UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II

Tesi di Dottorato di ricerca in
Scienze e Tecnologie delle produzioni Agro-alimentari
XVIII ciclo

ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AND THEIR COMPOUNDS AGAINST FOOD BORNE PATHOGEN AND SPOILAGE BACTERIA

Relatore

Prof. Giancarlo Barbieri

Correlatore

Dott. Gianluigi Mauriello

PhD student

Rosangela Di Pasqua

Co-ordinator

Prof. Salvatore Spagna Musso

ACKNOWLEDGEMENTS

It has been a very funny PhD course (both ah! ah! that ah! ah! ah! ah!), it started with tears and finished with a daughter, it couldn't finish better!!!!!!!!!!

Let's go on the acknowledgements:

My mother, my aunt and my two sisters deserve my biggest congratulations to have magnificently put up with me during these years.

Umberto, what to say!, you are the only living being to resist at pH level lower than 1, to the essential oils and their compounds, despite your fear of flying and despite your anxiety, you have magnificently got over this hard PhD course. Your labour has been longer than mine but mine gave birth to Sara.

Gianluigi Mauriello (il Fantastico), you gave me the possibility to do what I did with complete freedom, being a boss without doing it. THANK YOU!!!!!!!!!!!!

Danilo Ercolini deserve my acknowledgements to keep me informed about the packed lunch of the big couple during my staying in UK, and to keep the safety distance when it needed.

I apologize to Annalisa Casaburi, Carmelina Pennacchia and Federica Russo to have unexpectedly interrupt the acidity lessons, it'll come time to make up the lost time.

Athina Papadopoulou (Greek), Wendy Bezuit (French), Chantal Gilbert (Canadian) and Michi Baehr (German), thank you to have had with me good gossip about English life style.

To Nikki Hoskins, Annette Paish, Linda Everis, Rachel Batteridge, Nick Harvey (PPS lab. members) and to all the members of the Microbiology Department at CCFRA I express my thanks to have finally ignored the rule to perform the microbiological

analysis without the laminar flow, everything was carried out with complete freedom of movement.

My last and most important acknowledgement goes to Sara, my daughter, she is the biggest microorganism I've had to do with and will be the best results that a scientist could get.

CHAPTER 1: ESSENTIAL OILS AND THEIR COMPONENTS: ANTIBACTERIAL PROPERTIES, MODE OF ACTION AND POTENTIAL APPLICATION AS NATURAL PRESERVTIVES IN FOODS-AN OVERVIEW

CHAPTER 1

ESSENTIAL OILS AND THEIR COMPONENTS: ANTIBACTERIAL PROPERTIES, MODE OF ACTION AND POTENTIAL APPLICATION AS NATURAL PRESERVTIVES IN FOODS-AN OVERVIEW

1.1 INTRODUCTION

In spite of modern improvements in slaughter hygiene and food production techniques, food safety is an increasingly important public health issue (WHO, 2002a).

There is still a need for new methods of reducing or eliminating food borne pathogens, possibly in combination with existing methods (the hurdle principle; Leistner, 1978). At the same time, Western society appears to be experiencing a trend of 'green' consumerism (Tuley de Silva, 1996; Smid and Gorris, 1999), desiring fewer synthetic food additives and products with a smaller impact on the environment.

There is therefore scope for new 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; Guenther, 1948) 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 (Van de Braak and Leijten, 1999).

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* (Guenther, 1948). An estimated 3000 EOs are known, of which about 300 are commercially important—destined chiefly for the flavours and fragrances market (Van de Braak and Leijten, 1999). It has long been

recognised that some EOs have antimicrobial properties (Guenther, 1948; Boyle, 1955) and these have been reviewed in the past (Shelef, 1983; Nychas, 1995) as have the antimicrobial properties of spices (Shelef, 1983) but the relatively recent enhancement of interest in 'green' consumerism has lead to a renewal of scientific interest in these substances (Nychas, 1995; Tuley de Silva, 1996). Besides anti-bacterial properties (Deans and Ritchie, 1987; Carson *et al.*, 1995a; Mourey and Canillac, 2002), EOs or their components have been shown to exhibit antiviral (Bishop, 1995), antimycotic (Azzouz and Bullerman, 1982; Akgül and Kivanç , 1988; Jayashree and Subramanyam, 1999; Mari *et al.*, 2003), antitoxigenic (Akgül *et al.*, 1991; Ultee and Smid, 2001; Juglal *et al.*, 2002), antiparasitic (Pandey *et al.*, 2000; Pessoa *et al.*, 2002), and insecticidal (Konstantopoulou *et al.*, 1992; Karpouhtsis *et al.*, 1998) properties. These characteristics are possibly related to the function of these compounds in plants (Guenther, 1948; Mahmoud and Croteau, 2002).

1.2 HISTORICAL USE OF ESSENTIAL OILS

Although spices have been used for their perfume, flavour and preservative properties since antiquity (Bauer *et al.*, 2001), of the known EOs, only oil of turpentine was mentioned by Greek and Roman historians (Guenther, 1948). Distillation as a method of producing EOs was first used in the East (Egypt, India and Persia) (Guenther, 1948) more than 2000 years ago and was improved in the 9th century by the Arabs (Bauer *et al.*, 2001). The first authentic written account of distillation of essential oil is ascribed to Villanova (ca. 1235–1311), a Catalan physician (Guenther, 1948). By the 13th century EOs were being made by pharmacies and their pharmacological effects were described in pharmacopoeias (Bauer *et al.*, 2001) 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 (Crosthwaite, 1998). 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 (Guenther, 1948). 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 (Guenther, 1948). The use of tea tree oil for medical 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 (Carson and Riley, 1993). 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 (Boyle, 1955). 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 (Guenther, 1948).

1.3 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) (Bauer and Garbe, 1985; Van Welie, 1997; Van de Braak and Leijten, 1999). The well-known use of EO in aromatherapy constitutes little more than 2% of the total market (Van de Braak and Leijten, 1999).

Individual components of EOs are also used as food flavourings, either extracted from plant material or synthetically manufactured (Oosterhaven *et al.*, 1995).

The antibacterial properties of essential oils and their components are exploited in such diverse commercial products as dental root canal sealers (Manabe *et al.*, 1987), antiseptics (Bauer and Garbe, 1985; Cox *et al.*, 2000) and feed supplements for lactating sows and weaned piglets (Van Krimpen and Binnendijk, 2001; Ilsley *et al.*, 2002).

A few preservatives containing EOs are already commercially available. 'DMC Base Natural' is a food preservative produced by DOMCA S.A., Alhendý´n, Granada, Spain and comprises 50% essential oils from rosemary, sage and citrus and 50% glycerol (Mendoza-Yepes *et al.*, 1997).

Further physiological effects of EOs are made use of in widely differing products such as commercial potato sprout suppressants (Hartmans *et al.*, 1995) and insect repellents (Carson and Riley, 1993).

1.4 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 (Moyler, 1998). 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 (Packiyasothy and Kyle, 2002). 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.* (2001). Detailed compositional analysis is achieved by gas chromatography and mass-spectrometry of the EO or its headspace (Salzer, 1977; Scheffer and Baerheim Svendsen, 1981; Wilkins and Madsen, 1991; Daferera *et al.*, 2000; Juliano *et al.*, 2000; Jerkovic *et al.*, 2001; Delaquis *et al.*, 2002). Eos can comprise more than sixty individual components (Senatore, 1996; Russo *et al.*, 1998). Major components can constitute up to 85% of the EO, whereas other components are present only as a trace (Senatore, 1996; Bauer *et al.*, 2001). The phenolic components are chiefly responsible for the antibacterial properties of EOs (Cosentino *et al.*, 1999). The major antibacterial components of EOs with their structural formulae are presented in Fig. 1. 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 (Marino *et al.*, 2001), certain species of *Thymus* (Lattaoui and Tantaoui-Elaraki, 1994; Paster *et al.*, 1995; Marino *et al.*, 1999) and oregano (Paster *et al.*, 1995).

The composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources (Arras and Grella, 1992; Marotti *et al.*, 1994; McGimpsey *et al.*, 1994; Cosentino *et al.*, 1999; Marino *et al.*, 1999; Juliano *et al.*, 2000; Faleiro *et al.*, 2002). This can be explained, at least in part, by the formation of antibacterial substances from their precursors. p-Cymene (1-meth-yl-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* (Cosentino *et al.*, 1999;

Jerkovic *et al.*, 2001; Ultee *et al.*, 2002). 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 (Kokkini *et al.*, 1997) and to remain stable in plants harvested during different seasons (Jerkovic *et al.*, 2001). The same is true of *Thymus* vulgaris from Italy (Marino *et al.*, 1999). 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 *T. vulgaris* (Kokkini *et al.*, 1997). Generally, EOs produced from herbs harvested during or immediately after flowering possess the strongest antimicrobial activity (McGimpsey *et al.*, 1994; Marino *et al.*, 1999). Enantiomers of EO components have been shown to exhibit antimicrobial activity to different extents (Lis-Balchin *et al.*, 1999). 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 (Delaquis *et al.*, 2002).

1.5 IN VITRO TESTS OF ANTIBACTERIAL ACTIVITY

Tests of antimicrobial activity can be classified as diffusion, dilution or bioautographic methods (Rios *et al.*, 1988). The principles and practice of these test methods are explained in the literature (Barry, 1976; Davidson and Parish, 1989; Hodges and Hanlon, 1991) 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 (Davidson and Parish, 1989). The NCCLS method for antibacterial susceptibility testing, which is principally aimed at the

testing of antibiotics has been modified for testing EOs (Hammer *et al.*, 1999; NCCLS, 2000).

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 (Rios *et al.*, 1988), comparison of published data is complicated (Janssen *et al.*, 1987; Friedman *et al.*, 2002). A number of reviewers have surveyed the methods used for antibacterial activity studies carried out with Eos (Koedam, 1977a,b; Shelef, 1983; Janssen *et al.*, 1987; Rios *et al.*, 1988; Nychas, 1995). 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 anti-bacterial 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. In addition, the term 'minimum cidal concentration' has been used but is not defined (Hammer *et al.*, 1999).The terms 'minimum lethal dilution (or concentration)' (Janssen, 1989; Janssen *et al.*, 1987) and 'minimum inhibitory dilution' (Janssen, 1989) appear to have fallen out of use, at least in food microbiology literature.

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 (Deans *et al.*, 1993; Dorman and Deans, 2000). In order to make bacterial growth easier to visualise, triphenyl tetrazolium chloride may be added to the growth medium (Elgayyar *et al.*, 2001; Mourey and Canillac, 2002). 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 (Prudent *et al.*, 1995; Pintore *et al.*, 2002), different volumes of inoculum (1–100 µl) (Juven *et al.*, 1994; Prudent *et al.*, 1995), which is sometimes dotted (Pintore *et al.*, 2002) and sometimes streaked (Farag *et al.*, 1989) 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 (Farag *et al.*, 1989; Prudent *et al.*, 1995; Pintore *et al.*, 2002). 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 O.D. measurement and the method is more sensitive than the agar dilution method (Mann and Markham, 1998). A patented colour indicator based on resazurin has been used to determine the MICs for Methanolic extracts of plant materials (Salvat *et al.*, 2001) and EOs (Burt and Reinders, 2003) and the method can be automated by measuring the end point by fluorescence instead of visual means (Lancaster and Fields, 1996).

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 (Carson *et al.*, 1995b). 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) (Lambert *et al.*, 2001; Skandamis *et al.*, 2001; Burt and Reinders, 2003). 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 (Chaibi *et al.*, 1997). 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 (Marino *et al.*, 1999; Tassou *et al.*, 2000; Marino *et al.*, 2001).

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 (Ultee *et al.*, 1998; Skandamis *et al.*, 2000; Mejlholm and Dalgaard, 2002). A new method of calculating the MIC from OD measurements has been found suitable for testing combinations of antibacterial substances (Lambert and Pearson, 2000; Lambert *et al.*, 2001). 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 (Friedman *et al.*, 2002). 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 (Beuchat, 1976; Deans and Ritchie, 1987; Karapinar and Aktug, 1987; Aureli *et al.*, 1992; Moleyar and Narasimham, 1992; Juven *et al.*, 1994; Lattaoui and Tantaoui-Elaraki, 1994; Pandit and She- lef, 1994; Sivropoulou *et al.*, 1995, 1996; Ouattara *et al.*, 1997; Marino *et al.*, 1999; Pol and Smid, 1999; Marino *et al.*, 2001; Packiyasothy and Kyle, 2002), methanol (Onawunmi, 1989), Tween-20 (Kim *et al.*, 1995b; Mann and Markham, 1998; Hammer *et al.*, 1999), Tween-80 (Paster *et al.*, 1990; Juven *et al.*, 1994; Carson and Riley, 1995; Cosentino *et al.*, 1999; Mourey and Canillac, 2002; Bassole *et al.*, 2003; Wilkinson *et al.*, 2003), acetone in combination with Tween-80 (Prudent *et al.*, 1995), polyethylene glycol (Pintore *et al.*, 2002), propylene glycol (Negi *et al.*, 1999), n-hexane (Senatore *et al.*, 2000), dimethyl sulfoxide (Firouzi *et al.*, 1998) and agar (Mann and Markham, 1998;

Delaquis *et al.*, 2002; Gill *et al.*, 2002; Burt and Reinders, 2003). However, a number of researchers found it unnecessary to use an additive (Smith-Palmer *et al.*, 1998; Wan *et al.*, 1998; Cosentino *et al.*, 1999; Renzini *et al.*, 1999; Dorman and Deans, 2000; Tassou *et al.*, 2000; Canillac and Mourey, 2001; Elgayyar *et al.*, 2001; Lambert *et al.*, 2001; Cimanga *et al.*, 2002; Mejlholm and Dalgaard, 2002). One study employed vigorous shaking in phosphate saline buffer to suspend EOs (Friedman *et al.*, 2002). 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 (Remmal *et al.*, 1993b). 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 (Remmal *et al.*, 1993a,b). This is supported by the fact that Tween-80 has been recommended as a neutraliser of phenolic disinfectants (Cremieux *et al.*, 1981) and this has been confirmed in work on the action of thyme oil against *Salmonella typhimurium* (Juven *et al.*, 1994). Tween-80 has also been shown to protect *Listeria monocytogenes* from the antibacterial activity of an EO component during freeze–thaw cycles (Cressy *et al.*, 2003). 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 (Carson *et al.*, 1995b).

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.

1.6 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 (Board and Gould, 1991). Since then a fair number of trials have been carried out with EOs in foods. Reports of studies using diluted foods or food slurries (Pol *et al.*, 2001; Smith-Palmer *et al.*, 2001) and studies using dried herbs or spices or their extracts (Tassou *et al.*, 1996; Hao *et al.*, 1998 a,b) 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 (Shelef, 1983; Smid and Gorris, 1999). The ratio has been recorded to be approximately twofold in semi-skimmed milk (Karatzas *et al.*, 2001), 10-fold in pork liver sausage (Pandit and Shelef, 1994), 50-fold in soup (Ultee and Smid, 2001) and 25- to 100-fold in soft cheese (Mendoza-Yepes *et al.*, 1997). 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 (Stecchini *et al.*, 1993; Wan *et al.*, 1998). 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 (Gill *et al.*, 2002). 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 (Shelef, 1983; Tassou *et al.*, 1995). 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 (Tassou *et al.*, 1995, 1996; Skandamis and Nychas, 2000; Tsigarida *et al.*, 2000). 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 (Juven *et al.*, 1994).

