme essential oils. Rivista Italiana EPPOS 13:13-19.

25. **Mann, C. M., and J. L. Markham**. 1998. A new method for determining the minimum inhibitory concentration of essential oils. Journal of Applied Microbiology 84:538-544.
26. **Marino, M., C. Bersani, and G. Comi**. 1999. Antimicrobial activity of the essential oils of *Thymus vulgaris* L. measured using a bioimpedometric method. Journal of Food Protection 62:1017-1023.
27. **McGimpsey, J. A., M. H. Douglas, J. L. Van Klink, D. A. Beauregard, and N. B. Perry**. 1994. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris* L. in New Zealand. Flavour and Fragrance Journal 9:347-352.
28. **Mead, P. S., and P. M. Griffin**. 1998. *Escherichia coli* O157:H7. Lancet 352:1207-12.
29. **Moyler, D.** 1998. Presented at the International Federation of Essential Oils and Aroma Trades - 21st International Conference on Essential Oils and Aroma's., London, 8-12 November 1998.
30. **Nevas, M., A.-R. Korhonen, M. Lindström, P. Turkki, and H. Korkeala**. 2004. Antibacterial efficiency of Finnish spice essential oils against pathogenic and spoilage bacteria. Journal of Food Protection 67:199-202.
31. **Nostro, A., A. R. Blanco, M. A. Cannatelli, V. Enea, G. Flamini, I. Morelli, A. S. Roccaro, and V. Alonzo**. 2004. Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. FEMS Microbiology Letters 230:191-195.
32. **Olasupo, N. A., D. J. Fitzgerald, M. J. Gasson, and A. Narbad**. 2003. Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Letters in Applied Microbiology 36:448-451.
33. **Paster, N., M. Menasherov, U. Ravid, and B. Juven**. 1995. Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. Journal of Food Protection 58:81-85.
34. **Periago, P. M., B. Delgado, P. S. Fernandez, and A. Palop**. 2004. Use of carvacrol and cymene to control growth and viability of *Listeria monocytogenes* cells and predictions of survivors using frequency distribution functions. Journal of Food Protection 67:1408-1416.
35. **Ratledge, C., and S. G. Wilkinson**. 1988. An overview of microbial lipids, p. 3-22. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press Limited, London.
36. **Remmal, A., T. Bouchikhi, A. Tantaoui-Elaraki, and M. Ettayebi**. 1993. Inhibition of antibacterial activity of essential oils bij Tween 80 and ethanol in liquid medium. Journal de Pharmacie de Belgique 48:352-356.
37. **Rota, C., J. J. Carramiñana, J. Burillo, and A. Herrera**. 2004. In vitro antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. Journal of Food Protection 67:1252-1256.
38. **Sivropoulou, A., E. Papanikolaou, C. Nikolaou, S. Kokkini, T. Lanaras, and M. Arsenakis**. 1996. Antimicrobial and cytotoxic activities of *Origanum* essential oils. Journal of Agricultural and Food Chemistry 44:1202-1205.
39. **Skandamis, P., E. Tsigarida, and G.-J. E. Nychas**. 2000. Ecophysiological attributes of *Salmonella typhimurium* in liquid culture and within a gelatin gel with or without the addition of oregano essential oil. World Journal of Microbiology and Biotechnology 16:31-35.
40. **Smith-Palmer, A., J. Stewart, and L. Fyfe**. 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Food Microbiology 26:118-122.
41. **Ultee, A.** 2000. Bactericidal action of carvacrol towards the food pathogen *Bacillus cereus.* A case study of a novel approach to mild food preservation. Ph.D. thesis. Wageningen University, Wageningen, The Netherlands. ISBN 90-5808-219-9.

42. **Ultee, A., M. H. J. Bennink, and R. Moezelaar**. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 68:1561-1568.
43. **Ultee, A., L. G. Gorris, and E. J. Smit**. 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. Journal of Applied Microbiology 85:211-218.
44. **Ultee, A., R. A. Slump, G. Steging, and E. J. Smid**. 2000. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. Journal of Food Protection 63:620-624.
45. **Walsh, S. E., J.-Y. Maillard, A. D. Russell, C. E. Catrenich, D. L. Charbonneau, and R. G. Bartolo**. 2003. Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria. Journal of Applied Microbiology 94:240-247.
46. **WHO**. 2002. Food safety and foodborne illness, World Health Organization Fact sheet 237, revised January 2002, Geneva.

Chapter 4

Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7

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ABSTRACT

The essential oils of oregano and thyme are active against a number of foodborne pathogens, such as *Escherichia coli* O157:H7. Carvacrol is one of the major antibacterial components of these oils and *p*-cymene is thought to be its precursor in the plant. The effects of carvacrol and *p*-cymene on protein synthesis in *E. coli* O157:H7 ATCC 43895 cells were investigated. Bacteria were grown overnight in Mueller Hinton broth with a sub-lethal concentration of carvacrol or *p*-cymene and their protein composition was analyzed by SDS-PAGE and confirmed by Western blot. The presence of 1 mM carvacrol during overnight incubation caused *E. coli* O157:H7 to produce significant amounts of heat shock protein 60 (GroEL) ($p < 0.05$) and inhibited synthesis of flagellin highly significantly ($p < 0.001$), causing cells to be aflagellate and therefore non-motile. Amounts of HSP70 (DnaK) were not significantly affected. *p*-Cymene at 1 mM or 10 mM did not induce HSP60 or HSP70 in significant amounts and did not have a significant effect on flagellar synthesis. Neither carvacrol (0.3, 0.5, 0.8 or 1 mM) nor *p*-cymene (0.3, 0.5 or 0.8 mM) treatment of cells in mid-exponential growth phase induced significant amounts of HSP60 or HSP70 within 3 h, although numerical increases of HSP60 were observed. Motility decreased with increasing concentration of both compounds but existing flagella were not shed. This study is the first to demonstrate that essential oil components induce HSP 60 in bacteria and that overnight incubation with carvacrol prevents the development of flagella in *E. coli* O157:H7.

Keywords: *Escherichia coli* O157:H7, heat shock protein, flagellin, carvacrol, *p*-cymene

INTRODUCTION

Escherichia coli O157:H7 is a pathogen that causes serious foodborne infections and can lead to hemolytic uremic syndrome, particularly in children (4). The chief source is bovine feces and a variety of foods of both plant and animal origin such as meat and dairy products, vegetables and fruit can become contaminated (4, 27, 40).

Studies have shown that the essential oils (EOs) of the herbs oregano and thyme are effective against strains of *E. coli* (12, 33). The major antibacterial components of these oils are carvacrol and its isomer thymol (6). Both are approved food flavorings in the United States and Europe (8, 10) and have potential as antibacterial additives in food and feed (5, 32). A number of feed additives and food preservatives containing essential oils or carvacrol is already commercially available (21, 28, 38). *p*-Cymene is also a constituent of oregano and thyme oils but is less effective against food related pathogens (6, 12, 37) and is thought to be a precursor to carvacrol and thymol in the plant (35). The chemical structures of these compounds are shown in Figure 1.

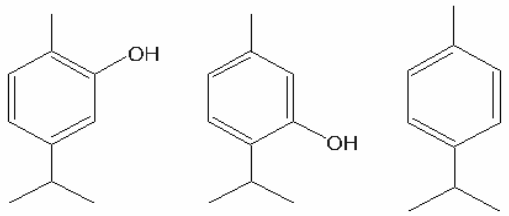

Figure 1. Structural formulae of the essential oil components (left to right) carvacrol, thymol and *p*-cymene.

The precise target(s) of the antibacterial action of EOs and their components have not yet been fully established. Changes in the fatty acid composition of bacterial cell membranes (an increase in unsaturated fatty acids) have been observed when cells are exposed to sub-lethal concentrations of EO components (11). Carvacrol and thymol damage the outer membrane of Gram-negative bacteria and increase the general permeability of the cytoplasmic membrane leading to leakage of ATP (14, 20). Carvacrol possesses ATPase inhibiting activity (14, 15); in any case it appears to dissipate the proton motive force (35). There are also indications for inhibition of other enzymes (39). *p*-Cymene has been shown to have lipolytic properties (9).

When bacteria are subjected to stress in the form of toxic substances, they generally increase their synthesis of stress proteins or heat shock proteins (HSPs). The HSP60 and HSP70 families of proteins are molecular chaperones and play a part in the assembly of newly synthesized polypeptides into their native conformation and in the folding and repair of cytosolic proteins (26). HSP60 (GroEL) and its cofactor GroES provide a compartment inside which proteins can fold whilst being protected from the cytosol. HSP70 (DnaK) holds nascent and newly synthesized polypeptides stable on the ribosomes (18, 26). The induction of HSPs has been observed for *E. coli* when subjected to stress in the form of ethanol, high osmotic stress, high temperature and the presence of phenol (24, 25). As yet, increased HSP production has not been reported for bacteria treated with carvacrol, thymol, *p*-cymene or other EO components.

The purpose of this study was to determine which changes in protein synthesis could be detected when *E. coli* O157:H7 cells were grown at sub-lethal concentrations of essential oil components carvacrol and *p*-cymene.

MATERIALS AND METHODS

Essential oil components.
Carvacrol and *p*-cymene were obtained from Sigma Aldrich Chemicals, Zwijndrecht, The Netherlands. Stock solutions in 99.8% ethanol were made on day of use. The final concentration of ethanol in the broth was never more than 1% v/v and in pilot experiments was shown to have no influence on the synthesis of HSPs, flagella or motility of cells (data not shown).

Bacterial strains and growth conditions.
Escherichia coli O157:H7 ATCC 43895 cells were tested for their reaction to the presence of EO components in two ways. Firstly, overnight (16 h) cultures were grown in Mueller Hinton Broth (MHB, Oxoid, Basingstoke, U.K.) at 37°C, with or without the addition of 1.0 mM carvacrol or *p*-cymene. Secondly, exponential phase cells for testing for a concentration-dependent effect of EO components were produced according to a growth curve previously carried out by growing cells in 100 ml MHB at 37°C with shaking to an optical density (OD) at 620 nm of 0.5 and a cell density of approx. $1x10^6$ cfu/ml. Aliquots of 10 ml were centrifuged at 2000 *g* for 5 min at room temperature and cells were resuspended in MHB with 0 mM, 0.3 mM, 0.5 mM, 0.8 mM or 1.0 mM of the relevant EO component. Incubation continued at 37°C for 3 h after which cells were harvested for protein analysis.

Protein extraction.
Whole cell protein extractions were made by separating *E. coli* cells from suspension by centrifugation in an Eppendorf 5415 C at 2000 *g* for 5 min at room temperature, washing twice in phosphate buffered saline (PBS, Cambrex Bioscience, Verviers, Belgium) and resuspension in sterile distilled water. Portions were kept apart for protein assay. Suspensions were mixed 1:1 with Laemmli buffer, heated at 95°C for 10 min and cooled on ice.

SDS-PAGE and protein identification.
Proteins were analyzed by electrophoresis on Tris-HCl Ready-Gels with 10% cross polymer in a Protean III electrophoresis system (Bio-Rad, Hercules, California) with pre-stained marker SeeBlue Plus2® (Invitrogen, Carlsbad, California) and molecular standard Benchmark Protein Ladder™ (Invitrogen). Protein concentrations were determined using Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Munich, Germany) and samples normalised for equal amounts of protein (approx. 2 μg/lane). Protein bands were made visible by staining with Coomassie blue R250 and bands of interest were identified by amino acid sequencing. Some were transferred to PVDF membrane by electroblotting and N-terminal sequence analysis was performed by using an Applied Biosystems-Perkin Elmer sequencer Model 476A at the Sequence Centre, Institute of Biomembranes, Utrecht University, The Netherlands. The sequences were screened for similarity to proteins in the NCBI database. Other bands of interest were excised and analyzed by trypsin digestion followed by MALDI-TOF MS by Gentaur, Brussels, Belgium. The peptide sequences in combination with the determined mass of the protein were used in a search of the NCBI database.

Western blotting.
After electrophoresis proteins were electroblotted at 100 V onto nitrocellulose membrane (0.2 μm) (Bio-Rad) and membranes were blocked with 0.5% blocking reagent (Roche Diagnostics, Mannheim, Germany). Membranes were then incubated for one hour with mouse antibodies against GroEL (mAb LK2) (3) or DnaK (mAb 7D9 prepared against mycobacterial HSP70 and cross-reactive with *E. coli* DnaK) or flagellin (MAb 15D8, Bioveris, Oxford, U.K.). After incubation with goat anti-mouse IgG conjugated with horseradish peroxidase (Invitrogen) the proteins were made visible by staining with a solution of dioctyl sodium sulfosuccinate and 3,3',5,5'-tetra methyl benzidine (Merck, Darmstadt, Germany). Positive controls for GroEL and DnaK were recombinant preparations of mycobacterial HSP60 and HSP70 respectively. *Salmonella enteritidis* flagellin provided by Dr Alphons van Asten was used as positive control. No blots were

carried out against the cofactor for GroEL (GroES). Blots were scanned with a GS-700 imaging densitometer (Bio-Rad, Veenendaal, The Netherlands) and the appropriate bands were quantified using Quantity One software (Bio-Rad). The ratio between test and control band density was calculated and is presented (means ± SD) in bar charts in the Figures.

Motility tests.
Motility of cells was determined by the hanging drop technique as follows: a droplet of the culture was suspended from a glass cover slip over a microscope slide with central concavity and observed under a light microscope. Bacterial cells were observed at 1000x magnification for 5 min and classified by a method established by Gill and Holley (14). Suspensions in which the majority of cells were actively moving and tumbling were classed as motile; suspensions in which a minority of cells were either moving or tumbling were classed as having reduced motility; and suspensions showing only Brownian movement were classed as non-motile.

Visualisation of flagella.
A 3 µl portion of the bacterial suspension was placed on a microscope slide and covered with a glass cover slip. One drop of flagella stain (Becton Dickinson, Sparks, MD, U.S.) was applied to the edge of the cover slip and allowed to diffuse through the suspension by capillary action. Slides were observed under differential interference contrast using a Leica DMRE light microscope with a x630 oil immersion objective and photographed with a Photometrics CoolSnap FX camera using IPLab software.