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 (Aureli *et al.*, 1992; Pandit and Shelef, 1994; Tassou *et al.*, 1995). 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 (Mejlholm and Dalgaard, 2002). 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 (Smith-Palmer *et al.*, 2001). Mint oil in the high fat products pâté and fish roe salad exhibited little antibacterial effect against *L. monocytogenes* and *S. enteritidis*, whereas in cucumber and yoghurt salad (low fat) the same EO was much more effective (Tassou *et al.*, 1995). 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 (Pol *et al.*, 2001). Protein content has also been put forward as a factor inhibiting the action of clove oil on *Salmonella enteritidis* in diluted low-fat cheese

(Smith-Palmer *et al.*, 2001). Carbohydrates in foods do not appear to protect bacteria from the action of EOs as much as and protein do (Shelef *et al.*, 1984). A high water and/or salt level facilitates the action of EOs (Shelef *et al.*, 1984; Wendakoon and Sakaguchi, 1993; Tassou *et al.*, 1995; Skandamis and Nychas, 2000).

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 gelatine 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 (Skanda-mis *et al.*, 2000). MICs for a particular EO on a particular bacterial isolate have been shown to be generally slightly lower in broth than in agar (Hammer *et al.*, 1999). Research into the growth characteristics of *L. 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 (Brocklehurst *et al.*, 1995). It is known that colonial growth restricts diffusion of oxygen (Wimpenny and Lewis, 1977) 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.

1.7 MODE OF ANTIBACTERIAL ACTION

Although the antimicrobial properties of essential oils and their components have been reviewed in the past (Koedam, 1977a,b; Shelef, 1983; Nychas, 1995), the mechanism of action has not been studied in great detail (Lambert *et al.*, 2001). 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 (Skandamis *et al.*, 2001; Carson *et al.*, 2002).

Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted.

An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Knobloch *et al.*, 1986; Sikkema *et al.*, 1994). Leakage of ions and other cell contents can then occur (Oosterhaven *et al.*, 1995; Gustafson *et al.*, 1998; Helander *et al.*, 1998; Cox *et al.*, 2000; Lambert *et al.*, 2001; Skandamis *et al.*, 2001; Carson *et al.*, 2002; Ultee *et al.*, 2002). 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 (Denyer and Hugo, 1991a). There is some evidence from studies with tea tree oil and *E. coli* that cell death may occur before lysis (Gustafson *et al.*, 1998).

Generally, the EOs possessing the strongest antibacterial properties against food borne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol (Farag *et al.*, 1989; Thoroski *et al.*, 1989; Cosentino *et al.*, 1999; Dorman and Deans, 2000; Juliano *et al.*, 2000; Lambert *et al.*, 2001). 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 (Denyer and Hugo, 1991b; Sikkema *et al.*, 1995; Davidson, 1997).

The chemical structure of the individual EO components affects their precise mode of action and antibacterial activity (Dorman and Deans, 2000). The importance of the presence of the hydroxyl group in phenolic compounds such as carvacrol and thymol has been confirmed (Knobloch *et al.*, 1986; Dorman and Deans, 2000; Ultee *et al.*, 2002). 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, *Staphylococcus aureus* and *Pseudomonas aeruginosa* appears to be comparable to that of carvacrol, for example (Lambert *et al.*, 2001; Ultee *et al.*, 2002). However, in one study carvacrol and thymol were found to act differently against gram-positive and gram-negative species (Dorman and Deans, 2000). The significance of the phenolic ring itself (destabilised electrons) is demonstrated by the lack of activity of menthol compared to carvacrol (Ultee *et al.*, 2002).

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 (Dorman and Deans, 2000). 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 is more active than p-cymene (Dorman and Deans, 2000).

Components of EO also appear to act on cell proteins embedded in the cytoplasmic membrane (Knobloch *et al.*, 1989). 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 (Juven *et al.*, 1994; Sikkema *et al.*, 1995).

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 (Conner and Beuchat, 1984). 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 (Wendakoon and Sakaguchi, 1995). Indications that EO components may act on proteins were also obtained from studies using milk containing different protein levels (Pol *et al.*, 2001).

1.7.1 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 (Lambert *et al.*, 2001).

Carvacrol and thymol are able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) 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 (Helander *et al.*, 1998).

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 (Ultee *et al.*, 2000a).

This distortion of the physical structure would cause expansion and destabilisation of the membrane, increasing membrane fluidity, which in turn would increase passive permeability (Ultee *et al.*, 2002).

Measurement of the average phase transition temperature of the bacterial lipids confirmed that membranes instantaneously became more fluid in the presence of carvacrol (Ultee *et al.*, 2000a).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 (Du) 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 (Ultee *et al.*, 1999). 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 (Ultee, 2000). Oregano EO, containing carvacrol as a major component, causes leakage of phosphate ions from *S. aureus* and *P. aeruginosa* (Lambert *et al.*, 2001).

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 (Ultee and Smid, 2001).

Juven *et al.* (1994) examined the working of thymol against *S. typhimurium* and *S. 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 there-fore more hydrophobic, and so may bind better to the hydrophobic areas of proteins and dissolve better in the lipid phase (Juven *et al.*, 1994).

1.7.2 EUGENOL

Eugenol is a major component (approximately 85%) of clove oil (Farag *et al.*, 1989). Sublethal 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 (Thoroski *et al.*, 1989). The hydroxyl group on eugenol is thought to bind to proteins, preventing enzyme action in *E. aerogenes* (Wendakoon and Sakaguchi, 1995).

1.7.3 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 (Ultee *et al.*, 2002). *p*-Cymene is not an effective antibacterial when used alone (Juven *et al.*, 1994; Dorman and Deans, 2000; Juliano *et al.*, 2000; Ultee *et al.*, 2000a), but when combined with carvacrol, synergism has been observed against *B. cereus* in vitro and in rice (Ultee *et al.*, 2000b). 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 (Ultee *et al.*, 2002).

1.7.4 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 (Oosterhaven *et al.*, 1995). 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 (Helander *et al.,* 1998).

1.7.5 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 (Helander *et al.*, 1998). The carbonyl group is thought to bind to proteins, preventing the action of amino acid decarboxylases in *E. aerogenes* (Wendakoon and Sakaguchi, 1995).

1.7.6 TERPINENE

γ-Terpinene did not antagonize growth of S. Typhimurium (Juven *et al.*, 1994),whereas α-terpinene inhibited 11 of the 25 bacterial species screened (Dorman and Deans, 2000).

1.8 SUSCEPTIBILITY OF GRAM-NEGATIVE AND GRAM-POSITIVE ORGANISMS

Most studies investigating the action of whole EOs against food spoilage organisms and food borne pathogens agree that, generally, EOs are slightly more active against gram-positive than gram-negative bacteria (Shelef, 1983; Shelef *et al.*, 1984; Farag *et al.*, 1989; Mendoza-Yepes *et al.*, 1997; Ouattara *et al.*, 1997; Smith-Palmer *et al.*, 1998; Marino *et al.*, 1999, 2001; Negi *et al.*, 1999; Juliano *et al.*, 2000; Ruberto *et al.*, 2000; Senatore *et al.*, 2000; Canillac and Mourey, 2001; Demetzos and Perdetzoglou, 2001; Lambert *et al.*, 2001; Marino *et al.*, 2001; Cimanga *et al.*, 2002; Delaquis *et al.*, 2002; Pintore *et al.*, 2002; Harpaz *et al.*, 2003). 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 (Ratledge and Wilkinson, 1988), which

restricts diffusion of hydro-phobic compounds through its lipopolysaccharide covering (Vaara, 1992). However, not all studies on EOs have concluded that gram-positives are more susceptible (Wilkinson *et al.*, 2003). *A. hydrophila* (gram-negative) appears in fact to be one of the most sensitive species (Deans and Ritchie, 1987; Stecchini *et al.*, 1993; Hao *et al.*, 1998 a,b; Wan *et al.*, 1998). 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 (Tassou *et al.*, 1995). 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 (Ouattara *et al.*, 1997). 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 (Deans and Ritchie, 1987).

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 (Dorman and Deans, 2000). It was postulated that individual components of EOs exhibit different degrees of activity against gram-positives and gram-negatives (Dorman and Deans, 2000) 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. 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 (Knobloch *et al.*, 1986; Deans and Ritchie, 1987; Paster *et al.*, 1990; Cosentino *et al.*, 1999; Lis-Balchin *et al.*, 1999; Dorman and Deans, 2000; Ruberto *et al.*, 2000; Senatore *et al.*, 2000; Tsigarida *et al.*, 2000; Pintore *et al.*, 2002; Wilkinson *et al.*, 2003).

1.9 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 (Dorman and Deans, 2000; Marino *et al.*, 2001; Delaquis *et al.*, 2002). 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 (Davidson and Parish, 1989).

Some studies have concluded that whole EOs have a greater antibacterial activity than the major components mixed (Gill *et al.*, 2002; Mourey and Canillac, 2002), 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 *S. aureus* and *P. aeruginosa* (Lambert *et al.*, 2001).

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 (Ultee *et al.*, 2000a).

Fractions of cilantro, coriander, dill and eucalyptus EOs (each containing several components), when mixed in various combinations, resulted in additive, synergistic or antagonistic effects (Delaquis *et al.*, 2002). 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 (Moleyar and Narasimham, 1992).

1.10 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 (Gould, 1996). 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 circum-stances with EOs and/or their components. Synergism between NaCl and mint oil against *S. enteritidis* and *L. monocytogenes* has been recorded in taramosalata (Tassou *et al.*, 1995). 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 (Wendakoon and Sakaguchi, 1993). 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) (Ultee *et al.*, 2000b). 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 (Ultee *et al.*, 2000b). Salt at 4% w/v in agar did not improve the antibacterial activity of cinnamaldehyde against gram-positive and gram-negative bacteria (Moleyar and Narasimham, 1992).

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 which germinate and sodium nitrite inhibiting the outgrowth of spores.

Both substances affect vegetative growth (Ismaiel and Pierson, 1990).

The simultaneous application of nisin (0.15 µg ml l^{-1}) and carvacrol or thymol (0.3 mmol l^{-1} or 45 µg ml l^{-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) (Periago *et al.*, 2001). 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 (Pol and Smid, 1999). 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 (Periago and Moezelaar, 2001).

The mechanism of synergy is not known. Previously, it was hypothesised that carvacrol may in-crease the number, size or duration of existence of the pores created by nisin in the cell membrane (Pol and Smid, 1999). 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 (Pol *et al.*, 2002).

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 (Karatzas *et al.*, 2000).

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 (Karatzas *et al.*, 2001).

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 (Paster *et al.*, 1990). The antibacterial activity of oregano and thyme EOs was greatly enhanced against *S. typhimurium* and *S. aureus* at low oxygen levels (Paster *et al.*, 1990). 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 log10 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 (Tsigarida *et al.*, 2000). Similarly, the lethal effect of clove and coriander EOs on *A. hydrophila* on pork loin steak stored at 2 and 10°C was more pronounced in vacuum packed pork than on samples stored in air (Stecchini *et al.*,

1993). 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% CO2 , 30% N2 and 30% O2) when, in contrast, no pronounced inhibition was evident in beef packed under air (Skandamis and Nychas, 2001).

1.11 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 (Commission Decision of 23 January, 2002). New flavourings may only be evaluated for registration after toxicological and metabolic studies have been carried out (Commission Decision of 23 Feb., 1999; Commission Regulation (EC) No. 1565/2000; Commission Regulation (EC) No. 622/2002; Regulation (EC) No. 2232/96), which could entail a considerable financial outlay.

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 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 (Smid and Gorris, 1999).

1.12 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 (Manabe *et al.*, 1987). 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 (Stammati *et al.*, 1999).

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 (Carson and Riley, 2001; Bleasel *et al.*, 2002).

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 (Lis-Balchin *et al.*, 1996; Madeira *et al.*, 2002).

Enantiomers of α-pinene have been shown to have very different spasmogenic effects (Lis-Balchin *et al.*, 1999).

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.

1.13 ORGANOLEPTIC ASPECTS

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 (Tsigarida *et al.*, 2000). 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 (Skandamis and Nychas, 2001). Oregano oil (0.05% v/w) on cod fillets produced a 'distinctive but pleasant' flavour, which decreased gradually during storage at 2°C (Mejlholm and Dalgaard, 2002). 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 (Harpaz *et al.*, 2003). 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 (Ouattara *et al.*, 2001). 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' (Kim *et al.*, 1995b). 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 (Roller and Seedhar, 2002).

1.14 FUTURE PERSPECTIVES

Arguably the most interesting area of application for EOs is the inhibition of growth and reduction in numbers of the more serious food borne 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 of view. 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 (Tuley de Silva, 1996). This applies to the food and cosmetic sectors but also to medicinal products (Bassett *et al.*, 1990). If EOs were to be required in much greater volumes than at present, bioengineering of their synthesis in plants could provide greater yields (McCaskill and Croteau, 1999; Mahmoud and Croteau, 2002).

International standardisation of the composition of commercially available EOs would be essential for reliable applications (Carson and Riley, 2001).

CHAPTER 2

IN VITRO ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS FROM MEDITERRANEAN *APIACEAE, VERBENACEAE* AND *LAMIACEAE* AGAINST FOODBORNE PATHOGENS AND SPOILAGE BACTERIA

2.1 INTRODUCTION

Antimicrobial activity of spices and herbs have been known and described for several centuries (Bagamboula *et al.*, 2003). In recent years, two consumer-driven demands have arisen in the food industry. The first is the provision of fresh and natural foods requiring minimal preparation while the second is the control of food safety (Knobloch *et al.*, 1989). Consumer demand has renewed the use of natural food antimicrobial agents for food preservation (Bagamboula *et al.*, 2003). Essential oils and/or their components are becoming increasingly popular as natural antimicrobial agents to be used for a wide variety of purposes, including food preservation, complementary medicine and natural therapeutics. At present, essential oils are used by the flavouring industry for flavour enhancement and for their antioxidant effect, while the potential use of these oils as natural antimicrobial agents has been less explored (Cosentino *et al.*, 2003). The essential oils arise from a secondary metabolism of the plant, normally formed in special cells or groups of cells or as glandular hair, found on many leaves and stems. Plant volatile oils are generally isolated from [non-woody] plant material by several methods, usually distillation and are variable mixture of principally terpenoids, specifically monoterpenes [C_{10}] and sesquiterpenes [C_{15}] although diterpenes [C_{20}] may also be present. A variety of other molecules may also occur such as aliphatic hydrocarbons, acids, alcohols, aldehyde, acyclic esters or lactones and exceptionally

nitrogen- and sulphur-containing compounds, coumarins and homologues of phenylpropanoids (Dorman and Deans, 2000).

Faleiro *et al.* (2003) have shown that the antimicrobial action is determined by more than one component. In such cases, the major component is not only responsible for the antimicrobial activity but a synergistic effect may take place.

The aim of this work was to investigate the antimicrobial activity of twelve essential oils against a large group of bacteria, including foodborne pathogens and spoilage microorganisms, for a potential use in food industry.

2.2 MATERIALS AND METHODS

2.2.1 PLANT MATERIAL

Plants belonging to *Verbenaceae*, *Lamiaceae* and *Apiaceae* family (table 1) were grown at the Garden of medicinal plants in the Campus of the State University of Salerno. Aerial parts of *Lamiaceae* and *Verbenaceae* species were collected at full flowering stage, in July-August 2003, and fruits of three *Apiaceae* species were collected at ripeness in August 2003. A voucher specimen of each plant was deposited in the herbarium of the Medical Botany Chair, Faculty of Pharmacy, State University of Salerno.

2.2.2 OIL ISOLATION

Twenty-five g of freshly picked aerial parts of each lamiaceous and verbenaceous species were cut into small pieces and then submitted to hydrodistillation for three h, according to the standard procedure reported in the European Pharmacopoeia (1975).

Twenty-five g of fruits of fennel, caraway and anise were submitted to the same procedure. The oil content of each plant, on a fresh weight basis, is reported in table 1.

2.2.3 BACTERIAL STRAINS AND GROWTH CONDITIONS

A pool of eleven bacterial strains, both Gram-positive and Gram-negative and generally recognised as foodborne pathogens and/or spoilage microorganisms, was used in this study (table 2).

For each experiment the strains were cultured as follows: lactic acid bacteria at 30°C (except for the strain *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072^T at 37°C) in MRS (Oxoid) for lactobacilli and M17 (Oxoid) for lactococci and enterococci; *Brochotrix thermosphacta* at 20°C in tryptone soy broth (Oxoid) supplemented with 0.5% yeast extract (Oxoid) (TSBYE); all other strains at 37°C in TSBYE. *Lb. delbrueckii* subsp. *lactis* and *Lb. plantarum* were grown in anaerobic conditions by using Anaerogen Kit (Oxoid).

The strains were routinely maintained at –30°C in media with 20% glycerol added.

2.2.4 ANTIMICROBIAL ASSAY

The antimicrobial activity of the essential oils was determined by agar diffusion method. Briefly, agar media were seeded with 2% of an overnight culture of each indicator strain and poured in Petri dishes (Ø 150 mm). The essential oils were diluted 1:5 (v/v) with methanol and aliquots of 5 µl were spotted onto the surface of the inoculated plates; the same volume of methanol was used as a control. After absorption

of essential oils the plates were incubated for 24 h at optimal temperature in order to detect growth inhibition zones.

2.2.5 Determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC)

Bacteriostatic and bactericidal concentrations of the oils were determined only against the microorganisms that exhibited sensitivity in the previous assay. Each strain was grown over-night at optimal temperature in liquid media added of increasing concentrations of essential oils ranging from 0% to 1% (1% was chosen as the maximum concentration because higher level would probably be unacceptable in food, Smith-Palmer *et al.,* 1998) and microbial growth was checked by spectrophotometric determination at 600 nm.

Minimum inhibitory concentration (MIC) was the lowest concentration at which bacteria failed to growth in liquid media, but yet viable when 100 µl samples were plated on agar media. Minimum lethal concentration (MLC) was the lowest concentration at which bacteria failed to growth in liquid media, but were not cultured after 100 µl samples were plated on agar media (Smith-Palmer *et al.,* 1998). The experiments were three times repeated.

2.3 Results

The MIC and MLC values of the essential oils against all the microorganisms tested are reported in table 2.

The Gram-positive bacteria that exhibited a higher sensitivity to the oils tested were *Staphylococcus aureus* and *B. thermosphacta*, while *Escherichia coli* O157:H7 and

Salmonella were the most sensitive among Gram-negatives. *Listeria monocytogenes* ATCC 7644 and *Lactococcus garvieae* DSM20684 were the least sensitive among the pathogens. Moreover, the spoilage microorganisms belonging to the genera *Lactobacillus* and *Lactococcus* as well as the strains of *Pseudomonas* and *Enterococcus faecium* showed the lowest sensitivity to the essential oils tested. In particular, *L. monocytogenes* ATCC 7644 and *Lactococcus* strains showed to be inhibited only by thyme, oregano, vervain and melissa oils, whereas oils extracted by marjoram, caraway, thyme and oregano inhibited the strain *Pseudomonas* P1. Finally, *Lactobacillus* strains and *E. faecium* B14S showed low sensitivity to the oils from thyme, oregano and vervain.

2.4 DISCUSSION

Essential oils extracted from 12 different aromatic plants were tested for their antimicrobial activity against foodborne pathogens and spoilage bacterial strains, both Gram-positives and Gram-negatives. Many authors, as highlighted below, report the antimicrobial activity of essential oils against foodborne pathogens. Instead, few attentions have been given to the effect of the essential oils on spoilage bacteria (Biavati *et al.*, 2004).

The marjoram essential oil exhibited a selective antimicrobial activity, inactivating only the Gram-negative bacteria tested in this study. Our results were in agreement with those of Friedman *et al.* (2002), who showed that the lethal concentration of marjoram essential oil for Gram-negatives was lower than Gram-positive bacteria. Daferera *et al.* (2003) performed gas-chromatographic analysis of marjoram essential oil and reported, out of a large number of constituents, the carvacrol as major component, a phenolic

compound that had a high bactericidal and bacteriostatic activity as reported by Dorman and Deans (2000). Both *E. coli* O157:H7 and *Salmonella* showed a MIC and a MLC of marjoram oil of 1% and greater than 1%, respectively. In contrast, *Pseudomonas* was more sensitive to this oil resulting inhibited or killed by 0.6% and 0.7%, respectively. This finding was in agreement with Smith-Palmer *et al.* (1998) who reported that bacteriostatic and bactericidal concentration of marjoram oil active against *E. coli* and *Salmonella enteritidis* was above 1%. Marjoram essential oil is also rich in monoterpenoids as γ-ocimene and terpinen-4-ol, as noticed by Daferera *et al.* (2003). Moreover, Carson and Riley (1995) found that, of eight major components of tea tree oil tested, only terpinen-4-ol was active against *Pseudomonas aeruginosa*. The antimicrobial activity of γ-ocimene and terpinen-4-ol rises in presence of substances increasing cell permeability such as phenolic compounds. Thus carvacrol, present in the marjoram and thyme essential oil, is able to increase cell membrane permeability and allows accumulation of γ-ocimene and terpinen-4-ol in cytoplasmic membranes to toxic levels (Mann *et al.*, 2000).

The principal components of thyme and oregano essential oils are carvacrol and thymol (Daferera *et al.*, 2003). Helander *et al.* (1998) described the cell outer membrane disintegrating property of thymol, which is similar to the above-mentioned action of carvacrol. The results of antimicrobial activity of thyme and oregano essential oils showed that the Gram-negatives were more sensitive than Gram-positives. In particular, *Salmonella* died in presence of 0.04% of thyme or oregano essential oils, while *E. coli* O157:H7 was killed by a concentration of 0.15% and 0.08% of the same oils. On the other hand, the concentration of these oils that determine the inactivation of lactic acid bacteria strains was above 1%. MLCs of thyme and oregano essential oils against *S.*

aureus DSM 20231 and *B. thermosphacta* 7R1 were above 0.19%. In agreement with our results Burt and Reinders (2003) and Sagadic *et al.* (2002) reported, among respectively four and seven tested essential oils, oregano and thyme as those with the strongest bactericidal and bacteriostatic effect against *E. coli* O157:H7.

The caraway oil, rarely tested in other studies, showed an antagonistic activity against *E. coli* O157:H7, *Salmonella, S. aureus* and *B. thermosphacta* strains at level above 1%, while *Pseudomonas* strain was inhibited at a concentration of 0.6%. The rest of the strains were not affected by the presence of caraway essential oil in the culture media. Hao *et al.* (1998) found that a population of *L. monocytogenes* inoculated on chicken breast, which was previously treated with caraway seeds essential oil, was not affected by the presence of the oil. On the other hand, Friedman *et al.* (2002) found that the 50% of the microbial population of two strains of *L. monocytogenes* were killed by 0.33% and 0.24% of caraway oil, respectively. In addition, the same authors found that *E. coli* and *Salmonella enterica* were killed by 0.46% and 0.47% of caraway oil, respectively.

Hyssopus officinalis essential oil appeared to have efficacy only against *E. coli* O157:H7, *Salmonella* and *B. thermosphacta* at concentrations above 1%. Mazzanti *et al.* (1998) found that the essential oil of *H. officinalis* from Italy showed a lower activity than one of French origin against different bacterial species. Furthermore, Marino *et al.* (2001) evidenced a weak antibacterial effect of this essential oil.

Fennel and anise essential oils displayed antimicrobial activity against few strains only at concentration above 1%. Likewise, Hammer *et al.* (1999) found that both essential oils were active only at concentrations above 1% against *Pseudomonas* and *Salmonella* while *E. coli* and *S. aureus* were more sensitive. The essential oil of *Verbena officinalis* appeared to have good antimicrobial performances. This essential oil was more active

against Gram-positive than Gram-negative bacteria, in particular *L. monocytogenes* and *S. aureus* were the most sensitive among Gram-positives. *B. thermosphacta* was killed by 0.9% of essential oil, while lactic acid bacteria and *E. coli* were less sensitive, in fact their inactivation occurred at concentrations greater than 1%. To our knowledge, no other author reported antimicrobial experiments using this oil, which may be effectively used as antimicrobials into spiced foods.

Our results showed that the essential oil of basil was weakly active against the tested microorganisms. However, Gram-negatives were more sensitive than Gram-positives as also reported by Koga *et al.* (1999). Furthermore, Baratta *et al.* (1998) reported that *B. thermosphacta* and *Pseudomonas aeruginosa* were resistant to undiluted basil oil, while in our study *B. thermosphacta* showed to be sensitive to basil oil at 0.8%. In contrast with our results, Smith-Palmer *et al.* (1998) found the basil oil as highly active against all five foodborne pathogens tested. The divergent results may be due to the origin of plant material that has an enormous influence on the chemical composition of basil oil (Suppakul *et al.*, 2003).

The oil of *Melissa officinalis* showed low antimicrobial activity against *Lactobacillus*, *Enterococcus*, *Pseudomonas* and *S. aureus* strains. Moreover, its activity against the other microorganisms tested was evidenced at concentrations above 1%. Sage and lavender essential oils also showed low antimicrobial activity. In fact, only *E. coli* O157:H7 and *Salmonella* were sensitive but at concentrations above 1%. In agreement with our results low activity of sage oil against *E. coli* and *Salmonella* also by Smith-Palmer *et al.* (1998) was evidenced. However, the same authors found *S. aureus* and *L. monocytogenes* sensitive at low concentration of this oil. Furthermore, Hammer *et al.*

(1999) and Friedman *et al.* (2002) found antibacterial activity of these oils against both foodborne pathogens and spoilage microorganisms.

In conclusion, the oils extracted by thyme, oregano and vervain showed to be the more effective being active against all the strains tested, with exclusion of vervain for *Pseudomonas* spp. strain. All the oils showed bacteriostatic and bactericidal activity against *E. coli* O157:H7 ATCC 35150 and *Salmonella* Typhimurium ATCC 6994, while eight of 12 tested oils showed to influence the growth of *B. thermosphacta* 7R1. Finally, the pathogenic microorganism *L. monocytogenes* ATCC 7644 and lactic acid bacteria strains were affected only by oils from thyme, oregano and vervain and occasionally by melissa oil. Thus, our results confirm that many essential oils possess *in vitro* antimicrobial activity against pathogens. Moreover, in this study the effect of some essential oils against spoilage bacteria was ascertained, suggesting that they may be used for the development of novel systems for food preservation. However, further studies are necessary to investigate the possible interaction between oils and food components for their use in food.

TABLES CHAPTER 2

Table 1 List of plant species and % (w/w) content of essential oil on a fresh weight basis

Botanical name	Family	Common name	Plant part	Oil content (%)
Carum carvi L.	Apiaceae	Caraway	Fruits	2.80
Foeniculum vulgare M.	Apiaceae	Fennel	“	2.30
Hyssopus officinalis L.	Lamiaceae	Hyssop	Aerial parts	0.41
Lavandula angustifolia L.	Labiateae	Lavender	“	0.49
Majorana hortensis L.	Labiateae	Marjoram	“	0.26
Melissa officinalis L.	Lamiaceae	Melissa	“	0.25
Ocimum basilicum L.	Lamiaceae	Basil	“	0.42
Origanum vulgare L.	Lamiaceae	Oregano	“	0.21
Pimpinella anisum L.	Apiaceae	Anise	Fruits	1.80
Salvia officinalis L.	Lamiaceae	Sage	Aerial parts	0.46
Thymus vulgaris L.	Lamiaceae	Thyme	“	0.26
Verbena officinalis L.	Verbenaceae	Vervain	“	0.39

Table 2 MIC and MLC (% v/v) of various essential oils against foodborne pathogenic bacteria (values are means of three repetitions)

Essential oils	Gram-negative						Gram-positive					
	Salmonella Thyphimurium ATCC 6994		*E. coli* O157:H7 ATCC 35150		*Pseudomonas* spp.		*L. monocytogenes* ATCC 7644		*S. aureus* DSM 20231		*Lc. garvieae* DSM 20684	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
Marjoram	1	> 1	1	> 1	0.6	0.7	-	-	-	-	-	-
Thyme	0.03	0.04	0.07	0.15	0.15	0.2	0.1	0.15	0.17	0.19	>1	>1
Caraway	> 1	> 1	> 1	> 1	0.6	0.7	-	-	1	> 1	-	-
Oregano	0.03	0.04	0.07	0.08	0.15	0.2	0.08	0.09	0.18	0.2	>1	>1
Hyssop	> 1	> 1	> 1	> 1	-	-	-	-	-	-	-	-
Anise	> 1	> 1	> 1	> 1	-	-	-	-	-	-	-	-
Fennel	> 1	> 1	> 1	> 1	-	-	-	-	> 1	> 1	-	-
Vervain	0.7	0.8	> 1	> 1	-	-	0.1	0.15	0.19	0.2	>1	>1
Basil	> 1	> 1	> 1	> 1	-	-	-	-	> 1	> 1	-	-
Melissa	> 1	> 1	> 1	> 1	-	-	> 1	> 1	-	-	>1	>1
Lavander	1	> 1	1	> 1	-	-	-	-	-	-	-	-
Sage	1	>1	> 1	> 1	-	-	-	-	-	-	-	-

continued on next page

Table 2 *(continued)*

Essential oils	Gram-positive									
	Lb. plantarum DSM 20174		*Lb. delbrueki* subsp. *lactis* DSM 20072		*Ec. faecalis* B14S		*Lc. lactis* subsp *lactis* DSM 20729		*B. thermosphacta* 7R1	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
Marjoram	-	-	-	-	-	-	-	-	-	-
Thyme	>1	>1	>1	>1	>1	>1	>1	>1	0.25	0.3
Caraway	-	-	-	-	-	-	-	-	1	> 1
Oregano	>1	>1	>1	>1	>1	>1	>1	>1	0.7	0.8
Hyssop	-	-	-	-	-	-	-	-	> 1	> 1
Anise	-	-	-	-	-	-	-	-	> 1	> 1
Fennel	-	-	-	-	-	-	-	-	> 1	> 1
Vervain	>1	>1	>1	>1	>1	>1	>1	>1	0.8	0.9
Basil	-	-	-	-	-	-	-	-	-	-
Melissa	-	-	-	-	-	-	>1	>1	1	> 1
Lavander	-	-	-	-	-	-	-	-	-	-
Sage	-	-	-	-	-	-	-	-	-	-

CHAPTER 3

ALTERATION IN CELLULAR FATTY ACIDS COMPOSITION, AN ADAPTIVE STRESS MECHANISM INDUCED BY SUBLETHAL CONCENTRATIONS OF ACTIVE COMPOUNDS FROM ESSENTIAL OILS.