Determination of viable counts.
Viable counts were carried out by serial tenfold dilution of samples in sterile physiological saline solution (0.85% NaCl) and plating out of appropriate dilutions in duplicate on Mueller-Hinton Agar (Oxoid). Plates were incubated at 37°C for 24 h.

Statistical analysis.
Densitometric data for the blots against HSPs and flagellin after overnight culture with carvacrol or *p*-cymene and after carvacrol treatment in the exponential phase were compared by a one-way analysis of variance and the Bonferroni post hoc test of significant difference. These data are based on three independent experiments.

RESULTS

Overnight culture with carvacrol or *p*-cymene. When *E. coli* O157:H7 cells were grown overnight in MHB in the presence of 1 mM carvacrol several protein bands were detected on an SDS-PAGE gel with higher or lower expression levels compared to the control sample (Figure 2). Two proteins of approximately 60 and 70 kDa (indicated by black arrows in Fig. 2) were identified by N-terminal amino acid sequencing as HSP60 and HSP70. A protein band of approximately 65 kDa that was down regulated in cells grown with carvacrol (also indicated with a black arrow) was identified as flagellin. The mass was determined as 59,918 Da and flagellin was identified as source protein for the peptide sequences with a probability-based mowse score of 294 ($p < 0.05$). In some experiments an additional carvacrol affected protein band was observed at 50 kDa (Fig. 2, white arrow) but this was not consistent.

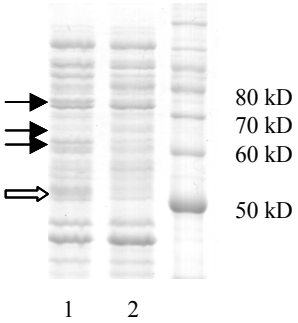

Figure 2. SDS-PAGE gel stained with Coomassie blue showing changes in protein synthesis by *E. coli* O 157:H7 strain ATCC 43895 cells when grown overnight in MHB with 1.0 mM carvacrol (lane 1) and in MHB only (lane 2). A molecular marker is shown in lane 3. The black arrows indicate increased amounts of 60 kDa and 70 kDa proteins and a decrease in 65 kDa protein. The white arrow indicates an increase in a 50 kDa protein, which did not appear consistently.

Western blot analysis was used to confirm these findings and representative blots are shown in Figure 3. The carvacrol-induced increase in the amount of HSP60 was significant ($p < 0.05$) but there was no significant change for HSP70. The reduction in the amount of flagellin formed after overnight culture with 1 mM carvacrol was very highly significant ($p < 0.001$) (Fig. 3).

Western blot analysis of proteins after overnight culture in the presence of the less antimicrobially active substance, *p*-cymene, was also carried out; the effect

of 1 mM and 10 mM *p*-cymene on protein levels is also shown in Fig. 3. *p*-Cymene at 1 mM produced no significant changes in amounts of HSP60, HSP70 or flagellin. Even when the concentration of *p*-cymene was increased to 10 mM no significant changes in HSPs or flagellin were detected (Fig. 3).

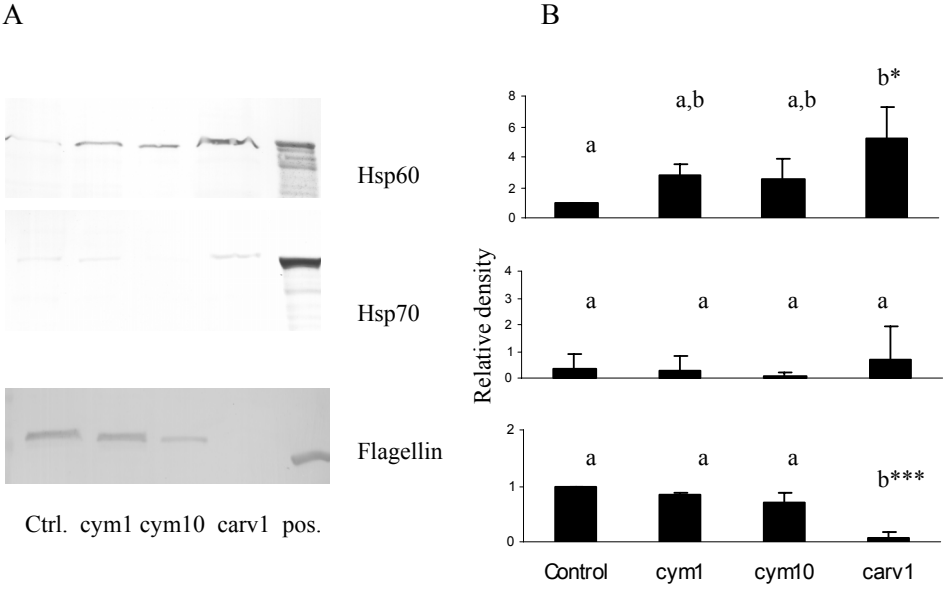

Figure 3. Effects on proteins in *E. coli* O157:H7 ATCC 43895 cells after overnight culture in presence of carvacrol or *p*-cymene. (A) Representative Western blot of HSP 60, HSP 70, and flagellin after 16 h culture in (from left to right) MHB (Ctrl.), MHB with 1.0 mM *p*-cymene (cym1), MHB with 10.0 mM *p*-cymene (cym10) and MHB with 1.0 mM carvacrol (carv1). The right-hand lane shows the positive control (pos.) containing purified preparations of the protein antigens. (B) Relative density results from three independent experiments, quantified by densitometric analysis of the Western blots represented in A. The bars represent the means ± SD of the results. Treatments that are significantly different from each other are indicated with different letters (*$p < 0.05$, ***$p < 0.001$).

Cells incubated with carvacrol were non-motile as determined by the hanging drop technique whilst cells grown in broth containing *p*-cymene were motile or had reduced motility, depending on the concentration present (Table 1). The lack of flagella after growth in carvacrol was also visualized using differential interference contrast microscopy. Representative pictures in Figure 4 show normal, flagellated cells grown in control broth (Fig. 4A) and cells lacking flagella after growth in broth containing 1 mM carvacrol (Fig. 4B). Cells grown in carvacrol

appeared longer and smoother than normal cells. Apparently normally flagellated cells grown in broth containing 1 mM *p*-cymene are shown in Fig. 4C.

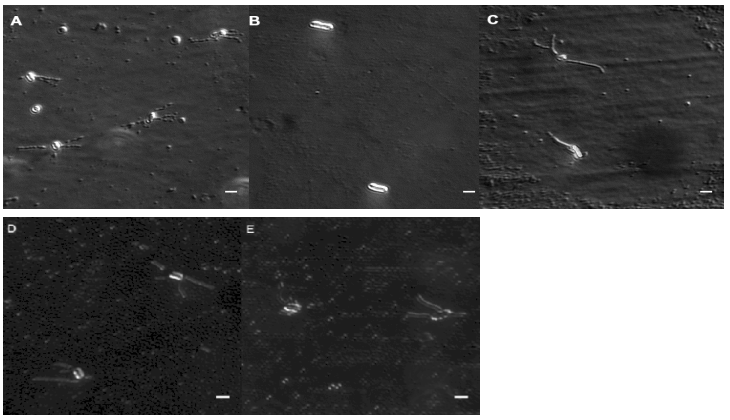

Figure 4. Differential interference contrast images of *E. coli* O157:H7 ATCC 43895 cells after overnight incubation in MHB showing: (A) normal cells with flagella; (B) aflagellate cells grown in MHB containing 1 mM carvacrol; and (C) flagellate cells grown in MHB containing 1 mM *p*-cymene. Cells retained their flagella when treated in the exponential phase with (D) 1 mM carvacrol or (E) 1 mM *p*-cymene. The bars represent 2 μm.

Table 1. Effect of carvacrol and *p*-cymene on the motility of *E. coli* O157:H7 ATCC 43895 cells[a].

			Motility classification		
Treatment during	Essential oil component added	Concn. of essential oil component (mM)	Motile	Reduced motility	Non-motile
Overnight incubation	Control	0	X		
	Carvacrol	1			X
	p-Cymene	1	X		
		10		X	
Mid-exponential growth phase	Control	0	X		
	Carvacrol	0.3		X	
		0.5			X
		0.8			X
		1.0			X
	p-Cymene	0.3		X	
		0.5			X
		0.8			X

[a] Cells were grown overnight in MHB in the presence of the substance or were treated in the exponential growth phase.

Carvacrol treatment in exponential phase.
Western blot analysis revealed that when cells in the exponential phase of growth were treated with increasing concentrations of carvacrol from 0, 0.3, 0.5, 0.8 and 1.0 mM for a period of three hours, no significant changes in HSP60, HSP70 or flagellin levels were detected (Fig.5). There was, however, a numerical increase in amounts of HSP60 produced with increasing concentration from 0, 0.3 to 0.5 mM carvacrol that leveled off at 0.8 mm and decreased again at 1.0 mM (Fig. 5).

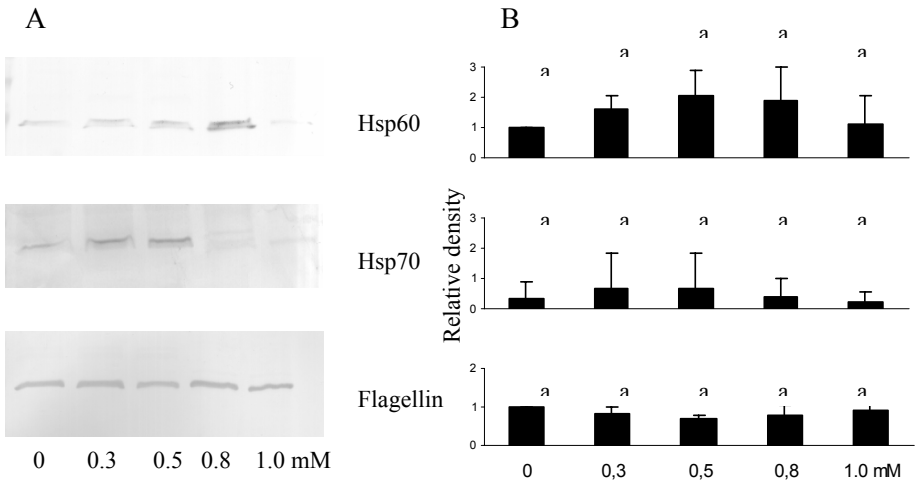

Figure 5. Effects on proteins in *E. coli* O157:H7 ATCC 43895 cells after addition of carvacrol to cells in exponential phase. (A) Representative Western blot of HSP60 (GroEL), HSP70 (DnaK) and flagellin after treatment with increasing concentrations of carvacrol. Cells were grown in MHB and resuspended in MHB containing carvacrol, and incubated for a further 3 h. (B) Relative density results from three independent experiments, quantified by densitometric analysis of the Western blots represented in panel A. The bars represent the means ± SD of the results. Treatments that are significantly different from each other are indicated with different letters ($p < 0.05$).

Colony counts carried out before treatment of the cells and at the moment of cell harvesting revealed that numbers of viable cells increased during the 3 h incubation period in the control and 0.5 mM carvacrol treatments and decreased with the 0.8 mM and 1.0 mM carvacrol treatments (Fig. 6). Adding carvacrol at 0.8 or 1.0 mM to cells in the mid-exponential growth phase apparently reduced numbers of viable cells and would therefore have reduced the number of cells available to make HSPs, which would account for the apparent leveling off in HSP60 amounts from 0.8 mM carvacrol.

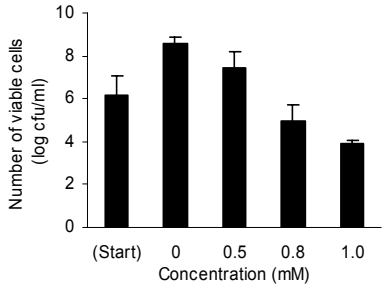

Figure 6. Effect of carvacrol on viability of *E. coli* O157:H7 ATCC 43895 cells in the exponential growth phase. Cells grown in MHB to an OD of 0.5 were centrifuged and resuspended in broth containing 0, 0.5, 0.8 or 1.0 mM carvacrol for 3 h. The bars show the means ± SD of the results of two experiments.

The decrease in the amount of flagellin due to carvacrol treatment in the exponential phase (Fig. 5) was not significant. This indicates that cells at this point in the growth curve already possessed flagella and that the flagella were not shed, as confirmed by the photos in Fig. 4D. A Western blot against flagellin using cells harvested throughout the exponential growth phase in normal broth confirmed that the amount of flagellin is at a high level before the culture reaches mid-exponential phase (OD = 0.5), which was the point at which carvacrol was added (Fig. 7). The motility of cells in the exponential phase of growth decreased with increasing concentration of carvacrol added (Table 1).

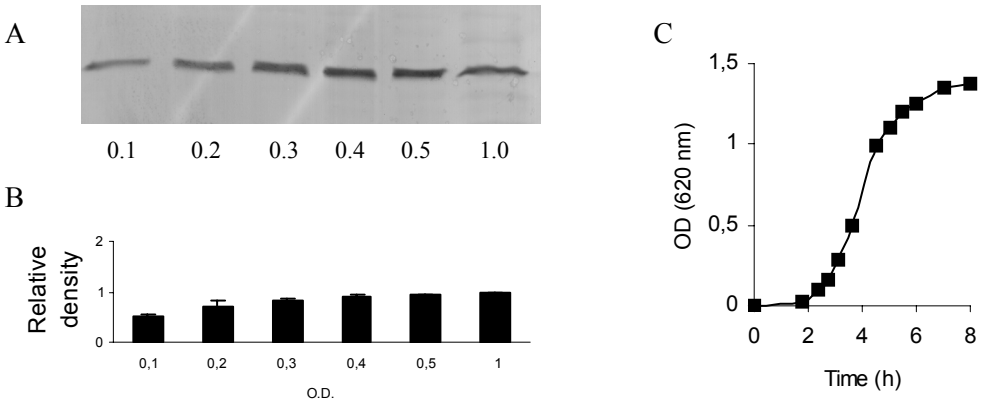

Figure 7 Amounts of flagellin protein in *E. coli* O157:H7 ATCC 43895 cells during exponential growth phase. (A) Representative Western blot of flagellin during growth in MHB. The OD of the bacterial culture is indicated. (B) Relative density results from two independent experiments, quantified by densitometric analysis of the Western blots represented in panel A. The bars represent the means ± SD of the reuslts (C) Growth curve for the strain in MHB. The data points are the means of two experiments.