3.1 INTRODUCTION

In the last twenty years much interest has been focused on understanding the mechanisms involved in adaptation of microbial cells to environmental conditions (Denich *et al.*, 2003).

With an increase in concerns of bacterial resistance in the clinical sector, pharmaceutical and food processing environments there is a need to understand these mechanisms and evaluate the potential for development of microbial resistance in these areas. It is well known that sublethal exposure to particular environmental conditions and antimicrobial substances may result the development of increased resistance and the promotion of cross-resistance to antimicrobial compounds (Russell, 1984; Sampathkumar *et al.*, 2004; Yuk and Marshall, 2004).

This work is exclusively focused on some foodborne pathogens and spoilage microorganisms and their ability to adapt the cytoplasmic membrane to sublethal environmental stress. Many of these microorganisms are capable of undertaking an adaptive response to sublethal stresses, enabling them to tolerate and survive subsequent exposure to normally lethal levels of the same stress or even a different type of stress (Roller, 2003).

The major adaptive response of the cells is to keep the fluidity of their membrane at a constant value, irrespective of actual environmental conditions. Such stabilisation of membrane fluidity, known as *homeoviscous* adaptation, constitutes the predominant

response of bacteria to membrane-active substances or changing environmental condition (Heipieper *et al.*, 2003), preventing the loss of the mechanical and chemical properties of the lipid bilayer (Russell and Fukanaga, 1990). If disturbance of membrane integrity occurs, then its functions as a barrier, as a matrix for enzymes and as an energy transducer is compromised (Heipieper *et al.*, 2003).

To decrease the effects of environmental changes on the membrane, the cell regulates its fluidity by changing the proportion of *iso* and *anteiso* branched fatty acids, isomerization of *cis* unsaturated fatty acids to corresponding *trans* isomers and altering the average fatty acid chain length, protein content and fatty acid composition (Mrozik *et al.*, 2004).

Lipids have manybiological functions in microbial cells and, consequently, a large body of research is devoted to lipids and their role in cell physiology (Guerzoni *et al.*, 2001).

Variations in temperature, pH, ethanol concentration and external osmolarity, the presence of substances able to affect the microbial growth, as well as transition to the stationary phase, lead to the alteration of fatty acid content to control membrane viscosity (Denich *et al.*, 2003; Russell, 1984).

The increase in unsaturation, on reducing the growth temperature, has been described for several microbes (Suutari *et al.*, 1990; Suutari and Laakso, 1994) and can be regarded as a universally conserved adaptation response (Keweloh and Heipieper, 1996). In particular, the way in which the fatty acid composition of membrane lipids is altered in response to growth temperature appears to depend on the mechanism of unsaturated fatty acid (UFA) synthesis utilised (Keweloh and Heipieper, 1996). In fact, the incorporation of more UFAs into the membrane tends to increase membrane fluidity (Ingram, 1976). Recent work suggests that monounsaturated membrane fatty acids are

necessary for maintenance of a ΔpH across the membrane (Fozo *et al.*, 2004; Fozo and Quivey, 2004) and an increase in fatty acid length is another important membrane alteration to increase survival in acidic environments (Fozo *et al.*, 2004).

The lipophilic character of the compounds used in this study suggests interaction with bacterial membranes, in fact the hydrophobicity of these molecules enables them to partition in the lipids of the bacterial cell membrane, disturbing the structure and rendering it more permeable (Knobloch *et al.*, 1986; Sikkema *et al.*, 1994).

Little is known about physiological changes in foodborne pathogens and spoilage bacteria exposed to a sublethal concentration of natural antimicrobial compounds.

The European Commission has registered a number of essential oil components 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 (Commission Decision of 23 January, 2002). However, the prospect of using natural antimicrobial compounds as preservatives in food raises the need for more information on the behaviour of spoilage and pathogenic microorganisms that may adapt to otherwise lethal concentrations of these compounds and subsequently exhibit altered survival or growth behaviour and increased resistance to other environmental stresses imposed by traditional preservation technologies. The aim of this study was to investigate alterations in the cellular fatty acid composition, an adaptive stress mechanism induced by sublethal concentrations of active compounds from essential oils.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIAL STRAINS.

Escherichia coli O157:H7 ATCC 43888 (non toxigenic), *Salmonella enterica* serovar Typhimurium ATCC 14028, *Pseudomonas fluorescens* NCIMB 10586, *Brochothrix thermosphacta* NCTC 10822 and *Staphylococcus aureus* NCTC 6571 were grown in Tryptone Soy Broth (Oxoid) supplemented with 0.5% Yeast Extract (Oxoid) (TSBYE) at optimal growth temperature for 18-20 h.

3.2.2 ANTIMICROBIAL COMPOUNDS.

All components were purchased from SIGMA; they were (R)-(+) Limonene 97%, Thymol 99%, Carvacrol 98%, Cinnamaldehyde 98% and Eugenol 98% and are referred to below as essential oil components. Each component was prepared as a 1M solution in methanol.

3.2.3 MAXIMUM SUBLETHAL CONCENTRATION (MSC) DETERMINATION AND CULTURE CONDITIONS.

To known quantities of antimicrobial solutions were added to TSBYE in order to obtain broths with different concentrations of each antimicrobial compound. The MSC was defined as the highest concentration of antimicrobial compound allowing an overnight growth at the optimal temperature. Each strain was cultivated in TSBYE without (control) and with antimicrobial compound at the MSC for 18-20 hours at the optimal growth temperature.

3.2.4 TOTAL LIPID EXTRACTION.

The culture broths of each microorganism were centrifuged for 10 min at 5000 g and the cell pellet was harvested and submitted for membrane fatty acid extraction. Extraction of fatty acid from cellular materials was carried out as described by Evans *et al.* (1998). Lipid samples were transmethylated for analysis of their acyl groups as fatty acid methyl esters (FAME). The samples of total lipid extract were evaporated to dryness in a round bottom flask using a boiling water bath. To the dried samples, heated under a reflux condenser (20-30 cm), were added 10 ml of KOH in methanol (2 M) and 1 ml of heptane. After 10 min, 5 ml of boron trifluoride (BF_3) were added followed, after 2 min, by 4ml of heptane. After waiting for 1 min, the samples were cooled. A saturated solution of Na_2SO_4 was added to the samples and after settling into a two-phase system, the upper layer was taken and transferred into a vial.

3.2.5 ANALYSIS OF THE FATTY ACID COMPOSITION.

Analytical gas chromatography was carried out on a Perkin-Elmer Auto System XL Gas Chromatograph, equipped with a flame ionisation detector and a capillary column BPX 70 (50 m x 0.32 mm, i.d.) (SGE Ltd, Milton Keynes);

1µl of sample was injected, using the following operating conditions: carrier gas helium flow 1 ml min^{-1}; injector temperature 220˚C; detector temperature 240˚C; initial oven temperature 70˚C for 3 min; ramp 1, 22˚C min^{-1} to 180˚C; ramp 2, 2˚C min^{-1} to 190˚C, hold for 10 min; ramp 3, 2.0°C/min to 220°C.

FAME peaks were identified by comparison of their retention times with those of a standard solution (SUPELCO 37 Component FAME Mix).

3.3 RESULTS

3.3.1 MSC OF ANTIMICROBIAL COMPOUNDS.

Table 1 reports the MSC of antimicrobial compounds added to the growth media. *Ps. fluorescens* and *Salmonella* Typhimurium strains were both able to grow in the presence of high concentrations of all tested compounds, showing more resistance than the other microorganisms.

3.3.2 CHANGES IN FATTY ACID COMPOSITION.

Membrane lipid composition changes were investigated by using gas-chromatographic analysis. Table 2 shows the concentrations of the UFAs and the variation of their values (ΔUFAs) following exposure to the stress. The results showed that the UFAs are always present at a higher amount than SFAs in the total lipid profile of the microorganisms used in this research. The fatty acids and their concentrations found in the lipid extracts of control and treated cells of each microorganism are reported in Tables 3-7.

The lipid profile of *E. coli* O157:H7 ATCC 43888 was affected by the presence of all antimicrobial compounds, as reported in Table 2. However, the UFA concentrations increased more when the cells grew in the presence of limonene and cinnamaldehyde. In both cases, the net increase of UFAs resulted from a decrease of palmitic acid (C16) concentration, and an increase in linoleaidic (C18:2 trans), docosanoic (C22) and eicosapentaenoic (C20:5 cis) acid concentrations. Additionally, cinnamaldehyde also resulted in an increase in concentration of palmitoleic acid (C16:1 cis). It is important to highlight the increase in oleic acid (C18:1 cis) in the lipid extract of *E. coli* cells grown in the presence of sublethal concentrations of eugenol.

For the control cells of *S.* Typhimurium ATCC 14028, approximately 60% of the fatty acids were UFAs. This percentage increased when carvacrol and eugenol were added to the growth media. In contrast, in the broth supplemented with thymol, the UFAs slightly decreased (Table 2). The increase of UFAs observed in the presence of eugenol and carvacrol is due to a balanced variation of the two classes of fatty acids. In fact, as observed in Table 4, the unsaturated *cis*-10-pentadecanoic ($C15:1_{cis}$), linoleaidic ($C18:2_{trans}$) and linolenic ($C18:3_{cis}$) acids underwent a greater increase than the other, while palmitic acid (C16), among the SFAs, showed a relative decrease.

The lipid profile of *Ps. fluorescens* NCIMB 10586 cultured in unsupplemented broth contained about 67% UFAs. All antimicrobial substances, with the exception of thymol, slightly affected the ratio S/UFAs. A relative net decrease of UFAs was induced by the presence of thymol in the growth media. It resulted principally from an increase in saturated myristic (C14), palmitic (C16) and stearic (C18) acids and, a decrease in unsaturated oleic (C18:1 cis), linoleaidic (C18:2 trans) and linolenic (C18:3 cis) acids (Table 5).

The results reported in Table 2 and Table 6 showed that the fatty acid composition of *B. thermosphacta* NCTC 10822 was strongly influenced by the presence of all antimicrobial compounds with the exception of thymol. In the case of eugenol and carvacrol, the net increase in UFAs was due to minor variations in the concentration of many acids. Limonene and cinnamaldehyde increased the concentration of unsaturated *cis*-10-pentadecanoic (C15:1 cis) and linolenic (C18:3 cis) acids and decreased the concentration of palmitic acid (C16). These variations were particularly evident when limonene was added to the culture media.

S. aureus NCTC 6571 had the highest concentration (71%) of UFAs when it was cultured in the unsupplemented medium. No substantial change in lipid composition of the extracts was observed in the presence of antimicrobial substances, except when the broth was supplemented with a sublethal concentration of cinnamaldehyde. In this case, the 17% UFAs decrease was mainly due to a decrease in myristoleic (C14:1cis) and oleic (C18:1 cis) acids and by an increase in the palmitic (C16) and stearic (C18) acids (Table 7).

3.4 DISCUSSION

Lipids are a functional part of cell membranes, so this investigation focused on the changes in fatty acid profile as a response of the cell to when challenged with an environmental disturbance: the presence of sublethal concentrations of natural antimicrobial compounds. In a previous study (Di Pasqua *et al.*, 2005) the antimicrobial activity of some essential oils was investigated and their chemical composition was determined (unpublished data). All the compounds used in this study are constituents of those essential oils. Thymol, eugenol and carvacrol are phenolic compounds, limonene is a terpene and cinnamaldehyde is an aliphatic aldehyde. The lipophilic character of these types of substances gives them the ability to penetrate the cytoplasmic membrane, or cause a change to its fluidity (Sikkema *et al.*, 1995).

As reported by Russell (1984), the mechanisms used by bacterial cells to alter the unsaturation ratio of membrane fatty acids depends on the mechanism of fatty acid synthesis. The author described two distinct and mutually exclusive unsaturated fatty acid synthetic pathways in bacteria: the anaerobic and the aerobic pathway. The former, used by anaerobes and some facultative aerobes, produces unsaturated fatty acids by *de*

novo synthesis through the action of a fatty acid synthetase (Russell, 1984; de Mendoza and Cronan, 1983). The latter produces only saturated fatty acids, employing the multi-component membrane desaturase enzymes (Russell, 1984, 1997). The only way for these bacteria to produce unsaturated fatty acids, most commonly palmitoleic and oleic acids, is by the action of the desaturase enzyme which creates a double bond in the saturated acyl chains by removing two hydrogen atoms and transferring them to oxygen (Russell, 1984, 1995).

The raised concentration of some UFAs detected in *E. coli* O157:H7, *Salmonella* and *B. thermosphacta* was not supported by a relative decrease in the same length SFAs. It is hypothesized that these microorganisms regulate their UFA synthesis by the anaerobic biosynthetic pathway. The findings of this study confirmed those of Chiou *et al.* (2004), who found that the presence of a sublethal concentration of ethanol during growth of *E. coli* O157:H7 resulted in extensive synthesis of lipids containing high amounts of unsaturated fatty acids. It seemed that, in both cases, the addition of a sublethal concentration of the antimicrobial compounds resulted in the cells using a similar adaptation mechanism to maintain membrane structure and function. Therefore, the cells responded to a sublethal concentration of antimicrobials by increasing the UFAs resulting in membrane fluidity changes. In fact, it is well known that UFAs give the membrane a high degree of fluidity, whereas those composed predominantly of saturated fatty acids tend to be relatively rigid (Russel *et al.*, 1984).

Yuk and Marshall (2004) and Sampathkumar *et al.* (2004) found that adaptation to pH, in *E. coli* O157:H7 and *Salmonella enterica* resulted in a decrease of UFA concentration, suggesting that the adaptation mechanism depends on the type of stress factor to which the cells have been exposed. Fozo *et al.* (2004) demonstrated that

increased fatty acid length is another important membrane alteration to increase survival in acidic environments; the same adaptation mechanism probably occurs when the cells grow in the presence of antimicrobial compounds. The results reported in this paper showed that, *E. coli* O157:H7, short-medium chain fatty acids (C4-C14) and long chain fatty acids (C20-C22) were either absent or present in low concentrations under control conditions. It is interesting to observe an increase of the C20-C22 level when the cells were grown in broths supplemented with the antimicrobials used in this study.

The same effect was found as an adaptation mechanism to a rise in growth temperature, where an increase in the proportion of long chain and saturated fatty acids within the membrane was observed (Denich *et al.*, 2003). Russell (1984) found that when bacteria anaerobically synthesize unsaturated fatty acids at low temperatures, they also synthesize longer chain fatty acids. However, the fluidizing effects of the unsaturated fatty acids chains dominate, over those due to change in chain length.

Ps. fluorescens and *S. aureus* showed generally small variations in their fatty acid composition when grown in the presence of the tested antimicrobials. It is probable that the high resistance of *Ps. fluorescens* and *S. aureus* to the tested compounds explains the unchanged composition of fatty acid profile. The findings discussed in this paper on *Ps. fluorescens* are in agreement with those found by other authors (Heipieper *et al.*, 2003; Mrozik *et al.*, 2004; Sikkema *et al.*, 1995), who reported a high resistance of this microorganism to several antimicrobials.

The biosynthetic pathway by which *Pseudomonas* synthesizes unsaturated fatty acids is through the action of the desaturase enzyme. The presence of antimicrobial compounds during growth presumably affects the action of this enzyme. However, the overall effect of the variations in lipid composition on the membrane fluidity is determined by the

sum of the effect of every single lipid. A small change in a particular lipid could have a greater impact than a larger change in another lipid (Ultee *et al.*, 2000).

It is believed that this is the first report on the effect that sublethal concentrations of antimicrobial compounds from essential oils have on the fatty acid profile of foodborne spoilage and pathogen microorganisms. Understanding how the cells adapt their functional structures upon exposure to antimicrobial compounds will enhance the understanding of the prevention of resistance mechanisms. However, further studies are necessary to see whether the modifications in membrane composition demonstrated in this research really lead to an increased resistance or are just part of a general adaptive response. These findings provide interesting information to any studies bridging the gap between mechanisms evaluated at the molecular level and observations at the organism level.

TABLES CHAPTER 3

TABLE 1: Maximum sublethal concentration of antimicrobial compounds

Strains[a]	Compounds [a] µl ml^{-1}				
	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
Escherichia coli O157:H7 (non toxigenic) ATCC 43888	0.8	10	3	0.8	2
Salmonella enterica ser. Typhimurium ATCC 14028	1	10	3	1	3
Pseudomonas fluorescens NCIMB 10586	3	10	2	2	3
Brochotrix thermosphacta NCTC 10822	0.6	2	2	0.8	0.8
Staphylococcus aureus NCTC 6571	1	2	2	1	2

a: Values are means of duplicate determinations

TABLE 2: Percentage of total fatty acids

Compounds	*E. coli* O157:H7		*S.* serovar Typhimurium		*Ps fluorescens*		*B. thermosphacta*		*S. aureus*	
	UFA[a]	ΔUFA[a, b]	UFA	ΔUFA	UFA	ΔUFA	UFA	ΔUFA	UFA	ΔUFA
Control	55.85	-	59.56	-	67.57	-	58.05	-	71.00	-
Thymol	62.61	6.76	57.93	-1.63	54.91	-12.66	61.26	3.21	72.31	1.31
Limonene	68.57	12.72	64.98	5.42	64.54	-3.03	81.74	23.69	78.55	7.55
Eugenol	56.15	10.31	70.20	10.63	65.96	-1.61	66.21	8.16	70.01	-1.00
Carvacrol	57.57	1.72	71.49	11.93	70.75	3.18	69.42	11.37	66.66	-4.35
Cinnamaldehyde	71.01	15.16	61.78	2.22	66.69	-0.88	70.19	12.15	53.76	-17.25

a: Values are means of duplicate determinations
b: UFAs control-UFAs treated cells

Table 3: Fatty acid profile of *Escherichia coli* O157:H7 ATCC 43888

FAME	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	2.18 ± 0.92[a]	2.78 ± 1.09	2.59 ± 0.78	N.D.[b]	1.88 ± 1.21	1.86 ± 1.18
C6:0	1.32 ± 0.66	0.13 ± 0.18	0.08 ± 0.11	0.63 ± 0.89	0.69 ± 0.75	0.13 ± 0.18
C8:0	1.69 ± 0.61	0.16 ± 0.20	0.07 ± 0.10	N.D.	0.66 ± 0.93	0.91 ± 0.80
C10:0	0.86 ± 1.22	0.11 ± 0.15	0.14 ± 0.19	N.D.	0.78 ± 0.45	0.15 ± 0.21
C12:0	1.62 ± 0.85	0.29 ± 0.11	0.20 ± 0.01	0.56 ± 0.80	1.55 ± 0.45	1.18 ± 1.21
C14:0	5.28 ± 1.37	2.51 ± 1.96	2.21 ± 1.17	1.29 ± 1.83	4.46 ± 0.96	4.51 ± 3.87
C14:1cis	5.03 ± 2.50	4.60 ± 1.46	7.56 ± 3.79	1.44 ± 2.03	0.44 ± 0.62	2.96 ± 2.47
C15:0	0.11 ± 0.15	0.21 ± 0.29	0.62 ± 0.88	N.D.	N.D.	N.D.
C15:1cis	6.49 ± 1.77	3.41 ± 4.82	3.37 ± 2.99	N.D.	3.74 ± 5.29	4.19 ± 1.32
C16:0	18.47 ± 2.67	12.42 ± 6.81	9.84 ± 3.58	15.91 ± 5.97	22.42 ± 11.04	8.39 ± 2.64
C16:1trans	0.14 ± 0.20	0.53 ± 0.49	0.21 ± 0.06	0.63 ± 0.89	0.06 ± 0.09	1.15 ± 1.23
C16:1cis	1.61 ± 1.03	2.59 ± 2.35	0.41 ± 0.58	2.27 ± 3.21	4.07 ± 1.96	8.58 ± 1.88
C17:0	3.57 ± 5.05	1.01 ± 1.43	4.10 ± 5.80	N.D.	N.D.	N.D.
C17:1cis	8.25 ± 1.96	1.82 ± 0.61	5.29 ± 2.12	1.77 ± 2.51	6.03 ± 1.83	3.69 ± 2.22
C18:0	4.14 ± 1.52	6.35 ± 5.49	2.26 ± 2.12	13.12 ± 3.02	4.85 ± 3.26	3.96 ± 3.57
C18:1trans	3.54 ± 0.63	1.90 ± 0.91	1.89 ± 0.19	1.17 ± 1.65	2.93 ± 0.28	2.12 ± 1.17
C18:1cis	7.07 ± 4.33	12.09 ± 4.57	6.83 ± 7.83	36.72 ± 8.39	7.63 ± 6.36	5.30 ± 4.13
C18:2trans	0.62 ± 0.87	0.62 ± 0.87	9.82 ± 6.33	1.08 ± 1.52	8.83 ± 12.18	10.88 ± 3.56
C18:2cis	2.28 ± 1.38	3.45 ± 1.10	0.79 ± 1.12	9.62 ± 8.99	1.38 ± 0.48	0.34 ± 0.48
C18:3 (gla)	3.11 ± 1.62	0.62 ± 0.87	2.01 ± 1.23	N.D.	2.71 ± 1.65	2.28 ± 2.14
C18:3cis	12.91 ± 4.61	8.96 ± 5.23	8.90 ± 3.51	3.30 ± 1.65	13.04 ± 3.95	9.17 ± 1.30
C20:0	0.93 ± 1.31	1.42 ± 1.34	0.23 ± 0.32	1.23 ± 1.74	0.88 ± 0.59	0.73 ± 1.03
C20:1cis	1.86 ± 0.86	2.66 ± 0.85	1.90 ± 0.03	N.D.	2.84 ± 0.97	2.14 ± 0.60
C20:2cis	N.D.	11.29 ± 4.29	10.05 ± 5.20	N.D.	N.D.	10.73 ± 2.11
C22:0	0.85 ± 1.21	8.03 ± 2.50	7.02 ± 2.55	1.08 ± 1.53	1.11 ± 0.21	5.68 ± 2.17
C20:5cis	0.63 ± 0.89	5.63 ± 3.26	5.33 ± 4.07	N.D.	0.12 ± 0.17	5.31 ± 1.67
C24:0	3.15 ± 1.41	1.98 ± 2.80	2.09 ± 2.95	N.D.	3.16 ± 1.43	1.49 ± 2.10
C22:6cis	2.32 ± 3.28	2.44 ± 1.68	4.19 ± 0.43	8.16 ± 5.05	3.75 ± 0.69	2.19 ± 1.64

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 4: Fatty acid profile of *Salmonella enterica* serovar Typhimurium ATCC 14028

FAME	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	1.87 ± 1.10[a]	3.09 ± 1.40	2.85 ± 1.37	4.11± 0.20	2.79 ± 2.01	3.77 ± 0.73
C6:0	0.51 ± 0.72	1.87 ± 1.96	0.63 ± 0.89	0.79 ± 0.69	0.28 ± 0.40	N.D.[b]
C8:0	0.12 ± 0.17	1.30 ± 1.20	0.16 ± 0.23	N.D.	0.20 ± 0.28	N.D.
C10:0	0.72 ± 0.73	0.18 ± 0.07	0.65 ± 0.47	0.21 ± 0.29	0.15 ± 0.22	0.12 ± 0.17
C12:0	1.06 ± 1.35	1.53 ± 1.94	0.81 ± 0.77	0.15 ± 0.21	0.60 ± 0.40	N.D.
C14:0	3.51 ± 1.52	4.15 ± 2.65	2.23 ± 0.50	1.97 ± 0.11	2.91 ± 0.49	1.88 ± 0.66
C14:1cis	N.D.	N.D.	0.39 ± 0.56	N.D.	N.D.	N.D.
C15:0	N.D.	0.12 ± 0.17	0.23 ± 0.32	0.28 ± 0.04	0.11 ± 0.15	0.21 ± 0.29
C15:1cis	4.00 ± 2.60	6.66 ± 2.01	6.32 ± 2.95	7.50 ± 1.59	9.11 ± 1.27	5.58 ± 0.81
C16:0	25.54 ± 3.92	18.80 ± 1.93	15.10 ± 3.02	11.64 ± 1.06	11.69 ± 2.37	18.06 ± 1.98
C16:1trans	0.31 ± 0.21	0.11 ± 0.15	0.60 ± 0.38	1.07 ± 1.11	0.61 ± 0.48	1.03 ± 1.22
C16:1cis	7.35 ± 1.25	3.81 ± 0.68	2.73 ± 0.75	3.88 ± 1.90	2.85 ± 0.57	1.94 ± 1.06
C17:0	1.77 ± 2.13	0.16 ± 0.23	2.91 ± 4.12	N.D.	N.D.	N.D.
C17:1cis	10.47 ± 0.82	2.50 ± 1.78	3.74 ± 1.22	2.40 ± 0.89	4.18 ± 1.69	2.94 ± 0.77
C18:0	2.51 ± 1.68	4.62 ± 3.73	3.22 ± 2.30	3.47 ± 1.13	2.74 ± 2.00	9.23 ± 4.40
C18:1trans	2.00 ± 0.64	6.15 ± 1.56	2.06 ± 1.14	3.71 ± 0.72	4.32 ± 1.16	7.95 ± 0.37
C18:1cis	2.80 ± 0.59	5.94 ± 4.68	9.47 ± 4.21	7.00 ± 3.23	5.66 ± 5.12	13.20 ± 3.07
C18:2trans	13.12 ± 4.29	14.54 ± 4.81	17.02 ± 8.15	20.43 ± 4.48	19.25 ± 4.55	13.73 ± 3.55
C18:2cis	1.31 ± 0.80	2.42 ± 2.44	2.99 ± 1.56	3.22 ± 2.88	1.13 ± 0.67	1.41 ± 0.25
C18:3 (gla)	6.14 ± 1.55	0.96 ± 1.01	1.03 ± 0.11	0.40 ± 0.56	2.32 ± 1.61	0.38 ± 0.54
C18:3cis	10.42 ± 3.09	11.94 ± 5.56	14.78 ± 4.15	15.83 ± 3.47	18.68 ± 1.94	10.56 ± 3.22
C20:0	0.16 ± 0.23	N.D.	N.D.	N.D.	0.23 ± 0.33	0.85 ± 1.20
C20:1cis	1.02 ± 1.44	2.45 ± 2.36	1.26 ± 1.60	2.41 ± 0.80	2.40 ± 1.92	1.28 ± 1.48
C22:0	0.06 ± 0.09	N.D.	0.12 ± 0.17	N.D.	N.D.	0.47 ± 0.66
C24:0	1.60 ± 2.26	3.80 ± 0.77	4.85 ± 0.35	4.78 ± 0.79	4.40 ± 1.62	2.36 ± 1.62
C22:6cis	1.65 ± 2.34	2.90 ± 2.34	3.84 ± 1.34	4.76 ± 1.53	3.37 ± 1.91	3.06 ± 1.41

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 5: Fatty acid profile of *Pseudomonas fluorescens* NCIMB 10586