***p*-Cymene treatment in exponential phase.**
Western blot analysis showed that cells in the exponential phase treated with increasing concentrations of 0, 0.3, 0.5 to 0.8 mM *p*-cymene for a period of three hours exhibited no significant changes in HSP60, HSP70 or flagellin levels (Fig. 8). There was, however, a numerical increase in amounts of HSP60 produced with increasing concentration of *p*-cymene (Fig. 8). Flagella were not shed from existing cells (Fig. 4E). Cell motility decreased with increasing concentration of *p*-cymene added (Table 1).

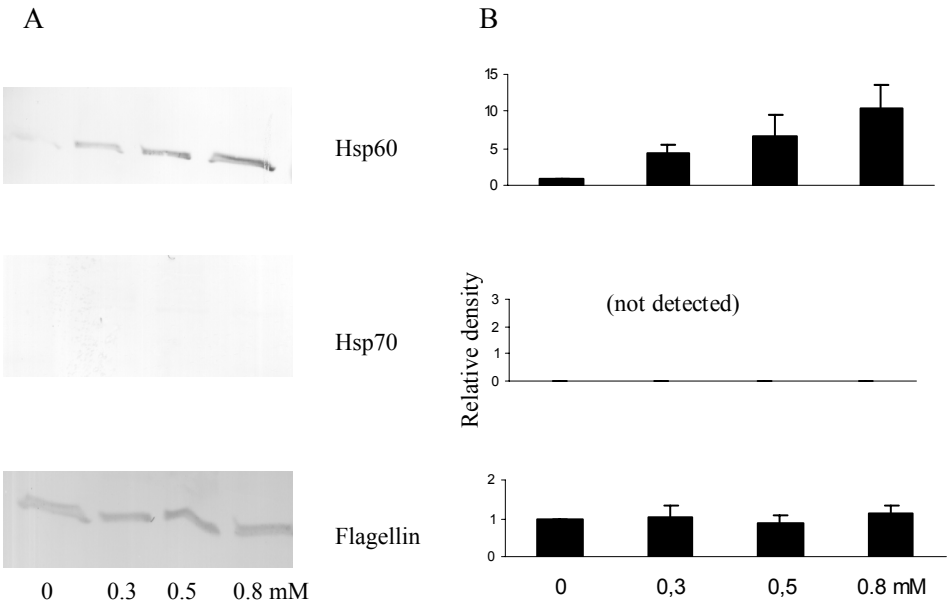

Figure 8 Effects on proteins in *E. coli* O157:H7 ATCC 43895 cells after addition of *p*-cymene to cells in exponential phase. (A) Representative Western blot of HSP 60 (GroEL), HSP 70 (DnaK) and flagellin synthesis after treatment with increasing concentrations of *p*-cymene. The cells were grown in MHB, resuspended in MHB containing *p*-cymene and incubated for a further 3 h. (B) Relative density from two independent experiments, quantified by densitometric analysis of the Western blots represented in panel A. The bars represent the means ± SD of the results.

DISCUSSION

In this report some effects of carvacrol and *p*-cymene on protein synthesis in *E. coli* O157:H7 ATCC 43895 are described. Although it has been shown that treatment with phenol caused *E. coli* to synthesize the heat shock protein HtpG (25), to our knowledge this is the first study to show that the essential oil component carvacrol, a methylated phenol, induces HSP60 in bacteria.

When cells were cultured overnight in the presence of 1.0 mM carvacrol almost no flagellin was synthesized and the cells were therefore aflagellate and non-motile. Flagellin can make up as much as 8% of total cell protein in *E. coli* and the synthesis of flagella requires a considerable amount of energy (24, 29). Being able to cease flagellin production when conditions are unfavorable (for instance when phenolic compounds are present in toxic amounts) and to conserve energy for other cell functions may therefore be a survival tactic (24). Salicylate, whose chemical structure also includes an aromatic ring, has also been shown to inhibit motility of *E. coli* in a concentration dependent manner and to block the synthesis of flagellin (23).

The greater surface smoothness of cells grown overnight with carvacrol observed in this study (Fig. 4B) has previously been reported in a study of the effects of oregano oil (of which carvacrol is one of the major components) on *E. coli* O157:H7 and may be due to modifications in the composition and relative percentages of peptides in the peptidoglycans in the cell wall (7).

That *p*-cymene had less effect than carvacrol on the induction of HSPs and on the inhibition of flagellin synthesis is consistent with it being far less toxic to *E. coli* (6, 12, 22). In overnight culture *p*-cymene did not have a significant influence even when added at a tenfold greater concentration than carvacrol (Fig. 3).

Under normal broth culture conditions this *E. coli* strain produced flagellin from early in the exponential phase of growth and production was at a very high level before an OD of 0.5 was reached (Fig. 7). Introduction of carvacrol in mid-exponential growth phase was therefore unlikely to cause a decrease in the total amount of flagellin, particularly since the presence of the higher concentrations of carvacrol reduced the ability of cells to replicate and reduced viable numbers of bacteria (Fig. 6). From the data provided by blots and photos (Figs. 4, 5 and 8) it is also apparent that adding carvacrol or *p*-cymene in the exponential growth phase did not cause bacteria to shed flagella. It appears that carvacrol must be present whilst cells are dividing in order to have an inhibitory effect on flagellin production.

In this study carvacrol and *p*-cymene added to exponentially growing cells reduced motility in a concentration dependent manner (Table 1). An earlier study also reported that the motility of exponential phase *E. coli* O157:H7 cells decreased

with increasing concentration of carvacrol (14). In that report, 1 mM carvacrol in broth was not enough to arrest motility, possibly because no ethanol was used to aid solution of the carvacrol. However, carvacrol at 5 mM caused an immediate reduction in motility and at 10 mM it caused an immediate cessation of motility and cell death (14). It was recently demonstrated that the speed of the flagellar rotary motor in *E. coli* is directly proportional to the proton motive force (PMF) (13). These observations on motility appear to back up the proposal that carvacrol in some way causes dissipation of the PMF (36).

The fact that *E. coli* cells grown in the presence of carvacrol have no flagella could have implications for the use of carvacrol as an antibacterial additive for foodstuffs or animal feeds. Bacterial flagella, specifically flagellin, activate the host immune response during infection (19, 30) and bacteria often repress the production of flagella after colonization (31). If the use of carvacrol as a food additive were to render any bacteria in the food aflagellate, these bacteria could more easily remain undetected by the immune system of people or animals that consumed the food, which would be undesirable. On the other hand, there could be advantages to rendering bacteria aflagellate because under certain circumstances cells without flagella have been shown to be significantly less able to adhere to epithelial cells and to be less invasive than flagellated cells. For example, flagella of enteropathogenic *E. coli* have been shown to be directly involved in adherence to epithelial cells in vitro (16). Flagella were found to be necessary to enable long-term infection and colonization by *E. coli* O157:H7 in poultry (2), although this was not the case in pigs or in ruminant models (1). The net effect of carvacrol may therefore be advantageous; aflagellate cells may be less able to invade the host and therefore detection by the host's immune system may not be so critical.

The maximum concentration of carvacrol used in this study (1 mM corresponding to approximately 0.015 % v/v) was chosen because, being sub-lethal, it would enable observations of the physiological changes in growing cells. Concentrations of essential oil components in foods and feed that have shown antimicrobial effects are as follows: 0.1-0.25 % essential oil components in soft cheese (28); 0.7-2.1 % v/w oregano oil (approx. 0.3-0.8 % v/w carvacrol equivalent) in eggplant salad (34); 500 ppm oregano oil (approx. 0.02 % carvacrol equivalent) in pig feed (38); and 0.1-1.0 % rubbed thyme leaves (approx. 0.001-0.01 % thymol equivalent) in feed for weanling piglets (17). The concentration of carvacrol used in this study is therefore lower than or in the same range as antimicrobially active concentrations of EO components that have been achieved in foodstuffs and animal feed. The question is whether carvacrol has the same effect on HSP levels and flagella when used in practice. This topic has significant implications for the practical application of carvacrol and merits further study.

In conclusion, this study shows that the presence of antibacterial essential oil component carvacrol (1 mM) during overnight incubation stimulates *E. coli* O157:H7 to produce significant amounts of HSP60 (GroEL) but not HSP70 (DnaK) and prevents synthesis of flagellin, causing cells to be aflagellate and therefore non-motile. The less active antimicrobial component *p*-cymene does not induce HSP60 or HSP70 or prevent the synthesis of flagellin, even when added at a tenfold higher concentration. During a three-hour treatment of exponentially growing cells neither carvacrol nor *p*-cymene induce significant amounts of HSP60 or HSP70, although numerical increases of HSP60 are observed. Both compounds reduce bacterial motility to an extent dependent on the concentration added to the growth medium.

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REFERENCES

1. **Best, A., R. M. La Ragione, D. Clifford, W. A. Cooley, A. R. Sayers, and M. J. Woodward**. 2006. A comparison of Shiga-toxin negative *Escherichia coli* O157 aflagellate and intimin deficient mutants in porcine in vitro and in vivo models of infection. Veterinary Microbiology 113:63-72.
2. **Best, A., R. M. La Ragione, A. R. Sayers, and M. J. Woodward**. 2005. Role for flagella but not intimin in the persistent infection of the gastrointestinal tissues of specific-pathogen-free chicks by Shiga toxin-negative *Escherichia coli* O157:H7. Infect. Immun. 73:1836-1846.
3. **Boog, C. J., E. R. De Graeff-Meeder, M. A. Lucassen, R. Van der Zee, M. M. Voorhorst-Ogink, P. J. Van Kooten, H. J. Geuze, and W. Van Eden**. 1992. Two monoclonal antibodies generated against human hsp60 show reactivity with synovial membranes of patients with juvenile chronic arthritis. Journal of Experimental Medicine 175:1805-1810.
4. **Buchanan, R. L., and M. P. Doyle**. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli.* Food Technology 51:69-76.
5. **Burt, S.** 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology 94:223-253.
6. **Burt, S. A., R. Vlielander, H. P. Haagsman, and E. J. A. Veldhuizen**. 2005. Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers. Journal of Food Protection 68:919-926.
7. **Caillet, S., F. Shareck, and M. Lacroix**. 2005. Effect of gamma radiation and oregano essential oil on murein and ATP concentration of *Escherichia coli* O157:H7. Journal of Food Protection 68:2571-2579.

8. **CFSAN**. 2006. http://www.cfsan.fda.gov/~dms/eafus.html, date consulted: 22 May 2006).
9. **Choi, H.-S.** 2006. Lipolytic effects of citrus peel oils and their components. Journal of Agricultural and Food Chemistry 54:3254-3258.
10. **Commission of the European Communities**. 1999. Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council on 28 October 1996. 1999/217/EC, Official Journal L084, 27/03/1999 p. 1-137. Commission of the European Communities,, Brussels, Belgium.
11. **Di Pasqua, R., N. Hoskins, G. Betts, and G. Mauriello**. 2006. Changes in membrane fatty acids composition of microbial cells induced by addition of thymol, carvacrol, limonene, cinnamaldehyde and eugenol in the growing media. Journal of Agricultural and Food Chemistry 54:2745-2749.
12. **Dorman, H. J. D., and S. G. Deans**. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88:308-316.
13. **Gabel, C. V., and H. C. Berg**. 2003. The speed of the flagellar rotary motor of *Escherichia coli* varies linearly with proton motive force. Proceedings of the National Academy of Sciences USA 100:8748-8751.
14. **Gill, A. O., and R. A. Holley**. 2006. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant essential oils aromatics. International Journal of Food Microbiology 108:1-9.
15. **Gill, A. O., and R. A. Holley**. 2006. Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. International Journal of Food Microbiology 111:170-174.
16. **Girón, J. A., A. G. Torres, E. Freer, and J. B. Kaper**. 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Molecular Microbiology 44:361-379.
17. **Hagmuller, W., M. Jugl-Chizzola, K. Zitterl-Eglseer, C. Gabler, J. Spergser, R. Chizzola, and C. Franz**. 2006. The use of *Thymi Herba* as feed additive (0.1%, 0.5%, 1.0%) in weanling piglets with assessment of the shedding of haemolysing *E. coli* and the detection of thymol in the blood plasma. Berliner und Munchener Tierarztliche Wochenschrift 119:50-54.
18. **Hartl, F.-U., and M. Hayer-Hartl**. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852-1858.
19. **Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem**. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410:1099-1103.
20. **Helander, I. M., H.-L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. Von Wright** 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.
21. **Jamroz, D., T. Wertecki, M. Houszka, and C. Kamel**. 2006. Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. Journal of Physiology and Animal Nutrition 90:255-268.
22. **Juliano, C., A. Mattana, and M. Usai**. 2000. Composition and *in vitro* antimicrobial activity of the essential oil of *Thymus herba-barona* Loisel growing wild in Sardinia. Journal of Essential Oil Research 12:516-522.