FAME	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	3.98 ± 1.36[a]	3.76 ± 0.96	6.83 ± 0.55	7.65 ± 0.84	7.65 ± 1.89	6.06 ± 0.89
C6:0	0.70 ± 0.99	1.06 ± 0.70	0.24 ± 0.34	0.24 ± 0.34	0.20 ± 0.29	0.56 ± 0.79
C8:0	0.93 ± 1.15	0.83 ± 0.37	0.26 ± 0.37	0.36 ± 0.51	0.25 ± 0.35	1.47 ± 0.19
C10:0	0.66 ± 0.93	0.29 ± 0.41	0.06 ± 0.08	0.23 ± 0.32	0.14 ± 0.20	0.49 ± 0.69
C12:0	1.53 ± 0.95	2.04 ± 1.05	1.62 ± 0.86	1.57 ± 0.62	2.91 ± 0.45	1.13 ± 0.95
C14:0	1.59 ± 0.80	4.09 ± 0.29	1.63 ± 1.03	2.98 ± 0.41	1.80 ± 0.60	3.19 ± 1.13
C14:1cis	6.51 ± 1.76	5.92 ± 0.36	6.42 ± 1.99	9.03 ± 1.20	9.14 ± 0.32	8.40 ± 1.61
C15:0	0.32 ± 0.45	0.25 ± 0.35	0.19 ± 0.26	0.35 ± 0.50	0.26 ± 0.36	N.D.[b]
C15:1cis	8.41 ± 0.72	8.40 ± 0.84	9.78 ± 1.41	7.77 ± 1.73	10.34 ± 0.98	9.89 ± 0.78
C16:0	11.45 ± 2.50	19.55 ± 1.58	17.45 ± 1.55	10.20 ± 1.22	8.17 ± 0.08	10.39 ± 2.11
C16:1trans	0.11 ± 0.15	0.56 ± 0.79	1.18 ± 1.68	N.D.	1.17 ± 1.65	N.D.
C16:1cis	N.D.	N.D.	1.78 ± 2.51	0.77 ± 1.10	1.39 ± 1.96	1.19 ± 1.69
C17:0	1.18 ± 1.67	N.D.	0.94 ± 1.33	N.D.	N.D.	N.D.
C17:1cis	0.51 ± 0.72	N.D.	0.74 ± 1.04	0.18 ± 0.26	N.D.	N.D.
C18:0	3.25 ± 1.37	9.54 ± 0.98	2.48 ± 1.06	5.39 ± 1.40	3.70 ± 1.69	5.89 ± 2.51
C18:1trans	N.D.	0.54 ± 0.77	N.D.	1.47 ± 2.08	N.D.	1.02 ± 1.45
C18:1cis	25.19 ± 2.89	16.95 ± 0.42	21.56 ± 1.07	22.38 ± 1.40	25.19 ± 1.92	22.99 ± 0.96
C18:2trans	3.09 ± 0.30	N.D.	0.06 ± 0.09	0.92 ± 1.30	N.D.	0.05 ± 0.08
C18:2cis	1.12 ± 0.61	5.79 ± 0.76	0.35 ± 0.49	2.49 ± 1.19	0.43 ± 0.61	0.70 ± 1.00
C18:3 (gla)	0.18 ± 0.25	N.D.	0.26 ± 0.37	0.23 ± 0.32	0.21 ± 0.29	0.22 ± 0.32
C18:3cis	18.49 ± 1.05	12.94 ± 2.90	16.56 ± 0.93	14.38 ± 2.84	16.55 ± 1.72	17.36 ± 1.39
C20:0	0.14 ± 0.20	0.16 ± 0.23	N.D.	0.07 ± 0.10	N.D.	0.06 ± 0.08
C20:3cis	N.D.	N.D.	1.40 ± 0.64	N.D.	N.D.	N.D.
C22:0	1.63 ± 0.74	0.19 ± 0.27	N.D.	0.06 ± 0.08	N.D.	0.49 ± 0.34
C22:1cis	0.12 ± 0.17	0.34 ± 0.48	0.28 ± 0.39	0.32 ± 0.46	0.37 ± 0.52	0.33 ± 0.46
C20:5cis	N.D.	N.D.	N.D.	1.53 ± 0.71	1.76 ± 2.49	0.47 ± 0.66
C24:0	5.07 ± 0.39	3.33 ± 1.71	3.77 ± 1.62	4.94 ± 0.22	4.18 ± 1.58	3.60 ± 0.80
C22:5cis	N.D.	N.D.	N.D.	0.29 ± 0.41	0.32 ± 0.45	0.39 ± 0.55
C22:6cis	3.85 ± 2.40	3.48 ± 0.87	4.17 ± 1.43	4.19 ± 1.52	3.89 ± 2.51	3.68 ± 1.43

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 6: Fatty acid profile of *Brochothrix thermosphacta* NCTC 10822

FAME	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyd
C4:0	2.51 ± 0.90[a]	3.31 ± 1.53	6.80 ± 0.69	4.04 ± 1.10	3.61 ± 0.83	5.63 ± 0.88
C6:0	0.67 ± 0.59	2.48 ± 1.10	0.27 ± 0.38	0.28 ± 0.40	N.D.[b]	0.24 ± 0.34
C8:0	1.05 ± 0.16	2.44 ± 0.78	N.D.	0.20 ± 0.29	0.20 ± 0.29	0.38 ± 0.17
C10:0	1.21 ± 0.49	2.36 ± 1.38	N.D.	0.84 ± 0.63	0.13 ± 0.19	0.17 ± 0.24
C12:0	2.30 ± 0.78	3.20 ± 1.60	0.54 ± 0.49	0.34 ± 0.47	0.67 ± 0.28	2.67 ± 1.70
C14:0	5.87 ± 1.95	4.51 ± 0.79	2.50 ± 1.09	3.43 ± 1.27	4.06 ± 1.35	4.29 ± 1.36
C14:1cis	2.05 ± 1.07	1.37 ± 0.10	1.56 ± 0.63	0.41 ± 0.58	1.84 ± 0.46	1.16 ± 0.38
C15:0	0.15 ± 0.21	N.D.	0.13 ± 0.18	0.12 ± 0.16	0.15 ± 0.21	0.18 ± 0.26
C15:1cis	5.81 ± 2.20	8.65 ± 0.46	13.37 ± 1.42	7.30 ± 1.48	8.82 ± 2.19	9.45 ± 1.70
C16:0	16.41 ± 2.60	10.39 ± 1.19	4.49 ± 2.47	12.21 ± 2.86	11.34 ± 1.45	9.56 ± 0.97
C16:1trans	0.21 ± 0.30	0.53 ± 0.51	0.20 ± 0.28	0.18 ± 0.25	1.18 ± 1.19	0.24 ± 0.33
C16:1cis	1.06 ± 0.28	1.80 ± 0.62	0.24 ± 0.34	1.32 ± 0.58	2.09 ± 1.32	0.25 ± 0.35
C17:0	0.16 ± 0.23	0.06 ± 0.08	N.D.	N.D.	N.D.	N.D.
C18:0	6.79 ± 2.12	4.50 ± 1.24	3.11 ± 1.63	8.29 ± 2.48	3.64 ± 1.72	4.19 ± 3.02
C18:1trans	1.80 ± 0.61	3.04 ± 0.27	3.80 ± 0.33	3.42 ± 1.19	3.88 ± 0.52	4.47 ± 1.05
C18:1cis	4.69 ± 0.92	3.16 ± 1.40	3.91 ± 1.85	6.95 ± 1.84	2.15 ± 1.23	3.84 ± 2.01
C18:2trans	21.64 ± 3.54	17.41 ± 2.95	23.31 ± 6.06	23.84 ± 2.15	20.63 ± 3.42	23.40 ± 1.21
C18:2cis	0.88 ± 0.88	1.80 ± 1.73	2.58 ± 1.99	1.59 ± 0.95	1.74 ± 1.80	0.76 ± 0.18
C18:3 (gla)	N.D.	0.15 ± 0.22	1.06 ± 0.71	0.23 ± 0.32	0.22 ± 0.31	0.91 ± 0.59
C18:3cis	12.67 ± 3.36	15.11 ± 3.56	22.60 ± 3.05	15.46 ± 2.91	18.53 ± 3.12	17.09 ± 3.93
C20:0	1.57 ± 0.64	1.98 ± 0.86	0.19 ± 0.27	0.29 ± 0.41	0.14 ± 0.19	N.D.
C20:1cis	2.33 ± 0.79	3.11 ± 0.17	3.25 ± 0.98	1.23 ± 1.51	3.84 ± 0.58	4.70 ± 0.78
C22:0	0.53 ± 0.75	0.65 ± 0.91	0.23 ± 0.33	0.19 ± 0.26	N.D.	N.D.
C20:5cis	N.D.	0.64 ± 0.57	N.D.	N.D.	N.D.	N.D.
C24:0	2.74 ± 1.01	2.87 ± 2.61	N.D.	3.56 ± 2.06	6.63 ± 0.59	2.49 ± 3.53
C22:6cis	4.91 ± 0.51	4.48 ± 0.84	5.88 ± 0.78	4.28 ± 1.74	4.49 ± 1.75	3.93 ± 2.51

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 7: Fatty acid profile of *Staphylococcus aureus* NCTC 6571

FAME	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	4.89 ± 1.22[a]	6.00 ± 0.42	7.01 ± 0.08	N.D.[b]	5.57 ± 0.43	3.90 ± 1.26
C6:0	1.15 ± 1.19	0.88 ± 0.21	0.22 ± 0.30	N.D.	1.19 ± 0.44	0.22 ± 0.31
C8:0	0.38 ± 0.14	0.87 ± 0.20	0.15 ± 0.21	N.D.	1.46 ± 0.61	0.31 ± 0.44
C10:0	0.34 ± 0.16	N.D.	0.03 ± 0.05	N.D.	1.16 ± 0.40	0.20 ± 0.29
C12:0	2.00 ± 0.20	1.75 ± 0.37	3.24 ± 0.44	N.D.	2.60 ± 0.59	1.54 ± 0.72
C14:0	1.65 ± 0.53	4.59 ± 0.79	1.00 ± 0.08	0.60 ± 0.26	6.71 ± 0.78	2.23 ± 0.26
C14:1cis	13.19 ± 2.74	10.79 ± 0.61	11.09 ± 0.21	5.64 ± 0.69	6.40 ± 7.57	5.93 ± 0.38
C15:0	0.26 ± 0.37	1.22 ± 1.12	0.41 ± 0.58	1.07 ± 1.52	1.20 ± 0.96	0.81 ± 0.62
C15:1cis	6.93 ± 0.94	10.64 ± 0.58	10.54 ± 0.67	7.21 ± 0.55	9.14 ± 0.41	6.08 ± 0.41
C16:0	3.59 ± 0.94	2.84 ± 0.88	3.46 ± 0.63	1.11 ± 0.37	4.12 ± 0.96	17.77 ± 0.79
C16:1trans	N.D.	N.D.	N.D.	0.06 ± 0.08	0.14 ± 0.20	N.D.
C16:1cis	0.62 ± 0.87	N.D.	N.D.	0.11 ± 0.16	0.44 ± 0.62	N.D.
C17:0	0.63 ± 0.89	0.97 ± 0.82	N.D.	0.13 ± 0.18	0.49 ± 0.70	N.D.
C17:1cis	N.D.	0.24 ± 0.33	N.D.	2.88 ± 0.42	N.D.	0.07 ± 0.10
C18:0	4.04 ± 0.66	2.05 ± 0.25	1.70 ± 0.63	2.43 ± 0.84	3.75 ± 0.94	13.73 ± 0.85
C18:1trans	N.D.	0.77 ± 1.09	N.D.	6.75 ± 0.41	0.44 ± 0.62	N.D.
C18:1cis	22.74 ± 1.17	21.38 ± 0.64	26.37 ± 2.11	21.50 ± 1.34	20.01 ± 0.49	17.91 ± 0.48
C18:2trans	N.D.	1.10 ± 1.35	N.D.	1.00 ± 0.50	N.D.	0.04 ± 0.06
C18:2cis	0.17 ± 0.25	0.65 ± 0.27	1.40 ± 0.63	0.27 ± 0.39	0.84 ± 0.29	1.69 ± 0.48
C18:3 (gla)	0.25 ± 0.36	0.92 ± 0.48	0.48 ± 0.03	0.76 ± 0.40	0.29 ± 0.41	0.16 ± 0.22
C18:3cis	15.42 ± 1.80	19.54 ± 0.89	18.91 ± 0.64	16.72 ± 1.17	17.59 ± 1.35	14.42 ± 1.19
C20:0	4.27 ± 1.07	1.98 ± 0.39	0.11 ± 0.15	0.84 ± 1.19	3.74 ± 1.17	1.52 ± 0.47
C20:1cis	N.D.	N.D.	N.D.	2.25 ± 0.08	N.D.	N.D.
C20:3cis	N.D.	0.45 ± 0.64	1.27 ± 0.39	N.D.	N.D.	0.93 ± 0.23
C22:0	N.D.	N.D.	N.D.	11.48 ± 1.73	N.D.	N.D.
C22:1cis	0.35 ± 0.49	0.44 ± 0.62	0.64 ± 0.23	0.35 ± 0.50	1.60 ± 0.77	0.29 ± 0.41
C20:5cis	4.19 ± 0.56	1.41 ± 1.80	3.00 ± 0.84	0.39 ± 0.55	1.38 ± 0.89	2.41 ± 0.64
C24:0	5.79 ± 0.81	4.55 ± 1.53	4.12 ± 1.23	12.34 ± 1.71	1.35 ± 0.74	4.02 ± 0.08
C22:5cis	0.35 ± 0.50	0.44 ± 0.62	N.D.	N.D.	4.53 ± 0.74	N.D.
C22:6cis	6.78 ± 1.08	3.54 ± 2.00	4.86 ± 0.70	4.12 ± 0.65	3.86 ± 0.44	3.84 ± 0.83

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

CHAPTER 4

MEMBRANE TOXICITY OF NATURAL ANTIMICROBIAL COMPOUNDS FROM ESSENTIAL OILS

4.1 INTRODUCTION

The antimicrobial activity of the essential oils constituents has already been fully discussed by many authors (Sikkema *et al.*, 1995; Helander *et al.*, 1998; Gill and Holley, 2004). The major active essential oil (EO) compounds are phenols, terpenes and aldehydes (Ceylan and Fung, 2004) and it is also well known that the action of these substances is principally performed against the cell cytoplasmic membrane (Knobloch *et al.*, 1989; Sikkema *et al.*, 1994; Ultee *et al.*, 2000a, 2002; Ceylan and Fung, 2004). The phenol compounds with a hydroxyl group (-OH) were found to posses the major antimicrobial activity among the EO constituents. It seems that the presence of the hydroxyl group is related to the inactivation of the enzymes. In fact, they interact with the membrane causing leakage of cellular components, change fatty acid and phospholipid constituents, impair energy metabolism and influence genetic material synthesis (Ceylan and Fung, 2004). Likewise to the phenol compounds, the site of action of the terpenes is the cell membrane. They permeate through the membranes causing them to swell, thus inhibiting respiratory enzymes and causing partial dissipation of the pH gradient and electrical potential (Sikkema *et al.*, 2004). Cinnamaldehyde has the highest antifungal activity among aliphatic aldehydes (Ceylan and Fung, 2004) and according to Helander *et al.*, (1998) it gain access to the periplasm and to the deeper parts of the cells, but did not result in the disintegration of outer membrane likewise carvacrol and thymol did. On the other hand, Gill and Holley (2004) hypothesized a mechanism for cinnamaldehyde action in which the interaction with the

cell membrane causes disruption sufficient to disperse the proton motive force by leakage of small ions without leakage of larger cell components, such as ATP.

In accordance with these findings, to evaluate the membrane damage, we have investigated the alteration of fatty acids profile caused by EO compounds, i.e. limonene, thymol, carvacrol, cinnamaldehyde and eugenol, after an exposition of the cells for two hours to a high concentration of these compounds.

4.2 MATERIALS AND METHODS

4.2.1 BACTERIAL STRAINS.

Escherichia coli O157:H7 ATCC 43888 (non toxigenic), *Salmonella enterica* serovar Typhimurium ATCC 14028, *Pseudomonas fluorescens* NCIMB 10586, *Brochothrix thermosphacta* NCTC 10822 and *Staphylococcus aureus* NCTC 6571 were grown in Tryptone Soy Broth (Oxoid) supplemented with 0.5% Yeast Extract (Oxoid) (TSBYE) at optimal growth temperature for 18-20 h.

4.2.2 ANTIMICROBIAL COMPOUNDS.

All components were purchased from SIGMA; they were (R)-(+) Limonene 97%, Thymol 99%, Carvacrol 98%, Cinnamaldehyde 98% and Eugenol 98% and are referred to below as essential oil components. Each component was prepared as a 1M solution in methanol.

4.2.4 CELLS TREATMENT.

The culture broths of each microorganism were centrifuged for 10 min at 5000 g and the cell pellet was harvested and resuspended in an equal volume of PBS. To each cellular suspension were added the compounds to have a final concentration of 0.2 M. The cells were left for two hours at 30° C and immediately submitted for membrane fatty acid extraction. Cells treated without antimicrobial were used as control.