23. **Kunin, C. M., H. H. Tong, and L. O. Bakaletz**. 1995. Effect of salicylate on expression of flagella by *Escherichia coli* and *Proteus*, *Providencia* and *Pseudomonas* spp. Infection and Immunity 63:1796-1799.
24. **Li, C., C. Louise, W. Shi, and J. Adler**. 1993. Adverse conditions which cause lack of flagella in *Escherichia coli*. Journal of Bacteriology 175:2229-2235.
25. **Mason, C. A., J. Dünner, P. Indra, and T. Colangelo**. 1999. Heat-induced expression and chemically induced expression of the *Escherichia coli* stress protein HtpG are affected by the growth environment. Applied and Environmental Microbiology 65:3433-3440.
26. **Mayhew, M., and F.-U. Hartl**. 1996. Molecular chaperone proteins, p. 922-937. *In* F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed, vol. I. ASM Press, Washington, D.C.
27. **Mead, P. S., and P. M. Griffin**. 1998. *Escherichia coli* O157:H7. Lancet 352:1207-12.
28. **Mendoza-Yepes, M. J., L. E. Sanchez-Hidalgo, G. Maertens, and F. Marin-Iniesta**. 1997. Inhibition of *Listeria monocytogenes* and other bacteria by a plant essential oil (DMC) en Spanish soft cheese. Journal of Food Safety 17:47-55.
29. **Neidhardt, F. C.** 1987. Chemical composition of *Escherichia coli*, p. 3-6. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. ASM, Washington, D.C.
30. **Ren, T., D. S. Zamboni, C. R. Roy, W. F. Dietrich, and R. E. Vance**. 2006. Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathogens 2:e18/1-9.
31. **Shen, A., and D. E. Higgins**. 2006. The MogR transcriptional repressor regulates nonhierarchical expression of flagellar motility genes and virulence in *Listeria monocytogenes*. PLoS Pathogens 2:e30/1-14.
32. **Si, W., J. Gong, R. Tsao, T. Zhou, H. Yu, C. Poppe, R. Johnson, and Z. Du**. 2006. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. Journal of Applied Microbiology 100:296-305.
33. **Skandamis, P., K. Koutsoumanis, K. Fasseas, and G.-J. E. Nychas**. 2001. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. Italian Journal of Food Science 13:65-75.
34. **Skandamis, P. N., and G.-J. E. Nychas**. 2000. Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs and oregano essential oil concentrations. Applied and Environmental Microbiology 66:1646-1653.
35. **Ultee, A., M. H. J. Bennink, and R. Moezelaar**. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 68:1561-1568.
36. **Ultee, A., E. P. W. Kets, and E. J. Smid**. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65:4606-4610.
37. **Ultee, A., R. A. Slump, G. Steging, and E. J. Smid**. 2000. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. Journal of Food Protection 63:620-624.
38. **Van Krimpen, M. M., and G. P. Binnendijk**. 2001. Ropadiar® as alternative for antimicrobial growth promoter in diets of weanling pigs. Report nr. 205. Praktijkonderzoek Veehouderij Lelystad, The Netherlands.

39. **Wendakoon, C. N., and M. Sakaguchi**. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. Journal of Food Protection 58:280-283.
40. **WHO**. 2002. Food safety and foodborne illness, World Health Organization Fact sheet 237, revised January 2002, Geneva.

Chapter 5

Inhibition of *Salmonella enterica* serotype Enteritidis on agar and raw chicken by carvacrol vapour

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ABSTRACT

Carvacrol is known to inhibit a number of foodborne pathogens. The activity of carvacrol vapour was evaluated against *S. enterica* serotype Enteritidis on tryptone soya agar and on pieces of raw chicken. On agar, the size of the inhibition zone increased with decreasing volume of agar, increasing vapour exposure period, increasing temperature, increasing volume of carvacrol used and with decreasing bacterial density ($p < 0.05$). Inhibition was equally effective under aerobic and anaerobic conditions. On chicken pieces (10 x 10 x 5 mm, UV-sterilized and inoculated with approx. 5 x 10^3 cfu) carvacrol vapour significantly reduced viable numbers of salmonellae at 4, 20 and 37°C and all viable cells were eliminated by a minimum of 3 h at 37°C ($p < 0.05$). A minimum concentration of 20% carvacrol (v/v) in ethanol was required to achieve a significant reduction and from 40% (v/v) no viable cells were recovered. In conclusion, carvacrol vapour is effective at inhibiting the growth of *S. enterica* serotype Enteritidis on agar and at inhibiting and eliminating these bacteria on the surface of raw chicken.

Keywords*: Salmonella enterica* serotype Enteritidis, carvacrol, vapour, chicken, meat, decontamination

INTRODUCTION

Salmonella enterica serotype Enteritidis is the most frequently reported zoonotic disease in many countries (21) and has been reported as causing more deaths than any other species of bacteria in the United States of America (11). Although over the last 40 years this serotype has been associated with the consumption of eggs (12), eating chicken prepared outside the home was recently identified as the greatest risk factor for infection in the U.S.A. (9). Measures to reduce the numbers of cases of illness due to infection include training food handlers, educating the public and developing techniques to reduce contamination of poultry during slaughter and processing (9, 10). Bacteria can be transferred to the surface of the carcase during defeathering and evisceration, and hygiene measures do not prevent contamination entirely (10). Post-processing decontamination of carcases, in the countries where it is allowed, reduces contamination of the outside of the carcase and should reduce the chance of infection (15, 22). This is particularly relevant for food intended for the young and the elderly since these groups have high mortality rates due to *Salmonella* infection (16).

The essential oils of certain herbs such as thyme and oregano are known to exhibit antimicrobial properties against certain foodborne pathogens (5, 13). These properties are due principally to the major constituents carvacrol and thymol, which have possibilities as food preservatives (3, 17). One limitation on the use of essential oils or their constituent in foods is their herbal aroma; in fact carvacrol and thymol are permitted food flavourings in the U.S. and Europe (http://www.cfsan.fda.gov/~dms/eafus.html, date consulted: 10 August 2006) (4, 6, 7). A possible method of achieving the antibacterial effect whilst reducing the flavour impact may be to apply the substances in the form of a vapour. There is some evidence to indicate that this could be achieved; oregano oil in the vapour phase has been shown to have a small effect on the microbial population on the surface of beef under certain conditions (14) and a more significant effect on the flora of sea bass (8). Carvacrol vapour showed antibacterial activity against a selection of food pathogens on agar (1) but did not produce a significant reduction of salmonellae on alfalfa seeds (20). No other studies on the effect of carvacrol vapour on food pathogens on the surface of food are known.

The aim of this study was to determine whether carvacrol vapour could significantly reduce viable numbers of *S. enterica* serotype Enteriditis on agar and on the surface of raw chicken.

MATERIALS AND METHODS

Chemicals.

Carvacrol was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade.

Bacterial cultures.

Salmonella enterica serotype Enteritidis 857, a Dutch field isolate (19), was stored on agar slants at 4°C. Overnight cultures were prepared freshly for each experiment by incubation at 37°C for 16 h in tryptone soya broth (TSB; Oxoid, Basingstoke, U.K.). Before use, the optical density (OD) of the suspension was measured using a Pharmacia Ultraspec III spectrophotometer at 620 nm and the suspensions were diluted in TSB to the bacterial density required.

Carvacrol vapour inhibition assay.

The standard experimental set-up was as follows: A 100 µl portion of a bacterial suspension containing approximately 10^5 cfu/ml was spread over the surface of a tryptone soya agar (TSA; Oxoid) plate and allowed to dry. A paper disc (diameter 45 mm, Schleicher and Schuell, Dassel, Germany) was laid on the inside surface of the lid and 10 µl carvacrol was placed on the disc. The plate inoculated with salmonella was immediately inverted on top of the lid and sealed with parafilm to prevent leakage of the vapour. Plates were incubated at 37°C for 24 h and the diameter of the resulting inhibition zone in the bacterial lawn was measured.

Per experiment, one of the following factors was varied: volume of carvacrol placed on the paper disc (1, 5, 10, 15, 20, 30, 40 or 50 µl); duration of carvacrol vapour treatment prior to removal of the carvacrol disc (30 min, 1, 2, 3, 4, 5 or 16 h); and bacterial load used to inoculate the agar (10^2, 10^3, 10^4, 10^5, 10^6, or 10^7 cfu). To determine whether carvacrol vapour had to diffuse into the agar to cause the antibacterial effect or whether the vapour itself was active, an experiment was performed to compare the use of 12.5 ml or 25 ml TSA per plate. Doubling the agar volume would result in a smaller airspace in the petridish and a shorter distance from the source of carvacrol vapour to the bacteria. If carvacrol exerts its antibacterial action in the vapour phase, doubling the agar volume would therefore increase the inhibition zone obtained. However if carvacrol needs to dissolve into the agar before inhibiting growth of salmonellae, a smaller inhibition zone would be observed because the vapour would diffuse into a larger volume of agar and away from the salmonella at the surface. Furthermore, an experiment was carried out with different holding temperatures during overnight vapour treatment (4, 20, 25 or 37°C) prior to the removal of the carvacrol disc and 24h incubation at 37°C. Aerobic and anaerobic conditions during vapour treatment and incubation were

also compared; anaerobic conditions being achieved by use of GasPak Plus (Becton Dickinson and Co., Sparks, MD, USA). Each experiment was carried out six times.

Chicken decontamination assay.
Chicken breasts were bought from a local supermarket and frozen on day of purchase. The meat was defrosted on day of use and pieces of approximately 10 x 10 mm and 5 mm thick were cut using sterilized equipment. The pieces were exposed to the UV lamp in a laminar flow cabinet for 15 min on both sides so that the natural bacterial flora should interfere as little as possible. In a pilot experiment in which the chicken pieces were not sterilized, the natural flora was treated by the standard carvacrol vapour treatment method described below and no viable cells were recovered (data not shown). Nevertheless, the pieces were sterilized by UV as a precaution.

A 50 µl portion of bacterial suspension containing approximately 10^5 cfu/ml was dotted onto the chicken in a petridish. A paper disc with carvacrol was placed inside the lid of the petridish and secured by means of a small piece of laboratory tape on the back. The dish was sealed with parafilm. As standard, 10 µl carvacrol was placed on the paper disc and the plates were stored at 4°C for 24 h followed by overnight incubation at 37°C. Per experiment, one of these factors was varied: treatment temperature (4, 20 or 37°C); duration held at 37°C before refrigerated storage (1, 2, 3 or 24 h); and concentration of the carvacrol used (0, 20, 40, 60, 80% (v/v) in ethanol or 100% carvacrol); After vapour treatment, chicken pieces were transferred to a sterile plastic bag containing 10 ml minimal TSB (a 100-fold dilution of TSB) and massaged for 90 s in a stomacher machine (Interscience bagmixer, St. Nom, France). Colony counts were carried out by decimal dilution in physiological salt solution (0.85% (w/v) NaCl) and plating out on TSA. Each experiment was carried out four or six times.

Statistical analyses.
SPSS version 12.0.1 software was used to compare group means by analysis of variance followed by post hoc tests of Least Significant Difference or Tukeys HSD test apart from the data for agar volume and anaerobic conditions, which were compared by T-test. All probabilities are quoted as significant at the 5% level ($p \leq 0.05$).

RESULTS AND DISCUSSION

Carvacrol vapour inhibition assay.

The inhibitory effect of carvacrol vapour on the growth of *S. enterica* serotype Enteritidis on agar is presented in Figures 1A to 1D. The inhibitive effect of the vapour increased with increasing volume of carvacrol used from 1 to 20 µl and levelled off from 30 µl carvacrol (Fig. 1A).

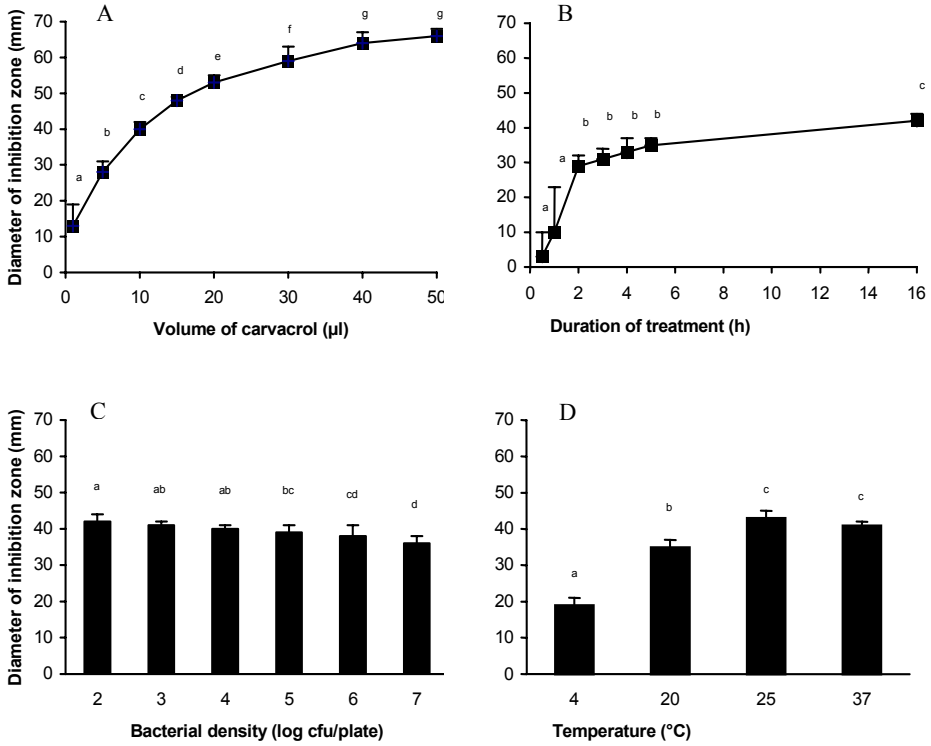

Figure 1. Effect of various conditions on the inhibition of *S. enterica* serotype Enteritidis on TSA at 37°C by carvacrol vapour. A: volume of carvacrol used; B: duration of vapour treatment; C: bacterial density; D: temperature. Data points with different letters are significantly different ($p \leq 0.05$).

The serial increases in diameter of the inhibition zone were significantly different from each other up to 40 µl. Based on the results of this first experiment, the rest of the study was carried out using 10 µl carvacrol in order to make any changes in inhibition zone easily visible. No other studies on the effect of carvacrol vapour on

salmonellae on agar could be found in the literature, however the minimum inhibitory dose of carvacrol vapour against a suspension of *Escherichia coli* dotted onto agar was determined as 5 mg per agar plate (1), which is approximately equivalent to 5 µl and in the same range as our results.