4.2.5 TOTAL LIPID EXTRACTION.

Extraction of fatty acid from cellular materials was carried out as described by Evans *et al.* (1998). Lipid samples were transmethylated for analysis of their acyl groups as fatty acid methyl esters (FAME). The samples of total lipid extract were evaporated to dryness in a round bottom flask using a boiling water bath. To the dried samples, heated under a reflux condenser (20-30 cm), were added 10 ml of 2 M KOH in methanol and 1 ml of heptane. After 10 min, 5 ml of boron trifluoride (BF_3) were added followed, after 2 min, by 4 ml of heptane. After waiting for 1 min, the samples were cooled. A saturated solution of Na_2SO_4 was added to the samples and after settling into a two-phase system, the upper layer was taken and transferred into a vial.

4.2.6 ANALYSIS OF THE FATTY ACID COMPOSITION.

Analytical gas chromatography was carried out on a Perkin-Elmer Auto System XL Gas Chromatograph, equipped with a flame ionisation detector and a capillary column BPX 70 (50 m x 0.32 mm, i.d.) (SGE Ltd, Milton Keynes).

1µl of sample was injected, using the following operating conditions: carrier gas helium flow 1 ml min^{-1}; injector temperature 220˚C; detector temperature 240˚C; initial oven temperature 70˚C for 3 min; ramp 1, 22˚C min^{-1} to 180˚C; ramp 2, 2˚C min^{-1} to 190˚C, hold for 10 min; ramp 3, 2°C/min to 220°C.

FAME peaks were identified by comparison of their retention times with those of a standard solution (SUPELCO 37 Component FAME Mix).

4.2.7 SAMPLE PREPARATION OF THE FOR THE SCANNING ELECTRONE MICROSCOPY (SEM) ANALYSIS

The cells, after being treated as above described, were harvested by centrifugation for 10 min at 5000 g, washed twice with PBS and finally resuspended in PBS containing 2,5% of glutharaldeyde to fix the cells. This suspension was filtered on a 25µm Nucleopore Track-Etch Membrane (Whatman), and after coated with gold under vacuum the membrane was ready for the SEM analysis.

Samples were visualized by using a Leica Cambridge S-360 scanning electron microscope.

4.3 RESULTS

4.3.1 ALTERATION OF MEMBRANE FATTY ACIDS PROFILE SUBSEQUENTLY THE EXPOSITION TO THE ANTIMICROBIAL COMPOUNDS.

Membrane lipid alterations were investigated by gas-chromatographic analysis of fatty acid methyl esters extracted from the cells after an exposition for two hours in presence of antimicrobial compounds.

As shown in table 1, the lipids of the tested strains were principally constituted by unsaturated fatty acids (UFAs), in particular *S. aureus* NCTC 6571 presented the highest concentration of UFAs. In the same table it is possible to notice that all the strains exhibited a reduction of the UFAs, observing the highest peaks of reduction when the strains were treated with limonene and cinnamaldehyde.

In the tables 2-6 are reported the fatty acids and their concentrations before and after the treatments with the compounds. It is interesting to observe that for all the strains most of the short chain fatty acids, even if at low concentrations in the control profile, were completely absent when the strains were treated with thymol, eugenol and cinnamaldehyde. On the contrary, when the strains were treated with limonene and carvacrol, the concentration of these fatty acids was unvaried or increased, in particular the caproic acid (C6:0) when *E. coli* and *S*. Typhimurium were treated with limonene raised 54.50% and 40.20%, respectively. The concentration of butyric acid (C4:0), when *B. thermosphacta* was treated with limonene, was found to be four times higher than the control value.

It is remarkable how the palmitic acid (C16:0) concentration homogeneously varied once the strains were treated with the different compounds, in particular it has to be highlight after the treatment of the cells with eugenol this fatty acid raised the highest value among the gram-negatives while among the gram-positives that occurred with thymol. The linoleaidic acid (C18:2trans) underwent a notable decrease contributing to the total decrease of the UFAs of all the strains.

A similar result, tables 3 and 6, has been found on linolenic (C18:3cis) acid when *S*. Typhimurium and *B. thermosphacta* were treated with all the compounds.

Noteworthy is the effect of the cinnamaldehyde on the heptadecanoic acid (C17:0), this fatty acid has been found at low concentrations in the control profiles but when the strains were treated with that compound, its concentrations raised values 70% higher than the control. Furthermore, most of the fatty acids present in the last part of the cell lipid profiles, after treatment with cinnamaldehyde, were not detected.

4.3.2 SCANNING ELECTRON MICROSCOPE

The figures 1-5 show the cells before (controls) and after the treatment with the natural antimicrobial compounds. By the photos it is possible to assert that the action of some antimicrobials is most probably performed on the membrane of the cells.

Figure 1a shows the control cells of *E coli* O157:H7, comparing them with the cells treated with the compounds (fig. 1 b-f) it can be seen notable differences on the membrane of the cells. In particular it is interesting the alteration due to the presence of eugenol when compared with those due to the other compounds, in fact it seems that eugenol is able to disrupt the membrane allowing the leakage of intracellular constituents, while the other compounds determined just structural alteration of the membrane.

S. Typhimurium and *Pseudomonas* spp., after treatment with cinnamaldehyde and limonene, presented modifications on the membrane, probably because these compounds penetrate in the membrane altering the structure (fig. 2 b-c and 3 b-c). In addition *S*. Typhimurium membrane was altered also by carvacrol and by thymol (fig. 2 d-e), in particular with thymol some of the cells underwent a swelling as it is possible to see in the photo.

Cinnamaldehyde is able to alter the external structure of all the strains tested, but in *S. aureus* was noticed cell wall deterioration and a high degree of cell lysis (fig. 4 b).

B. thermosphacta reported visible membrane alterations when treated with eugenol, cinnamaldehyde and limonene (fig. 5 b-d). In particular the eugenol determined a swelling of the cells and sometime disruption of the membrane.

4.4 DISCUSSION

Due to the hydrophobicity character of the essential oils constituents, the primary site of their toxicity is the membrane. Thymol, eugenol and carvacrol are phenolic compounds, limonene is a terpene and cinnamaldehyde is an aliphatic aldehyde. The action of these compounds on the cells is, most probably, in some cases related to the decrease of the membrane unsaturated fatty acids and in other cases to an alteration of its structure.

In the previous study (Chp 3), the adaptation of the cells to the presence of these compounds resulted by a sensitive modification of the percentage of the UFAs, which are well known to give a high degree of fluidity to the membrane (Bayer *et al.*, 2000). In this research the cells were treated with a high concentration of antimicrobials.

The high amount of fatty acids relived by GC analysis is probably due to their major availability caused by the outer-membrane disintegrating properties of some compounds, making more easily extractable the fatty acids (Ingram, 1977). Another intriguing hypothesis is the possibility that the action of these compounds could principally take place on UFAs, determining their dispersion throughout the suspension and preventing their extraction from the cells.

Helander *et al.* (1998) found that thymol and carvacrol both had prominent outer-membrane disintegrating properties, besides to increase the permeability of cytoplasmic membrane to ATP. Fitzgerald *et al.* (2004) found that the addition of carvacrol to the cultural broth resulted in the rapid depletion of intracellular ATP levels and a corresponding increase in extracellular ATP levels indicating leakage of ATP in *E. coli* and *L. innocua*. Helander *et al.* (1998) also observed a similar change in ATP levels when *E. coli* cells were exposed to either carvacrol or thymol. Ultee *et al.* (2002) proposed that carvacrol acts as a trans-membrane carrier of monovalent cations by exchanging its hydroxyl H^+ for another ion such as K^+.

Burt and Reinders (2003) found, by electron microscopy observation, that treating *E. coli* O157:H7 with oregano essential oil, which is rich in thymol and carvacrol, the cells collapsed after loss of contents. We did not observe a similar effect on *E. coli* O157:H7 and on *S.* Typhimurium strains after being treated with thymol and carvacrol, but the SEM observations showed on the membrane important surface alteration, not involving, as the mechanism of action, a particularly high degree of alteration on fatty acid profile, as found by chromatographic analysis.

Limonene, a cyclic terpene, determined higher fatty acid changes than carvacrol and thymol, except for *B. thermosphacta*, and most probably the membrane alteration observed by SEM are strictly related to those changing. Studies with liposome model systems confirmed that cyclic terpene hydrocarbons accumulate in the membrane, which causes a loss of membrane integrity and dissipation of the proton motive force (Sikkema *et al.,* 1994).

In our findings eugenol caused a noteworthy change only in *Ps. fluorescens* fatty acids profile, cell wall deterioration and a high degree of cell lysis in *E. coli* and deformity in cellular shape of many *B. thermosphacta* cells.

The hydroxyl group of eugenol is thought to bind to proteins, so most probably the slight effect of eugenol on fatty acids and on the membrane is due to a different mechanism of action, which probably involves membrane proteins not visible by SEM.

In a study on *Saccharomyces cerevisiae* it was demonstrated that *trans*-cinnamaldehyde (5 mM) caused a partial collapse of the integrity of the cytoplasmic membrane, leading to excessive leakage of metabolites and enzymes from the cell and finally loss of viability (Smid *et al.*, 1996).

Helander *et al.* (1998) suggested that a possibility for the *trans*-cinnamaldehyde, an aliphatic aldehyde, to penetrate into the cells was through OM-traversing porin proteins.

The high variation of UFAs in all the strain tested in presence of cinnamaldehyde, suggest that this compound acts on the membrane altering its lipid profile, increasing the surface areas of the membrane, altering its structure, but probably it is also able to penetrate in the deeper part of the cell, leading them to death. So the action of cinnamaldehyde does not result closely implicated in the disintegration of the membrane, in fact this results, by SEM observation, true just for *S. aureus* while the other cells show swelling on the surface, perfectly visible on *B. thermosphacta* treated with cinnamaldehyde.

In the light of our findings, we can asses that the antimicrobial action of the tested compounds takes place in different way, each one is involved in increasing the membrane disorder. The mechanism of action implicate the alteration, in different

measure, of fatty acids profile, the damage, alteration or disintegration of cell membrane structure, but in any case these substances are able to lead the cell to death.

Thanks to their hydrophobicity the compounds are able to penetrate and diffuse into the cell membrane, thus more attention should be dedicated to the interactions of the compounds with the elements constituting the substrate. The understanding of these interactions can be helpful to avoid that the compounds could be bound by other material without performing their antimicrobial action.

TABLES AND FIGURES CHAPTER 4

Table 1: Percentage of total fatty acids

Compounds	*E. coli* O157:H7		*S.* serovar Typhimurium		*Ps fluorescens*		*B. thermosphacta*		*S. aureus*	
	UFA[a]	ΔUFA[a, b]	UFA	ΔUFA	UFA	ΔUFA	UFA	ΔUFA	UFA	ΔUFA
Control	55,71	-	59,56	-	67,46	-	58,05	-	70,66	-
Thymol	42,80	-12,91	47,50	-12,06	30,95	-36,52	34,23	-23,82	39,73	-30,92
Limonene	18,38	-37,33	25,59	-33,98	9,80	-57,67	64,70	6,66	32,23	-38,42
Eugenol	43,57	-12,14	44,21	-15,35	1,66	-65,80	48,23	-9,82	53,19	-17,47
Carvacrol	30,99	-24,72	49,38	-10,19	42,64	-24,82	39,66	-18,39	19,23	-51,43
Cinnamaldehyde	14,12	-41,58	7,75	-51,81	1,13	-66,33	2,74	-55,31	3,27	-67,38

a: Values are means of duplicate determinations
b: UFAs control-UFAs treated cells

Table 2: Fatty acid profile of *Escherichia coli* O157:H7 ATCC 43888

F.A.	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	2.18 ± 0.92[a]	N.D.[b]	N.D.	N.D.	0.85 ± 1.20	N.D.
C6:0	1.32 ± 0.66	N.D.	54.50 ± 1.99	N.D.	3.08 ± 2.08	N.D.
C8:0	1.69 ± 0.61	N.D.	1.67 ± 0.48	N.D.	2.33 ± 3.30	N.D.
C10:0	0.86 ± 1.22	3.52 ± 0.65	0.57 ± 0.81	N.D.	2.82 ± 1.63	4.24 ± 1.06
C12:0	1.62 ± 0.85	N.D.	0.89 ± 1.26	N.D.	4.55 ± 1.84	N.D.
C14:0	5.28 ± 1.37	7.69 ± 0.49	3.13 ± 0.67	6.90 ± 0.82	7.91 ± 0.17	2.51 ± 1.82
C14:1cis	5.03 ± 2.50	N.D.	N.D.	N.D.	N.D.	N.D.
C15:0	0.11 ± 0.15	N.D.	N.D.	4.76 ± 0.88	N.D.	N.D.
C15:1cis	6.49 ± 1.77	N.D.	N.D.	N.D.	N.D.	N.D.
C16:0	18.47 ± 2.67	33.66 ± 1.74	17.53 ± 0.25	37.78 ± 1.22	24.95 ± 0.13	7.37 ± 0.61
C16:1cis	1.61 ± 1.03	3.21 ± 0.21	1.83 ± 0.14	4.96 ± 0.87	2.60 ± 0.85	2.39 ± 0.03
C17:0	3.57 ± 5.05	N.D.	N.D.	N.D.	N.D.	68.79 ± 6.76
C17:1cis	8.25 ± 1.96	15.04 ± 0.61	9.17 ± 0.40	20.80 ± 2.55	11.24 ± 4.24	2.25 ± 0.18
C18:0	4.28 ± 1.32	11.65 ± 0.31	2.71 ± 0.46	7.00 ± 2.49	10.46 ± 1.73	2.97 ± 0.29
C18:1trans	3.54 ± 0.63	N.D.	N.D.	N.D.	1.09 ± 0.33	0.51 ± 0.46
C18:1cis	7.07 ± 4.33	15.88 ± 0.68	3.40 ± 0.74	9.79 ± 1.50	13.78 ± 2.17	5.68 ± 0.84
C18:2trans	0.62 ± 0.87	0.64 ± 0.90	0.42 ± 0.11	N.D.	N.D.	0.12 ± 0.17
C18:2cis	2.28 ± 1.38	2.95 ± 0.19	0.69 ± 0.10	N.D.	N.D.	2.83 ± 2.51
C18:3 (gla)	3.11 ± 1.62	4.18 ± 0.61	2.64 ± 0.16	6.45 ± 5.70	2.28 ± 1.20	0.36 ± 0.51
C18:3cis	12.91 ± 4.61	0.90 ± 1.28	0.22 ± 0.31	N.D.	N.D.	N.D.
C20:0	0.93 ± 1.31	N.D.	0.61 ± 0.87	N.D.	5.11 ± 1.07	N.D.
C20:1cis	1.86 ± 0.86	N.D.	N.D.	1.58 ± 2.23	N.D.	N.D.
C22:0	0.85 ± 1.21	0.69 ± 0.97	N.D.	N.D.	3.27 ± 0.57	N.D.
C20:5cis	0.63 ± 0.89	N.D.	N.D.	N.D.	N.D.	N.D.
C24:0	3.15 ± 1.41	N.D.	N.D.	N.D.	3.67 ± 0.94	N.D.
C22:6cis	2.32 ± 3.28	N.D.	N.D.	N.D.	N.D.	N.D.