To determine the effect of changes in the duration of vapour exposure on growth of salmonellae, periods from 30 min up to 16 h were evaluated and the results are presented in Figure 1B. A sharp and significant increase in the diameter of the inhibition zone was found during the first two hours of treatment and from 2 h to 16 h further small increases were seen. The difference between 5 h and 16 h was also significant. Longer periods allowed evaporation of more carvacrol and more time for diffusion of vapour through the air and/or agar, enabling interaction with bacterial cells. This is in agreement with a study on the treatment of salmonellae on alfalfa seeds with carvacrol or thymol vapour. Greater reductions in numbers of viable salmonellae were achieved in the order 1 h < 3 h < 7 h (20).

The effect of increasing the bacterial density from 10^2 to 10^7 cfu per plate is shown in Figure 1C. In this range, a total decrease in the average inhibition zone of 6 mm was measured. Although the difference was statistically significant from 10^5 cfu/plate, the total effect of increasing bacterial density was small. This indicates that the method of applying carvacrol vapour to inhibit bacterial growth is fairly insensitive to the magnitude of bacterial contamination.

The effect of temperature during exposure of salmonellae to carvacrol vapour on agar is shown in Figure 1D. Growth was clearly inhibited at 4°C and the efficacy of the vapour improved significantly at 20°C and again at 25°C, presumably due to the increase in volatility of carvacrol with increasing temperature. No further significant improvement in efficacy was seen at 37°C.
Increasing the volume of agar used in the petridish from 12.5 ml to 25 ml caused a significant decrease in the inhibition zone from a mean of 62 ± 3 mm to 44 ± 2 mm. This suggests that it was the concentration of carvacrol vapour diffused into the agar rather than the concentration gradient of carvacrol vapour in the air that had the greatest influence on the inhibition zone. This is confirmed by the fact that fresh bacterial cultures inoculated within the inhibition zones and incubated without carvacrol vapour for a further 24 h did not grow (data not shown).

In standard experiments under aerobic and anaerobic conditions, mean inhibition zones were 40 ± 2 mm and 42 ± 2 mm respectively. This difference was not significant. No other data for testing of carvacrol vapour under anaerobic conditions has been found in the literature. However, vapour of oregano oil, which generally contains a large proportion of carvacrol (2), had a limited effect on the

numbers of *Enterobacteriaceae* on beef and the effect did not differ after packaging in 100% carbon dioxide (14).

Chicken decontamination assay.
The inhibitory effect of carvacrol vapour on the growth of *S. enterica* serotype Enteritidis on pieces of raw chicken is presented in Figures 2 to 4. Treatment with carvacrol vapour significantly reduced viable numbers of salmonellae on chicken relative to the controls at 4°C, 20°C and 37°C (Fig. 2).

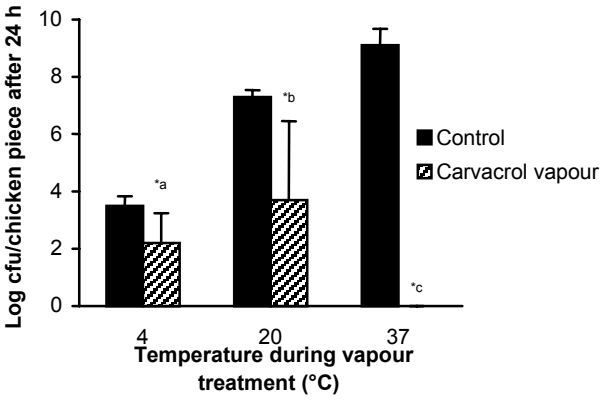

Figure 2. Effect of temperature on *S. enterica* serotype Enteritidis during carvacrol vapour treatment of chicken pieces. Data points with different letters are significantly different and those with asterisks are significantly different to the controls ($p \leq 0.05$).

The possibility of significantly reducing numbers of viable salmonellae on chicken at refrigeration temperature offers perspectives for commercial applications. Other workers have also found that essential oil components can be effective bacterial inhibitors at refrigeration temperatures. A study of the inhibition of mesophilic aerobes on packaged grapes by thymol in air found it to be effective at 1°C; total viable counts were markedly lower than in control packs (18). Similarly, oregano or thyme essential oil vapour in the packaging for sea bass produced a marked inhibition of total viable count for up to 33 d at 2°C (8). A study on the effect of oregano oil vapour on bacterial populations on pieces of beef showed no clear inhibition during 4 d at 15°C and a marginal improvement compared to the control at 5°C (14).

A significant interaction was found between temperature and treatment group; the higher the temperature, the greater was the reduction relative to untreated controls. At 37°C no viable salmonellae were recovered from treated chicken. In an experiment to determine how long a period at 37°C was necessary to achieve elimination of all viable bacteria it was found that a significant reduction was achieved after 1 h of vapour treatment and from 3 h no viable cells were recovered (Fig. 3). A holding temperature of 37°C would not be an obvious choice for application in the poultry-processing sector since it would be generally conducive to the growth of pathogens and a number of spoilage organisms. However it is conceivable that a vapour treatment process could be developed whereby newly slaughtered chicken pieces are decontaminated by carvacrol vapour to eliminate *S. enterica* before refrigeration. Particularly the safety of chicken destined for consumption by immunodeficient individuals would be improved by such a treatment. Further testing on meat with the intact natural bacterial flora would be necessary first.

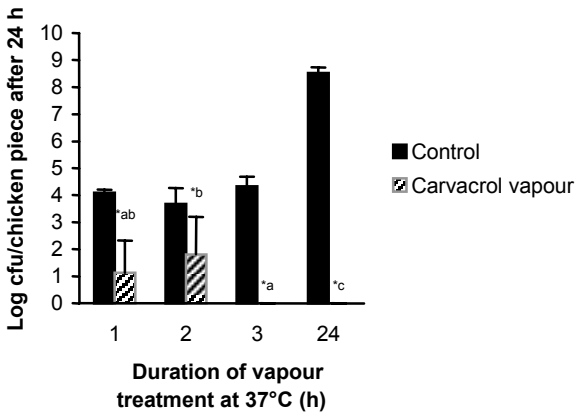

Figure 3. Effect of duration of the holding period at 37°C on *S. enterica* serotype Enteritidis during carvacrol vapour treatment of chicken pieces. Data points with different letters are significantly different and those with asterisks are significantly different to the controls ($p \leq 0.05$).

The minimum concentration of carvacrol necessary to achieve a reduction in salmonellae on raw chicken was determined and the results are presented in Figure 4. Concentrations from 20% (v/v) carvacrol in ethanol were significantly effective and from 40% (v/v) no viable salmonellae were recovered. Since the standard volume of carvacrol solution used was 10 µl, these concentrations are

equivalent to using 2 and 4 µl respectively of pure carvacrol. It is therefore possible that the cost of decontamination of chicken by means of carvacrol vapour could be reduced by the use of a smaller volume or a less concentrated vapour product.

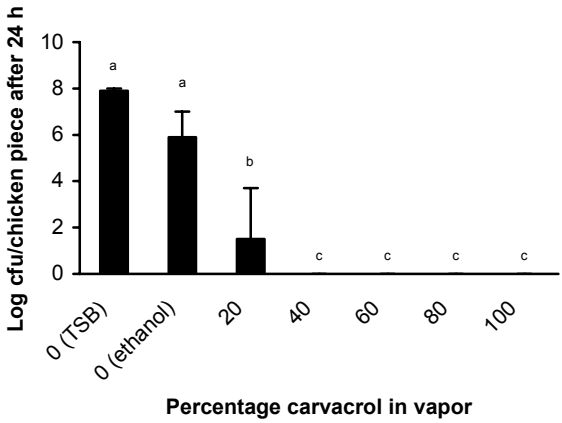

Figure 4. Effect of concentration of carvacrol in the vapour on *S. enterica* serotype Enteritidis during vapour treatment of chicken pieces at 37°C. Data points with different letters are significantly different ($p \leq 0.05$).

Since it appears that carvacrol vapour diffused into the agar in the first set of experiments, it is likely that the vapour also diffused into the layer of moisture and/or cell membranes on the surface of the chicken and inhibited bacterial growth by that route. This could mean that the flavour and aroma of the meat may also be affected and this would limit commercial applications to products where a herbal flavour is desirable.

In conclusion, carvacrol vapour is effective at inhibiting the growth of *S. enterica* serotype Enteritidis on agar and at inhibiting and eliminating these bacteria on the surface of raw chicken under experimental conditions. The size of the inhibition zone on agar is dependent on time, temperature, amount of carvacrol and slightly on bacterial density, but is not dependent on aerobic conditions. On chicken breast meat, carvacrol vapour significantly reduced viable numbers of salmonellae in a temperature range from 4 to 37°C and all viable cells (approx. 5 x 10^3 cfu) were eliminated by a minimum holding period of 3 h at 37°C.

REFERENCES

1. **Ben Arfa, A., S. Combes, L. Preziosi-Belloy, N. Gontard, and P. Chalier**. 2006. Antimicrobial activity of carvacrol related to its chemical structure. Letters in Applied Microbiology 43:149-154.
2. **Burt, S.** 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology 94:223-253.
3. **Burt, S. A., R. Vlielander, H. P. Haagsman, and E. J. A. Veldhuizen**. 2005. Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers. Journal of Food Protection 68:919-926.
4. **Commission of the European Communities**. 1999. Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council on 28 October 1996. 1999/217/EC, Official Journal L084, 27/03/1999 p. 1-137. Commission of the European Communities,, Brussels, Belgium.
5. **Dorman, H. J. D., and S. G. Deans**. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88:308-316.
6. **European Commission**. 1999. Commission Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council on 28 October 1996. 1999/217/EC, Official Journal L084, 27/03/1999 p. 1-137.
7. **European Parliament**. 1996. Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996 laying down a Community procedure for flavouring substances used or intended for use in or on foodstuffs, Official Journal L299 23/11/1996 p.1-4.
8. **Harpaz, S., L. Glatman, V. Drabkin, and A. Gelman**. 2003. Effects of herbal essential oils used to extend the shelf life of freshwater-reared Asian sea bass fish (*Lates calcarifer*). Journal of Food Protection 66:410-417.
9. **Kimura, A. C., V. Reddy, R. Marcus, P. R. Cieslak, J. C. Mohle-Boetani, H. D. Kassenborg, S. D. Segler, F. P. Hardnett, T. J. Barrett, D. L. Swerdlow, and Emerging Infections Program Foodnet Working Group.** 2004. Chicken consumption is a newly identified risk factor for sporadic *Salmonella enterica* serotype Enteritidis infections in the United States: a case-controlled study in FoodNet sites. Clinical Infectious Diseases 38:S244-252.
10. **Mead, G. C., and R. W. A. W. Mulder**. 1997. Management in poultry processing before and after harvest, 20, Microbial control in the meat industry; 2. Concerted Action CT94-1456. University of Bristol Press, Bristol.
11. **Olsen, S. J., L. C. MacKinnon, J. S. Goulding, N. H. Bean, and L. Slutsker**. 2000. Surveillance for foodborne disease outbreaks - United States, 1993-1997. Morbidity and Mortality Weekly Report CDC Surveillance Summary 49:1-62.
12. **Rabsch, W., B. M. Hargis, R. M. Tsolis, R. A. Kingsley, K. H. Hinz, H. Tschäpe, and A. J. Bäumler**. 2000. Competitive exclusion of *Salmonella* Enteritidis by *Salmonella* Gallinarum in poultry. Emerging Infectious Diseases 6:443-448.
13. **Skandamis, P., K. Koutsoumanis, K. Fasseas, and G.-J. E. Nychas**. 2001. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. Italian Journal of Food Science 13:65-75.
14. **Skandamis, P., and G.-J. E. Nychas**. 2002. Preservation of fresh meat with active and modified atmosphere packaging conditions. International Journal of Food Microbiology 79:35-45.

15. **Smulders, F. J. M.** 1995. Preservation by microbial decontamination, the surface treatment of meats by organic acids, p. 253-282. *In* G. W. Gould (ed.), New methods of food preservation. Blackie Academic and Professional, London.
16. **Thorns, C. J.** 2000. Bacterial food-borne zoonoses. Revues Scientifiques et Techniques 19:226-239.
17. **Ultee, A., E. P. W. Kets, and E. J. Smid**. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65:4606-4610.
18. **Valverde, J. M., F. Guillen, D. Martinez-Romero, S. Castillo, M. Serrano, and D. Valero**. 2005. Improvement of table grapes quality and safety by the combination of modified atmosphere packaging (MAP) and eugenol, menthol or thymol. Journal of Agricultural and Food Chemistry 53:7458-7464.
19. **Van Asten, A. J. A. M., K. A. Zwaagstra, M. F. Baay, J. G. Kusters, J. H. J. Huis in 't Veld, and B. A. M. Van der Zeijst**. 1995. Identification of the domain which determines the g,m-serotype of the flagellin of *Salmonella enteritidis*. Journal of Bacteriology 177:1610-1613.
20. **Weissinger, W. R., K. H. McWatters, and L. R. Beuchat**. 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. Journal of Food Protection 64:442-450.
21. **World Health Organization**. 2002. Future Trends in Veterinary Public Health Report nr. Technical report nr. 907. WHO Study Group on Future Trends in Veterinary Public Health Geneva.
22. **Yang, Z., Y. Li, and M. Slavik**. 1998. Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken cracasses. Journal of Food Protection 61:829-832.

Chapter 6

Summarising discussion

Summarising discussion

Essential oils are aromatic oils derived from plants, usually by hydrodistillation. Due to the recent trend in 'green' consumerism, there is increasing interest in the antimicrobial properties of essential oils and the possibility of using them to replace existing 'synthetic' preservatives in food. Other potential uses are as decontaminants for fresh meat or carcases at slaughter and as antimicrobial feed additives for cattle, all with the aim of reducing viable numbers of pathogens in the food chain.

In this thesis experiments are described that were carried out to select plant essential oils that have an inhibitory action on the foodborne pathogen *Escherichia coli* O157:H7, to identify the major active components, and to determine the concentrations and conditions required to inhibit growth and to reduce viable numbers of bacterial cells. Oregano oil was found to be the most effective essential oil and carvacrol and thymol were found to be the major phenolic components, which possess antibacterial activity. The effect of the food additives agar, carrageenan and lecithin on the antibacterial activity of oregano oil and carvacrol were tested and the effect of carvacrol on the synthesis of bacterial proteins was analysed. Lastly, a method of applying carvacrol to fresh meat in the form of a vapour was examined and its efficacy in inhibiting *Salmonella enteritidis* was evaluated.

This chapter discusses the main findings of this research in the context of possible applications for antibacterial essential oils in the food production chain. In addition, suggestions are made for further research.

In **chapter 1** an overview of the literature on the antibacterial activity of plant essential oils is presented (8). Essential oils are volatile aromatic oils derived from part of a plant by hydrodistillation of the dried material. Particularly oils obtained from herbs and spices are known to be inhibitive or lethal to microorganisms, depending on the concentration used. Essential oils are active against Gram negative and Gram positive bacteria at a wide range of pHs and salt concentrations and are composed of several substances representing different chemical groups. The pleasant herbal aroma and flavour of the oils and their major constituents are favourable to applications in food and drink. A number of the constituents that

have antimicrobial properties are approved food flavourings in Europe and the USA.

More recent research has shown that oregano oil and its main component carvacrol may inhibit ATPase activity when added in sub-lethal concentrations and this may play a role in the inhibition of bacterial growth (13, 19). When added at bactericidal concentrations, carvacrol appears to cause irreversible damage to bacterial membranes and this leads to cell death (18, 23). A study of the structural requirements for the antimicrobial activity of carvacrol demonstrated that the hydroxyl group is not essential to antibacterial activity, but that a reactive substituent is required at that position on the benzene ring. The methyl and isopropyl groups are not essential to the antimicrobial action, but probably aid the interaction of the molecule with the bacterial membrane (42).

The results presented in **chapter 2** show that the essential oils of the herbs oregano and thyme appear most active against the selected strain of *E. coli* O157:H7, which was isolated from bovine faeces (10). Oregano oil exhibited its bactericidal effect very quickly on bacteria in broth. Starting with a suspension containing approximately 10^6 cfu/ml, treatment with 156 ppm oregano oil led to no viable cells being recovered after 5 min; after treatment with 625 ppm oregano oil no viable cells were recovered after 1 min. Cells treated with 625 ppm oil and observed under an electron microscope were lysed and apparently empty of cell contents. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for oregano oil were in the same range of concentration as some synthetic food preservatives. For example, the preservatives sorbate and benzoate combined are permitted up to 1500 mg/kg (1500 ppm) in prepared salads and potassium nitrite is permitted up to 150 mg/kg (150 ppm) in meat products (14). The conclusions from this chapter are that oregano oil is a fast and effective inhibitor of *E. coli* O157:H7 and can be lethal when used in high concentrations. This presents possibilities for its use as an antimicrobial additive or decontaminant for food.

In **chapter 3** the chemical composition of two thyme oils and two oregano oils was determined and the relative contribution of the four main components to the antibacterial action of the essential oils was calculated (12). Carvacrol and its isomer thymol, both phenolic compounds, appeared to be almost entirely responsible for the antibacterial activity. The other two major components *p*-cymene and γ-terpinene were found to be inactive against the strain of *E. coli* used and no interactions such as synergy or antagonism between the four compounds could be detected. However, in one oregano oil the activity was much lower than could be expected on the basis of its carvacrol and thymol content. Possibly one of

the other components was interacting with carvacrol and/or thymol, thereby reducing its activity. It is clear from these results that there are large variations in composition between essential oils derived from the same species of plant. The composition of essential oil from a particular species varies according to a number of factors such as season, geographical location, soil status and post-harvest handling (chapter 1). If an essential oil from a particular plant were to be used as an ingredient in food products on a continuous basis, it would be very difficult to obtain a product of a constant composition. At the moment there is no system of quality assurance that guarantees a particular standard of composition or the absence of contaminants. It may be more feasible to determine which specific components in which ratios are responsible for the antibacterial activity of an essential oil and to combine these purified or synthetically derived compounds in the precise ratio desired.

The interaction between essential oils and certain food additives was considerable and has a significant impact on their practical use. In chapters 2 and 3 is described how the stabiliser agar improved the antibacterial action of oregano oil in broth and how the action of carvacrol was improved by agar and carrageenan. The observed enhancement is presumably due to the delayed separation of the essential oil or carvacrol droplets from suspension. Several other adjuvants are mentioned in the literature for aiding dissolution or dispersal of essential oils but not all are approved food additives and some, such as polysorbate 80 (Tween 80), have been shown to neutralise phenolic compounds (15). The emulsifier lecithin markedly reduced the efficacy of oregano oil. In the discussion in chapter 2, this was attributed to fatty acid impurities in the lecithin, which could have been taken up by *E. coli* for membrane repair (25). However, it is more likely that carvacrol, due to its hydrophobic nature, became enclosed within lecithin micelles and therefore came less into contact with bacterial cells. A study on the action of carvacrol on *Listeria monocytogenes* in steak tartare included an investigation of the influence of egg yolk and bovine serum albumin (BSA) on efficacy. It was concluded that carvacrol very likely is inhibited by lipids and fatty acids and it was demonstrated that carvacrol binds to BSA (41). These interactions pose considerable limitations on the use of essential oils as antibacterial additives in food as some lipids and proteins in foods may effectively neutralise the effect of the essential oil on the bacterial cell. To compensate for this, a higher concentration of essential oil may be required, which would lead to a marked flavour increase.

Changes in protein synthesis in *E. coli* O157:H7 brought about by overnight incubation with a sub-lethal concentration of carvacrol were demonstrated in **chapter 4**. The results showed that heat shock protein 60 (HSP60) was induced in significant amounts and flagellin synthesis was almost completely inhibited,

resulting in aflagellate cells (11). Incubation of the same strain with *p*-cymene did not result in significant induction of HSP60 or reduction in flagellin synthesis, even when added at a 10-fold higher concentration, and this is consistent with it being non-toxic to *E. coli* (chapter 3). The function of HSP60 is to fold newly synthesised polypeptides and to catalyse the refolding or degradation of proteins that have become misformed during synthesis or due to unfolding in response to stress (33). These results suggest that the presence of carvacrol during cell division causes increased amounts of proteins to be formed (substrates of HSP60) and/or causes changes to the quaternary structure of proteins during their synthesis.

The repression of flagellin synthesis in *E. coli* O157:H7 by carvacrol is an interesting and potentially useful property. Since flagellin can represent up to 8% of total cell protein in *E. coli* (33) the assembly of flagella from flagellin monomers requires a considerable amount of the available energy (27). Disruption of the proton motive force (PMF) and consequent depletion of the ATP pool has been proposed as a mechanism by which carvacrol may act when present in sub-lethal concentrations (19, 39). The inhibition of flagellar formation could therefore be a mechanism that aids survival of bacteria under toxic circumstances by redirecting the limited amount of energy available to more essential cell functions (27) and is therefore probably a consequence of the dissipation of the PMF.

If carvacrol-induced inhibition of flagellin synthesis can be reproduced in vivo it may be a useful property for application in animal feed. There are already feed additives on the market which contain carvacrol (4, 24, 29, 40), the main purpose of which is said to be improvement of palatability and feed intake. However, feed efficiency performance of these products and additives containing thymol is variable and often not comparable to antibiotic feed additives or organic acids (4, 28). Inhibition of flagella formation could be a useful purpose for plant-derived feed additives. Flagella are essential to bacterial motility and there is evidence to indicate that flagella mediate adherence of pathogenic *E. coli* to epithelial cells in the gut and urinary tract (20, 35, 43). For example, flagella were involved in adhesion of *E. coli* O157:H7 to gut epithelium in poultry (7). However, not all research confirms this; in porcine and ruminant models flagella were not necessary for adhesion (6) nor were flagella necessary for colonisation with a strain of *E. coli* O157:H7 in bovines (16). It would be interesting to study whether carvacrol at an appropriate concentration in intestinal contents can inhibit adhesion and colonisation by pathogenic strains of *E. coli* in the real life situation. Such pathogens picked up from feed, via direct contact with other animals or from the farm environment may then pass through the animal host in the faeces instead of adhering to the gut and causing (sub-clinical) infection. In turn, this would reduce the numbers of pathogens in the gut contents at slaughter, which would reduce the chance of cross-contamination and improve meat safety (5). One obstacle to this

process would be the observed inactivation of carvacrol due to binding with proteins and lipids (chapter 2 and (41)). This problem could be addressed by developing a delivery system (such as encapsulation) or finding an effective synergist. Without such an aid it will be difficult to achieve a physiologically relevant concentration of essential oils in the gut contents. No information was found in the literature on the concentrations attained in the animal gut by feeding these additives. In a trial in which weanling piglets were fed up to 1% (w/w) thyme leaves in the diet (equivalent to 200 ppm thymol), significant increases of thymol levels in blood plasma (mean 500 ng/ml) were found, but faeces were not analysed for thymol. Numbers of haemolysing *E. coli* excreted in the faeces were not significantly different between control and treated groups and growth performance of the piglets was comparable to that of the control group (21).

During bacterial infection, the mammalian host immune system detects flagellin via the Toll-like receptor (TLR) 5 present in gut epithelium, which triggers pro-inflammatory and adaptive immune responses in order to combat infection (22, 30). It is presumed that both detection of flagellin by TLR5 and the detection of virulence proteins by co-receptors are required to trigger the inflammatory response, since flagellated non-pathogens do not elicit such a response in the host (36, 45). However, some pathogenic species are known to repress the production of flagella or to produce mutated flagellin after colonisation, which impairs TLR5 activation (2, 37). It is uncertain whether, if pathogens present in the contents of the gut were to be rendered aflagellate by the introduction of carvacrol into the diet, the immune system of the host would still 'recognise' the bacteria as pathogens and whether the protective immunological reactions would ensue. Further research may reveal whether carvacrol can indeed render *E. coli* O157:H7 aflagellate in vivo and whether this is a beneficial property. If commensal species were also to lose their flagella and become less able to colonise, this would be an undesirable outcome, so this would require further evaluation.

Since essential oils are volatile, their application as a vapour instead of as a liquid may be a feasible method of application to fresh foods as a decontaminant. If the vapour were to act on bacteria on the surface of the food without first coming into contact with the food itself, the flavour impact of essential oils on the food may be reduced. The action of carvacrol vapour against *S. enteritidis* was evaluated in **chapter 5**. The vapour was effective at preventing growth of salmonellae on agar and in significantly reducing viable numbers on raw chicken at temperatures ranging from 4°C to 37 °C (9). The fact that the vapour is effective at refrigeration temperature appears promising for practical applications in the food sector since many foods are stored and transported at low temperatures. Possible uses could be the inclusion of a carvacrol carrier within the packaging for fresh products.

Alternatively, small carcases or pieces of meat could be decontaminated by vapour before further processing. Chapter 5 also reports that carvacrol vapour apparently acts on the salmonellae after first diffusing into the surface of the agar instead of acting directly via the airspace. This could mean that the flavour of the food will be affected to some extent.

Future perspectives and further research

It seems clear that oregano oil and carvacrol show non-selective permeabilisation of the membranes of *E. coli* O157:H7 when introduced at bactericidal concentrations (13, 18, 23). The precise mode of interaction of carvacrol with the bacterial cell membranes has not yet been confirmed. It is generally accepted that both carvacrol and *p*-cymene, being hydrophobic, partition in the bacterial membrane and distort the physical structure. This makes the membrane more fluid and therefore more permeable, leading to the leakage of cations (18, 39). It has been suggested that the carvacrol molecule achieves this effect by aligning between the acyl chains of the phospholipids in the cell membrane (38). *p*-Cymene, lacking the charged hydroxyl group, is more hydrophobic than carvacrol and causes markedly more swelling of bacterial cell membranes than carvacrol (39). However, since *p*-cymene is less antimicrobially active than carvacrol (12, 39), it would appear that not only the disruption of the membrane is necessary to inhibit bacterial growth and survival, but also an interaction between the hydroxyl group and components of the membrane. In this, the hydroxyl group may be replaced by a similarly reactive moiety such as an amino group without greatly reducing the activity (42). It is possible that interaction by means of hydrogen-bonding to proteins located in the cell membrane or in the cytosol contributes to the antibacterial activity of carvacrol. The necessity of the hydroxyl or other reactive moiety to the activity of carvacrol could be related to the described hydrogen-bonding of carvacrol to DNA (32). Further research on interactions between carvacrol and bacterial proteins would seem valid.

More information on the way in which carvacrol interacts with lipids in the bacterial membrane could be obtained by testing unilamellar vesicles made from various lipid mixtures based on the lipid composition of *E. coli* membranes. Measurement of leakage of fluorescent agents from vesicles composed of individual lipids, combinations of lipids and *E. coli* whole lipid under the influence of carvacrol should reveal with which lipids the compound preferably interacts and the contribution of lipid composition to the permeability of bacterial membranes.

It appears likely that the use of oregano and thyme essential oils and/or their individual constituents as antibacterial additives in food and as feed additives will continue in the near future. The use of essential oils as a food preservative,

particularly in foods with a herbal flavour may increase. The use of essential oils for vapour treatment of food may be promising for certain niches in the food industry to prevent growth of spoilage organisms or to reduce viable numbers of pathogens. A method of vaporising essential oil into the air has been developed (3, 17); perhaps a similar technique could be used to vapour-treat fresh meat or carcases within a closed unit. Another method of using the vapour could be the addition of a carvacrol-releasing device within the packaging. The greatest limitation to the use of essential oils and their components, both in vapour and liquid form, will be the interactions with food components and the strong flavour and aroma. These factors may be partially alleviated if synergists can be identified.

The observation that carvacrol inhibits the development of flagella in *E. coli* O157:H7 in vitro leads to the question as to whether carvacrol could inhibit attachment of *E. coli* O157:H7 to epithelial cells in vivo. The progression of this line of research would be firstly to test this in vitro with gut epithelial cell lines. Particularly if this effect is observed on other pathogens too, carvacrol could have a newly recognised function in its role as a feed additive with the aim of preventing adhesion, infection and/or invasion of pathogens in the intestinal mucosa of food animals and thereby indirectly improving meat safety. There is a need for an inexpensive method of pathogen control at farm level; up to now, *E. coli* O157:H7 control in cattle on the farm has always been less cost-effective than decontamination at the slaughterhouse (1). However, in order to achieve effective on-farm use in animals, inactivation of carvacrol by lipids and proteins would need to be reduced by technological means, such as developing methods of encapsulation or controlled release. In this way, the active ingredient could be delivered or released at the location in the alimentary tract where it is required to act.

Many Gram negative pathogens, including enterohaemorrhagic *E. coli* and *S. typhimurium*, use a type III secretory system (TTSS) to secrete toxins and to inject host cells with virulence proteins. These systems are structurally and functionally related to the flagellar export system (26, 44). In chapter 4, carvacrol was shown to inhibit the formation of flagella in *E. coli* O157:H7. It would be interesting to investigate whether carvacrol has any effect on the formation of TTSS and thereby the secretion of other virulence proteins. As mentioned in the discussion of chapter 4, pathogenic bacteria are recognised by host cells by the presence of monomeric flagellin and other virulence proteins. If both the development of flagella and the secretion of virulence proteins were to be inhibited in pathogens present in the gut, the inflammatory reaction by the host cells may be suppressed. However, it has been suggested that the down regulation of flagellar synthesis could be the result of competition with TTSS for the secretion of proteins and for cellular energy (31). Cells lacking flagella may therefore be more virulent

than the flagellated phenotype. Recently, the theory was put forward that it is the anti-inflammatory effect of antimicrobial growth promoters that brings about the advantage in feed efficiency and not the inhibition of microbial growth itself (34). The balance between activation of the immune system in order to prevent infection and the suppression of inflammation to conserve energy could be a productive area for further research with essential oil components, and may contribute to an explanation of the observed variable effects of essential oils on animal feed efficiency performance.

In conclusion, there are several possible applications for oregano and thyme essential oils, and the constituent carvacrol in particular, in improving food safety. The addition of essential oil or carvacrol to food products could inhibit growth and reduce viable numbers of spoilage bacteria and pathogens. The use of carvacrol vapour within food packaging to extend the shelf life and improve safety or as a novel decontaminant for fresh meat is another possibility. A further potential application is in limiting colonisation of certain pathogens in the gut of food animals. However, considering current knowledge of the mechanism of action there remain certain limitations to the effective use of essential oils. Much has still to be learned about the physiological effects of the main components on bacteria. Furthermore, methods of delivering a physiologically relevant dose to the point where the active component is required need to be further developed.

References

1. **Ahmadi, B. V.** 2007. Cost-effectiveness of *Escherichia coli* O157:H7 control in the beef chain. PhD thesis. Wageningen University, Wageningen, The Netherlands. ISBN 978-90-8504-641-7.
2. **Andersen-Nissen, E., K. D. Smith, K. L. Strobe, S. L. Barret, B. T. Cookson, S. M. Logan, and A. Aderem**. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. Proceedings of the National Academy of Sciences USA 102:9247-9252.
3. **Anonymous**. 2007. Essential oils 'combat superbug'. http://news.bbc.co.uk/2/hi/uk_news/england/manchester/6471475.stm: (Date accessed: 27 March 2007).
4. **Benchaar, C., H. V. Petit, R. Berthiaume, T. D. Whyte, and P. Y. Chouinard**. 2006. Effects of addition of essential oils and monensin premix on digestion, ruminal fermentation, milk production and milk composition in dairy cows. Journal of Dairy Science 89:4352-4364.
5. **Berends, B. R., F. Van Knapen, D. A. A. Mossel, S. A. Burt, and J. M. A. Snijders**. 1998. Salmonella spp. on pork at cutting plants and at the retail level and the influence of particular risk factors. International Journal of Food Microbiology 44:207-217.
6. **Best, A., R. M. La Ragione, D. Clifford, W. A. Cooley, A. R. Sayers, and M. J. Woodward**. 2006. A comparison of Shiga-toxin negative *Escherichia coli* O157 aflagellate

and intimin deficient mutants in porcine in vitro and in vivo models of infection. Veterinary Microbiology 113:63-72.

7. **Best, A., R. M. La Ragione, A. R. Sayers, and M. J. Woodward**. 2005. Role for flagella but not intimin in the persistent infection of the gastrointestinal tissues of specific-pathogen-free chicks by Shiga toxin-negative *Escherichia coli* O157:H7. Infect. Immun. 73:1836-1846.
8. **Burt, S.** 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology 94:223-253.
9. **Burt, S. A., M. J. Fledderman, H. P. Haagsman, F. Van Knapen, and E. J. A. Veldhuizen**. 2007. Inhibition of *Salmonella enterica* serotype Enteritidis on agar and raw chicken by carvacrol vapour. International Journal of Food Microbiology (in press).
10. **Burt, S. A., and R. D. Reinders**. 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Letters in Applied Microbiology 36:162-167.
11. **Burt, S. A., R. Van der Zee, A. P. Koets, A. M. De Graaff, F. Van Knapen, W. Gaastra, H. P. Haagsman, and E. J. A. Veldhuizen**. 2007. Carvacrol induces HSP60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7. Applied and Environmental Microbiology 73:4484-4490.
12. **Burt, S. A., R. Vlielander, H. P. Haagsman, and E. J. A. Veldhuizen**. 2005. Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers. Journal of Food Protection 68:919-926.
13. **Caillet, S., F. Shareck, and M. Lacroix**. 2005. Effect of gamma radiation and oregano essential oil on murein and ATP concentration of *Escherichia coli* O157:H7. Journal of Food Protection 68:2571-2579.
14. **Commission of the European Communities**. 1995. European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners., Official Journal L61, 18/03/1995 p. 1-57. European Parliament and Council, Brussels, Belgium.
15. **Cremieux, A., H. Guirard-Dauriac, and G. Dumenil**. 1981. Neutralisation des antiseptiques et désinfectants. Journal de Pharmacie de Belgique 36:223-226.
16. **Dobbin, H. S., C. J. Hovde, C. J. Williams, and S. A. Minnich**. 2006. The *Escherichia coli* O157 flagellar regulatory gene *flhC* and not the flagellin gene *fliC* impacts colonization of cattle. Infect. Immun. 74:2894-2905.
17. **Edwards-Jones, V., R. Buck, S. G. Shawcross, M. M. Dawson, and K. Dunn**. 2004. The effect of essential oils on methicillin-resistant Staphylococcus aureus using a dressing model. Burns 30:772-777.
18. **Gill, A. O., and R. A. Holley**. 2006. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant essential oils aromatics. International Journal of Food Microbiology 108:1-9.
19. **Gill, A. O., and R. A. Holley**. 2006. Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. International Journal of Food Microbiology 111:170-174.
20. **Girón, J. A., A. G. Torres, E. Freer, and J. B. Kaper**. 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Molecular Microbiology 44:361-379.
21. **Hagmüller, W., M. Jugl-Chizzola, K. Zitterl-Eglseer, C. Gabler, J. Spergser, R. Chizzola, and C. Franz**. 2006. The use of *Thymi Herba* as feed additive (0.1%, 0.5%, 1.0%) in weanling piglets with assessment of the shedding of haemolysing *E. coli* and the detection of thymol in the blood plasma. Berliner und Munchener Tierarztliche Wochenschrift 119:50-54.

22. **Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem**. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410:1099-1103.
23. **Helander, I. M., H.-L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. Von Wright** 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46:3590-3595.
24. **Jamroz, D., A. Wiliczkiewicz, T. Wertelecki, J. Orda, and J. Skorupinska**. 2005. Use of active substances of plant origin in chicken diets based on maize and locally grown cereals. British Poultry Science 46:485-493.
25. **Keweloh, H., R. Diefenbach, and H.-J. Rehm**. 1991. Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. Archives of Microbiology 157:49-53.
26. **Lee, S. H., and J. E. Galan**. 2004. Salmonella type III secretion-associated chaperones confer secretion-pathway specificity. Molecular Microbiology 51:483-495.
27. **Li, C., C. Louise, W. Shi, and J. Adler**. 1993. Adverse conditions which cause lack of flagella in *Escherichia coli*. Journal of Bacteriology 175:2229-2235.
28. **Manzanilla, E. G., M. Nofrarias, M. Anguita, M. Castillo, J. F. Perez, S. M. Martin-Orue, C. Kamel, and J. Gasa**. 2006. Effects of butyrate, avilamycin, and a plant extract combination on the intestinal equilibrium of early-weaned pigs. J. Anim Sci. 84:2743-2751.
29. **Manzanilla, E. G., J. F. Perez, M. Martin, C. Kamel, F. Baucells, and J. Gasa**. 2004. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. Journal of Animal Science 82:3210-3218.
30. **Miyamoto, Y., M. Iimura, J. B. Kaper, A. G. Torres, and M. F. Kagnoff**. 2006. Role of Shiga toxin versus H7 flagellin in enterohaemorrhagic Escherichia coli signalling of human colon epithelium in vivo. Cellular Microbiology 8:869-879.
31. **Monday, S. R., S. A. Minnich, and P. C. H. Feng**. 2004. A 12-Base-Pair Deletion in the Flagellar Master Control Gene flhC Causes Nonmotility of the Pathogenic German Sorbitol-Fermenting Escherichia coli O157:H- Strains. J. Bacteriol. 186:2319-2327.
32. **Nafisi, S., A. Hajiakhoondi, and A. Yektadoost**. 2004. Thymol and carvacrol binding to DNA: model for drug-DNA interaction. Biopolymers 74:345-351.
33. **Neidhardt, F. C.** 1987. Chemical composition of *Escherichia coli*, p. 3-6. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. ASM, Washington, D.C.
34. **Niewold, T. A.** 2007. The Nonantibiotic Anti-Inflammatory Effect of Antimicrobial Growth Promoters, the Real Mode of Action? A Hypothesis. Poult Sci 86:605-609.
35. **Ramos, H. C., M. Rumbo, and J. C. Sirard**. 2004. Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. Trends in Microbiology 12:509-517.
36. **Rumbo, M., C. Nempont, J.-P. Kraehenbuhl, and J. C. Sirard**. 2006. Mucosal interplay among commensal and pathogenic bacteria: lessons from flagellin and Toll-like receptor 5. FEBS Letters 580:2976-2984.
37. **Shen, A., and D. E. Higgins**. 2006. The MogR transcriptional repressor regulates nonhierarchical expression of flagellar motility genes and virulence in *Listeria monocytogenes*. PLoS Pathogens 2:e30/1-14.
38. **Ultee, A.** 2000. Bactericidal action of carvacrol towards the food pathogen *Bacillus cereus.* A case study of a novel approach to mild food preservation. Ph.D. thesis. Wageningen University, Wageningen, The Netherlands. ISBN 90-5808-219-9.

39. **Ultee, A., M. H. J. Bennink, and R. Moezelaar**. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 68:1561-1568.
40. **Van Krimpen, M. M., and G. P. Binnendijk**. 2001. Ropadiar® as alternative for antimicrobial growth promoter in diets of weanling pigs. Report nr. 205. Praktijkonderzoek Veehouderij Lelystad, The Netherlands.
41. **Veldhuizen, E. J. A., T. O. Creutzberg, S. A. Burt, and H. P. Haagsman**. 2007. Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against *Listeria monocytogenes* in steak tartare. Journal of Food Protection 70:2127-2132.
42. **Veldhuizen, E. J. A., J. L. M. Tjeerdsma-van Bokhoven, C. Zweijtzer, S. A. Burt, and H. P. Haagsman**. 2006. Structural requirements for the antimicrobial activity of carvacrol. Journal of Agricultural and Food Chemistry 54:1874-1879.
43. **Wright, K. J., P. C. Seed, and S. J. Hultgren**. 2005. Uropathogenic *Escherichia coli* flagella aid in efficient urinary tract colonization. Infection and Immunity 73:7657-7668.
44. **Yip, C. K., B. B. Finlay, and N. C. J. Strynadka**. 2005. Structural characterization of a type III secretion system filament protein in complex with its chaperone. Nature Structural and Molecular Biology 12:75-81.
45. **Zeng, H., A. Q. Carlson, Y. Guo, Y. Yu, L. S. Collier-Hyams, J. L. Madara, A. T. Gewirtz, and A. S. Neish**. 2003. Flagellin Is the Major Proinflammatory Determinant of Enteropathogenic Salmonella. J Immunol 171:3668-3674.

Samenvatting

Antibacteriële activiteit van etherische oliën: mogelijke toepassingen in voedingsmiddelen

Etherische oliën zijn aromatische oliën die verkregen zijn door middel van destillatie uit plantendelen. Ze zijn al lang in gebruik als geur- en smaakstoffen en als geneesmiddelen. Recentelijk is de interesse van wetenschappers voor de chemische samenstelling en de biologische effecten van etherische oliën toegenomen. Vooral de mogelijkheid om bacterieremmende stoffen uit deze oliën te isoleren, krijgt tegenwoordig veel aandacht. Een voorbeeld is het ontwikkelen van conserveringsmiddelen met een 'natuurlijk' karakter voor gebruik in voedingsmiddelen. Andere toepassingen die als doel hebben het terugdringen van het aantal ziekteverwekkende bacteriën in de voedselproductieketen, zijn ook interessant.

Het doel van het onderzoek beschreven in dit proefschrift was om de antibacteriële eigenschappen van etherische oliën afkomstig van kruiden nader te definiëren. In verschillende experimenten is nagegaan bij welke concentraties en onder welke omstandigheden etherische oliën en hun bestanddelen werkzaam zijn tegen bepaalde ziekteverwekkende bacteriën die in voedingsmiddelen kunnen voorkomen (*Escherichia coli* O157:H7 en *Salmonella enterica* serotype Enteritidis).

In deze samenvatting worden de belangrijkste bevindingen uit dit proefschrift gepresenteerd en wordt een aantal suggesties gedaan voor toekomstig onderzoek.

In **hoofdstuk 1** wordt een overzicht van de wetenschappelijke literatuur op dit gebied gepresenteerd. Experimenten in het laboratorium hebben aangetoond dat etherische oliën actief zijn tegen verschillende voedsel gerelateerde ziekteverwekkende bacteriën, waaronder *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus* en *Staphylococcus aureus*. Gram negatieve organismen zijn in het algemeen iets minder gevoelig dan Gram positieve organismen. Van een aantal componenten van etherische oliën is de antibacteriële werking aangetoond, bijvoorbeeld van

carvacrol, thymol, eugenol, perillaldehyde en cinnamaldehyde, met minimum inhibiërende (remmende) concentraties (MICs) van 0.05-5 microliter per milliliter onder laboratorium omstandigheden. Een twee- tot honderd keer hogere concentratie is nodig om hetzelfde effect te bereiken in voedingsmiddelen. Onderzoek met vers vlees, vleesproducten, vis, melk, zuivelproducten, groenten, fruit en gekookte rijst heeft aangetoond dat de concentratie die nodig is om een significant antibacterieel effect te bereiken rond de 0,5-20 microliters per gram ligt in voedingsmiddelen en rond de 0,1-10 microliters per milliliter in oplossingen voor het schoonspoelen van groente of fruit. Etherische oliën bevatten een groot aantal bestanddelen met verschillende chemische eigenschappen. Het is daarom mogelijk dat het werkingsmechanisme van een bepaalde etherische olie berust op een effect op meerdere aangrijpingspunten in de bacteriecel. De hydrofobische eigenschappen van de oliën leiden er waarschijnlijk toe dat zij worden opgenomen in het vettige gedeelte van het celmembraan. De membraan wordt daardoor permeabel en celinhoud kan weglekken. Het is ook mogelijk dat de energievoorziening van de cel wordt aangetast. Een aantal bestanddelen van etherische oliën is goedgekeurd als smaakstoffen in het EU en de Verenigde Staten. Bij gebruik voor antibacteriële doeleinden kunnen ongewenste sterke smaakafwijkingen worden beperkt door zorgvuldige selectie van etherische oliën, afhankelijk van het soort voedingsmiddel. Geconcludeerd kan worden dat een aantal etherische oliën actief is tegen voedsel gerelateerde ziekteverwekkende bacteriën, maar dat een hogere concentratie nodig is in voedingsmiddelen om hetzelfde effect te bereiken in vergelijking met laboratorium omstandigheden. De fenol-achtige bestanddelen lijken het meest actief tegen bacteriën.

Het doel van de experimenten die worden beschreven in **hoofdstuk 2** was om de antibacteriële eigenschappen van vijf etherische oliën te kwantificeren met en zonder de toevoeging van een verdikkingsmiddel (agar) en een emulgator (lecithine) en bij drie verschillende temperaturen. Vijf etherische oliën, waarvan bekend was dat ze enige antibacteriële werking hebben, werden gescreend. Oliën van oregano (*Origanum vulgare*) en tijm (*Thymus vulgaris*) (lichte en rode tijmolie) vertoonden de sterkste antimicrobiële werking, gevolgd door West Indische laurier (*Pimenta racemosa*) en kruidnagel (*Eugenia caryophyllata* / *Syzygium aromaticum*). Oregano-olie was in staat *E. coli* O157:H7 te doden bij een concentratie van 625 microliters per liter bij 10, 20 en 37°C. Reeds na één minuut was deze concentrate in staat alle aanwezige bacteriën te doden. De toevoeging van 0,05% agar (een verdikkingsmiddel) versterkte de antibacteriële eigenschappen, vooral bij 10°C, terwijl 0,25% lecithine (een emulgator) daarentegen de antibacteriële activiteit verzwakte. Foto's gemaakt met behulp van een elektronenmicroscoop toonden aan dat bacteriecellen die behandeld waren met

oregano-olie aanzienlijke beschadigingen vertoonden. De conclusie van dit onderzoek was dat oregano- en tijmoliën onder laboratorium omstandigheden goed in staat zijn om de groei en levensvatbaarheid van *E. coli* O157:H7 tegen te gaan. Deze eigenschappen werden aangetoond bij verschillende temperaturen en werden versterkt door toevoeging van een verdikkingsmiddel en verzwakt door de toevoeging van een emulgator.

In **hoofdstuk 3** wordt beschreven hoe de componenten van oregano- en tijmolie zijn bepaald door middel van *high-performance liquid chromatography* (HPLC) met UV detectie en *liquid chromatographic* tandem massa spectrometrie (LC-MS). De minimum inhibiërende (remmende) concentratie (MIC) en minimum biocidische (dodelijke) concentratie (MBC) van de bestanddelen carvacrol, thymol, *p*-cymeen en γ-terpineen werden bepaald voor een *E. coli* O157:H7, die uit rundermest was geïsoleerd. Voor de meest actieve stoffen, carvacrol en thymol (beide fenol-achtigen) was de MIC 1,2 millimol per liter. *p*-Cymeen en γ-terpineen vertoonden geen antibacteriële werking tot en met 50 millimol per liter. Er werd geen interactie (synergie of antagonisme) tussen de verschillende stoffen vastgesteld. Het effect van de toevoeging van de verdikkingsmiddelen agar en carrageenan op toxische, maar niet dodelijke, concentraties van carvacrol werd onderzocht. Beide toevoegingen versterkten de werking van carvacrol, waarschijnlijk doordat de afscheiding van de vetachtige carvacrol bolletjes uit de waterachtige bouillon door de dikkere consistentie werd vertraagd. Een verdikkingsmiddel verbeterde de werking van carvacrol niet indien carvacrol voorafgaand aan het experiment in alcohol werd opgelost.

In **hoofdstuk 4** worden de resultaten van een onderzoek naar de invloed van carvacrol, een component van oregano-olie, op de eiwitproductie van de bacteriecel beschreven. Na incubatie gedurende 16 uur met een toxische maar niet dodelijke concentratie carvacrol, werden cellen van *E. coli* O157:H7 ATCC 43895 (een referentiestam) geoogst en onderzocht op eiwit samenstelling. Dit werd gedaan door middel van *polyacrylamide gel electroforese* (PAGE) en z.g. Western blotting. De aanwezigheid van carvacrol bleek bacteriën aan te sporen significante hoeveelheden van een bepaald 'stress-eiwit' (*heat shock protein*) HSP60 aan te maken. Het was al bekend dat dit eiwit door de bacterie wordt aangemaakt bij blootstelling aan ongunstige omstandigheden, zoals hoge temperaturen etc. Dat carvacrol dit effect ook bewerkstelligt was nog niet eerder bekend. Een andere opmerkelijke bevinding in dit onderzoek was dat door de aanwezigheid van carvacrol de aanmaak van het eiwit 'flagelline' werd stopgezet. Dit had als resultaat dat de bacteriën geen flagella (zweephaartjes) konden ontwikkelen en dus

niet langer beweeglijk waren. Bacteriën gebruiken flagella om zich voort te bewegen. Van een aantal bacteriesoorten is bekend dat zij met gebruik van flagella makkelijker de darm kunnen koloniseren en ziekteverschijnselen kunnen veroorzaken. Wanneer een praktische manier kan worden gevonden om carvacrol zodanig in voedingsmiddelen toe te passen dat ziekteverwekkende bacteriën geen flagella kunnen ontwikkelen, zou de kans op infecties kunnen worden verkleind. Dit zou zowel bij de mens als bij landbouwhuisdieren van toepassing kunnen zijn. De niet-antimicrobiële stof *p*-cymeen had geen significant effect op de aanmaak van HSPs of flagelline, hetgeen lijkt te suggereren dat deze effecten gerelateerd zijn aan de antibacteriële werking van carvacrol. Cellen behandeld met carvacrol verkeren in een stress-toestand, waarbij de aanmaak van flagelline en daardoor de ontwikkeling van flagella worden verhinderd.

Een mogelijke toepassing van carvacrol kan zijn om het oppervlak van vers vlees te ontdoen van bacteriën. Omdat carvacrol heel makkelijk verdampt, zou het mogelijk zijn deze stof in die vorm toe te passen in plaats van in vloeibare vorm. Wellicht kan op deze wijze ook de geur- en smaakoverdracht van carvacrol op het vlees worden verminderd. In **hoofdstuk 5** wordt een experiment beschreven, waarbij de effectiviteit van carvacroldamp eerst werd getest op bacteriën groeiend op petrischalen en vervolgens op bacteriën die waren aangebracht op stukjes rauwe kipfilet. De bacterie *Salmonella enterica* serotype Enteritidis werd hiervoor gebruikt, omdat deze bacteriestam (vaker dan *E. coli* O157:H7*)* is geassocieerd met voedselinfecties na het eten van kipgerechten. In het experiment met bacteriën op petrischalen, nam de grote van de remmingszone toe naarmate de blootstellingsperiode, de temperatuur of de hoeveelheid carvacroldamp toenam. De aan- of afwezigheid van zuurstof had geen invloed op de remming. Carvacroldamp was ook op kipfilet effectief in het remmen van de uitgroei van salmonella's bij 4°C, hetgeen aangeeft dat de techniek mogelijkheden biedt voor toepassing in de praktijk. Vlees wordt immers bij lage temperatuur opgeslagen, vervoerd en verkocht. Het was mogelijk om de behandeling zo uit te voeren dat geen levende bacteriën werden teruggevonden op de kip. Hiervoor was wel een dampbehandeling bij hogere temperaturen nodig, namelijk gedurende minimaal drie uur bij 37°C. Geconcludeerd kan worden dat, afhankelijk van de geselecteerde temperatuur- en tijdcombinatie, het met behulp van carvacroldamp mogelijk is *Salmonella enterica* serotype Enteritidis op stukjes rauwe kip in de groei te remmen of volledig uit te schakelen.

Toekomstig onderzoek. Van de voor dit onderzoek geselecteerde etherische oliën bleek oregano-olie de meest effectieve antibacteriële olie te zijn; carvacrol bleek een van de belangrijkste antibacteriële componenten van deze olie. Vervolgstudies

zijn gepland om meer te weten te komen over het preciese werkingsmechanisme waardoor carvacrol de groei van bacteriën verhindert. De manier waarop het molecuul een verbinding aangaat met het celmembraan van de bacterie is een onderwerp voor studie. Verder is het van belang na te gaan welke specifieke componenten binnen de bacteriecel door carvacrol worden beschadigd. Bovendien is het interessant te onderzoeken of carvacrol onder praktijkomstandigheden (bijvoorbeeld toegevoegd aan voedingsmiddelen of veevoeder) de vorming van flagella bij ziekteverwekkende bacteriën kan voorkomen. Wellicht kan dit leiden tot het ontwikkelen van middelen die de kolonisatie van *E. coli* O157:H7 en salmonella's in de darm kunnen verminderen, en daarbij ontstekingreacties in de darm en de kans op infecties met deze bacterie bij mensen en landbouwhuisdieren verkleinen.

Curriculum Vitae

Sara Ann Burt was born on 21 December 1961 in Swindon, England and was educated at Faringdon County Grammar School in Oxfordshire. She received her Bachelor of Science (with honours) degree in Food Science from Nottingham University in 1983. After several years working in new product development and as hygiene officer for Associated British Foods (Burton's Biscuits Limited, Cwmbrân, Wales), Sara trained as a teacher. She gained her Post-Graduate Certificate in Education specialising in the teaching of adults at Cardiff University in Wales. In 1988 Sara moved to The Netherlands and in 1989 she joined the Department of the Science of Food of Animal Origin (now the Division of Veterinary Public Health of the Institute for Risk Assessment Sciences, IRAS), teaching veterinary students and assisting with research projects. During the 1990's Sara also developed the department's consultancy work for industry and government concerning veterinary public health. In 2002 she wrote a proposal for a PhD project studying the antibacterial activity of essential oils and their potential applications in improving the safety of foods of animal origin. Since then Sara has combined teaching with her research project, which has resulted in this thesis.

List of publications

Berends, B.R., F. van Knapen, D.A.A. Mossel, **S.A. Burt**, J.M.A. Snijders (1998). "*Salmonella* spp. on pork at cutting plants and at the retail level and the influence of particular risk factors." *International Journal of Food Microbiology* 44(3): 207-217.

Berends, B.R., F. van Knapen, D.A.A. Mossel, **S.A. Burt**, J.M.A. Snijders (1998). "Impact on human health of *Salmonella* spp. on pork in The Netherlands and the anticipated effects of some currently proposed control strategies." *International Journal of Food Microbiology* 44(3): 219-229.

Burt, S.A. and R.D. Reinders (2003). "Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7." *Letters in Applied Microbiology* 36(3): 162-167.

Burt, S. (2004). "Essential oils: their antibacterial properties and potential applications in foods - a review." *International Journal of Food Microbiology* 94(3): 223-253.

Burt, S.A., R. Vlielander, H.P. Haagsman, E.J.A. Veldhuizen (2005). "Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers." *Journal of Food Protection* 68(5): 919-926.

Burt, S.A., R. van der Zee, A.P. Koets, A.M. De Graaff, F. van Knapen, W. Gaastra, H.P. Haagsman, E.J.A. Veldhuizen (2007). "Carvacrol induces HSP60 and inhibits synthesis of flagellin in *Escherichia coli* O157:H7." *Applied and Environmental Microbiology* 73(14): 4484-4490.

Burt, S.A., M.J. Fledderman, H.P. Haagsman, F. van Knapen, E.J.A. Veldhuizen (2007). "Inhibition of *Salmonella enterica* serotype Enteritidis on agar and raw chicken by carvacrol vapour." *International Journal of Food Microbiology* (in press).

Veldhuizen, E.J.A., J. L. M. Tjeerdsma-van Bokhoven, C. Zweijtzer, **S.A. Burt**, H.P. Haagsman (2006). "Structural requirements for the antimicrobial activity of carvacrol." *Journal of Agricultural and Food Chemistry* 54: 1874-1879.

Veldhuizen, E.J.A., T.O. Creutzberg, **S.A. Burt**, H.P. Haagsman (2007). "Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against Listeria monocytogenes in steak tartare." *Journal of Food Protection* 70(9): 2127-2132.

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