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 3: Fatty acid profile of *Pseudomonas fluorescens* NCIMB 10586

F.A.	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	3.98 ± 1.36[a]	N.D.[b]	3.66 ± 0.93	N.D.	1.43 ± 2.02	N.D.
C6:0	0.70 ± 0.99	N.D.	7.54 ± 0.73	N.D.	4.04 ± 2.10	N.D.
C8:0	1.04 ± 0.99	N.D.	8.27 ± 0.37	N.D.	3.19 ± 3.38	N.D.
C10:0	0.66 ± 0.93	5.87 ± 0.20	5.97 ± 1.35	N.D.	6.30 ± 2.52	10.90 ± 0.36
C12:0	1.53 ± 0.95	N.D.	9.85 ± 1.15	1.86 ± 0.35	5.72 ± 4.15	N.D.
C14:0	1.59 ± 0.80	8.74 ± 1.01	10.91 ± 0.83	12.75 ± 0.71	7.29 ± 0.52	N.D.
C14:1cis	6.51 ± 1.76	N.D.	N.D.	N.D.	N.D.	N.D.
C15:0	0.32 ± 0.45	N.D.	N.D.	N.D.	N.D.	1.26 ± 0.06
C15:1cis	8.41 ± 0.72	N.D.	N.D.	N.D.	N.D.	N.D.
C16:0	11.45 ± 2.50	44.86 ± 0.97	10.12 ± 0.13	65.31 ± 0.30	11.98 ± 1.60	0.86 ± 0.08
C16:1cis	N.D.	N.D.	N.D.	N.D.	5.75 ± 1.68	0.52 ± 0.08
C17:0	1.18 ± 1.67	N.D.	N.D.	N.D.	N.D.	85.63 ± 0.62
C17:1cis	0.51 ± 0.72	N.D.	N.D.	N.D.	28.60 ± 10.86	N.D.
C18:0	3.25 ± 1.37	9.59 ± 0.25	9.91 ± 0.58	14.03 ± 0.46	6.79 ± 0.55	0.23 ± 0.01
C18:1trans	N.D.	8.64 ± 1.15	N.D.	N.D.	N.D.	N.D.
C18:1cis	25.19 ± 2.89	18.94 ± 0.73	4.22 ± 0.10	N.D.	N.D.	N.D.
C18:2trans	3.09 ± 0.30	3.37 ± 0.43	2.91 ± 1.04	N.D.	N.D.	N.D.
C18:2cis	1.30 ± 0.36	N.D.	N.D.	N.D.	N.D.	N.D.
C18:3cis	18.49 ± 1.05	N.D.	2.67 ± 0.77	1.66 ± 0.02	2.64 ± 1.44	N.D.
C20:0	0.14 ± 0.20	N.D.	10.44 ± 1.80	4.41 ± 0.53	4.09 ± 3.09	N.D.
C22:0	1.63 ± 0.74	N.D.	8.89 ± 0.09	N.D.	3.18 ± 2.31	N.D.
C22:1cis	0.12 ± 0.17	N.D.	N.D.	N.D.	5.65 ± 2.08	0.61 ± 0.87
C24:0	5.07 ± 0.39	N.D.	4.61 ± 0.36	N.D.	3.36 ± 2.08	N.D.
C22:6cis	3.85 ± 2.40	N.D.	N.D.	N.D.	N.D.	N.D.

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 4: Fatty acid profile of *Salmonella enterica* serovar Typhimurium ATCC 14028

F.A.	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	1.87 ± 1.10[a]	N.D.[b]	N.D.	N.D.	1.04 ± 1.48	N.D.
C6:0	0.51 ± 0.72	N.D.	40.20 ± 4.13	N.D.	N.D.	N.D.
C8:0	0.12 ± 0.17	N.D.	0.90 ± 1.27	N.D.	N.D.	N.D.
C10:0	0.72 ± 0.73	N.D.	0.86 ± 1.21	N.D.	N.D.	2.74 ± 0.84
C12:0	1.06 ± 1.35	0.80 ± 0.30	1.22 ± 1.72	N.D.	N.D.	N.D.
C14:0	3.51 ± 1.52	7.73 ± 0.85	4.48 ± 0.31	7.34 ± 0.19	6.47 ± 0.72	1.19 ± 0.54
C15:0	N.D	N.D.	N.D.	N.D.	N.D.	1.13 ± 0.10
C15:1cis	4.00 ± 2.60	N.D.	N.D.	N.D.	N.D.	N.D.
C16:0	25.54 ± 3.92	39.25 ± 0.55	21.95 ± 1.18	43.22 ± 0.61	39.34 ± 1.36	3.17 ± 0.98
C16:1trans	0.31 ± 0.21	0.13 ± 0.19	0.10 ± 0.14	0.75 ± 0.13	0.45 ± 0.63	0.16 ± 0.15
C16:1cis	7.35 ± 1.25	4.62 ± 0.49	2.07 ± 0.21	4.69 ± 0.58	5.10 ± 0.32	1.81 ± 1.87
C17:0	1.77 ± 2.13	N.D.	N.D.	N.D.	N.D.	82.51 ± 4.97
C17:1cis	10.47 ± 0.82	20.50 ± 0.39	11.54 ± 0.51	19.08 ± 0.11	23.11 ± 0.76	2.66 ± 0.62
C18:0	2.51 ± 1.68	4.71 ± 0.31	2.59 ± 0.15	5.23 ± 0.01	3.78 ± 0.12	1.52 ± 1.75
C18:1trans	2.00 ± 0.64	6.85 ± 0.30	2.20 ± 0.41	3.59 ± 0.17	3.35 ± 0.88	N.D.
C18:1cis	2.80 ± 0.59	6.31 ± 0.18	4.05 ± 0.51	7.08 ± 0.34	6.26 ± 0.37	1.46 ± 1.09
C18:2trans	13.12 ± 4.29	N.D.	N.D.	N.D.	N.D.	N.D.
C18:2cis	1.31 ± 0.80	N.D.	N.D.	N.D.	N.D.	N.D.
C18:3 (gla)	6.14 ± 1.55	9.10 ± 0.26	5.63 ± 0.99	9.01 ± 0.39	11.11 ± 1.60	1.66 ± 0.67
C18:3cis	10.42 ± 3.09	N.D.	N.D.	N.D.	N.D.	N.D.
C20:0	0.16 ± 0.23	N.D.	0.84 ± 1.19	N.D.	N.D.	N.D.
C20:0	1.02 ± 1.44	N.D.	N.D.	N.D.	N.D.	N.D.
C22:0	0.06 ± 0.09	N.D.	0.87 ± 1.23	N.D.	N.D.	N.D.
C24:0	1.60 ± 2.26	N.D.	0.52 ± 0.73	N.D.	N.D.	N.D.
C22:6cis	1.65 ± 2.34	N.D.	N.D.	N.D.	N.D.	N.D.

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 5: Fatty acid profile of *Staphylococcus aureus* NCTC 6571

F.A.	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	4.89 ± 1.22[a]	N.D.[b]	N.D.	N.D.	N.D.	N.D.
C6:0	1.15 ± 1.19	N.D.	4.65 ± 1.20	N.D.	5.72 ± 0.40	N.D.
C8:0	0.38 ± 0.14	N.D.	3.50 ± 0.44	N.D.	5.44 ± 0.77	N.D.
C10:0	0.34 ± 0.16	0.73 ± 0.04	3.21 ± 0.02	N.D.	4.32 ± 0.64	3.54 ± 0.08
C12:0	2.00 ± 0.20	1.98 ± 0.08	5.64 ± 0.52	N.D.	7.66 ± 0.64	N.D.
C14:0	1.65 ± 0.53	7.84 ± 0.00	5.74 ± 0.20	8.69 ± 0.88	7.19 ± 0.13	0.45 ± 0.01
C14:1cis	13.19 ± 2.74	12.46 ± 0.24	20.03 ± 0.89	28.23 ± 2.69	16.39 ± 1.05	1.50 ± 0.10
C15:0	0.26 ± 0.37	N.D.	N.D.	N.D.	N.D.	0.48 ± 0.08
C15:1cis	6.93 ± 0.94	2.06 ± 0.11	2.42 ± 0.70	N.D.	2.84 ± 0.59	0.25 ± 0.02
C16:0	3.59 ± 0.94	24.61 ± 0.70	9.35 ± 0.42	10.68 ± 0.68	9.88 ± 0.93	1.72 ± 0.04
C16:1cis	0.62 ± 0.87	2.96 ± 0.09	2.40 ± 0.49	3.97 ± 0.24	N.D.	0.27 ± 0.01
C17:0	0.63 ± 0.89	0.72 ± 0.03	N.D.	N.D.	N.D.	87.97 ± 0.16
C17:1cis	N.D.	1.14 ± 0.17	1.51 ± 0.38	N.D.	N.D.	N.D.
C18:0	4.04 ± 0.66	16.19 ± 0.72	15.06 ± 0.54	8.82 ± 4.20	14.94 ± 0.91	1.47 ± 0.02
C18:1trans	N.D.	0.93 ± 0.15	N.D.	N.D.	N.D.	0.05 ± 0.02
C18:1cis	23.00 ± 0.81	15.67 ± 0.37	2.34 ± 0.48	3.83 ± 0.21	N.D.	1.06 ± 0.16
C18:2trans	N.D.	0.80 ± 0.09	1.36 ± 0.48	N.D.	N.D.	0.14 ± 0.01
C18:2cis	0.17 ± 0.25	2.15 ± 0.07	2.17 ± 0.53	3.38 ± 0.56	N.D.	N.D.
C18:3cis	15.42 ± 1.80	N.D.	N.D.	4.51 ± 0.49	N.D.	N.D.
C20:0	4.27 ± 1.07	8.19 ± 0.05	13.50 ± 0.39	18.62 ± 1.85	15.43 ± 0.12	1.10 ± 0.08
C20:1cis	N.D.	N.D.	N.D.	3.27 ± 0.45	N.D.	N.D.
C20:2cis	N.D.	N.D.	N.D.	4.60 ± 0.48	N.D.	N.D.
C22:0	N.D.	N.D.	4.05 ± 0.23	N.D.	4.82 ± 1.06	N.D.
C20:5cis	4.19 ± 0.56	N.D.	N.D.	N.D.	N.D.	N.D.
C24:0	6.14 ± 0.32	N.D.	3.08 ± 0.08	N.D.	5.36 ± 0.28	N.D.
C22:6cis	7.14 ± 0.58	1.55 ± 2.19	N.D.	1.39 ± 1.96	N.D.	N.D.

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

Table 6: Fatty acid profile of *Brochothrix thermosphacta* NCTC 10822

F.A.	Control	Thymol	Limonene	Eugenol	Carvacrol	Cinnamaldehyde
C4:0	2.51 ± 0.90[a]	N.D.[b]	11.80 ± 0.80	N.D.	N.D.	N.D.
C6:0	0.67 ± 0.59	N.D.	N.D.	N.D.	N.D.	N.D.
C8:0	1.05 ± 0.16	N.D.	1.43 ± 0.17	N.D.	N.D.	N.D.
C10:0	1.21 ± 0.49	3.17 ± 0.29	N.D.	N.D.	8.64 ± 1.41	1.62 ± 0.19
C12:0	2.30 ± 0.78	2.81 ± 2.07	2.28 ± 0.37	4.20 ± 3.26	6.98 ± 1.01	0.24 ± 0.01
C14:0	5.87 ± 1.95	6.07 ± 0.15	2.25 ± 0.40	8.48 ± 0.39	4.89 ± 0.62	0.18 ± 0.02
C14:1cis	2.05 ± 1.07	4.84 ± 0.43	6.57 ± 0.91	5.48 ± 0.21	5.07 ± 0.85	0.37 ± 0.23
C15:0	0.15 ± 0.21	7.16 ± 0.27	N.D.	N.D.	N.D.	0.72 ± 0.12
C15:1cis	5.81 ± 2.20	1.84 ± 0.52	7.06 ± 1.25	4.30 ± 0.36	3.30 ± 0.42	0.07 ± 0.10
C16:0	16.41 ± 2.60	37.91 ± 1.20	11.79 ± 0.71	30.00 ± 5.11	29.38 ± 4.97	1.00 ± 0.07
C16:1trans	0.21 ± 0.30	N.D.	N.D.	N.D.	N.D.	0.64 ± 0.45
C16:1cis	1.06 ± 0.28	N.D.	1.56 ± 0.13	N.D.	N.D.	0.09 ± 0.01
C17:0	0.16 ± 0.23	N.D.	0.31 ± 0.44	N.D.	N.D.	93.30 ± 1.45
C17:1cis	N.D.	N.D.	N.D.	N.D.	2.57 ± 0.01	N.D.
C18:0	6.79 ± 2.12	5.98 ± 0.49	2.42 ± 0.25	3.06 ± 0.47	7.42 ± 1.39	0.20 ± 0.02
C18:1trans	1.80 ± 0.61	N.D.	0.07 ± 0.10	N.D.	N.D.	N.D.
C18:1cis	4.69 ± 0.92	8.19 ± 0.82	19.15 ± 0.92	10.84 ± 1.69	7.69 ± 3.22	0.87 ± 0.59
C18:2trans	21.64 ± 3.54	0.35 ± 0.49	0.08 ± 0.05	1.14 ± 0.21	0.41 ± 0.58	N.D.
C18:2cis	0.88 ± 0.88	N.D.	0.84 ± 0.73	N.D.	N.D.	N.D.
C18:3 (gla)	N.D.	N.D.	0.62 ± 0.45	N.D.	N.D.	N.D.
C18:3cis	12.67 ± 3.36	7.80 ± 0.05	7.86 ± 7.96	9.71 ± 1.61	9.27 ± 2.35	0.68 ± 0.47
C20:0	1.57 ± 0.64	1.08 ± 0.04	0.23 ± 0.17	1.56 ± 0.33	1.34 ± 0.30	N.D.
C20:1cis	2.33 ± 0.79	N.D.	2.32 ± 0.35	1.27 ± 1.79	N.D.	N.D.
C20:2cis	N.D.	8.56 ± 0.68	12.90 ± 0.75	11.01 ± 1.36	8.14 ± 2.76	N.D.
C20:3cis	N.D.	N.D.	0.81 ± 0.21	N.D.	N.D.	N.D.
C22:0	0.53 ± 0.75	1.62 ± 0.05	N.D.	1.70 ± 0.50	1.70 ± 0.66	N.D.
C22:1cis	N.D.	N.D.	0.73 ± 0.21	N.D.	N.D.	N.D.
C20:5cis	N.D.	N.D.	0.90 ± 0.17	N.D.	N.D.	N.D.
C24:0	2.74 ± 1.01	N.D.	2.78 ± 0.15	2.78 ± 0.50	N.D.	N.D.
C22:6cis	4.91 ± 0.51	2.64 ± 0.14	3.23 ± 0.77	4.48 ± 0.24	3.20 ± 0.15	N.D.

a: Values are means of duplicate determinations ± S.D.
b: Not Detected

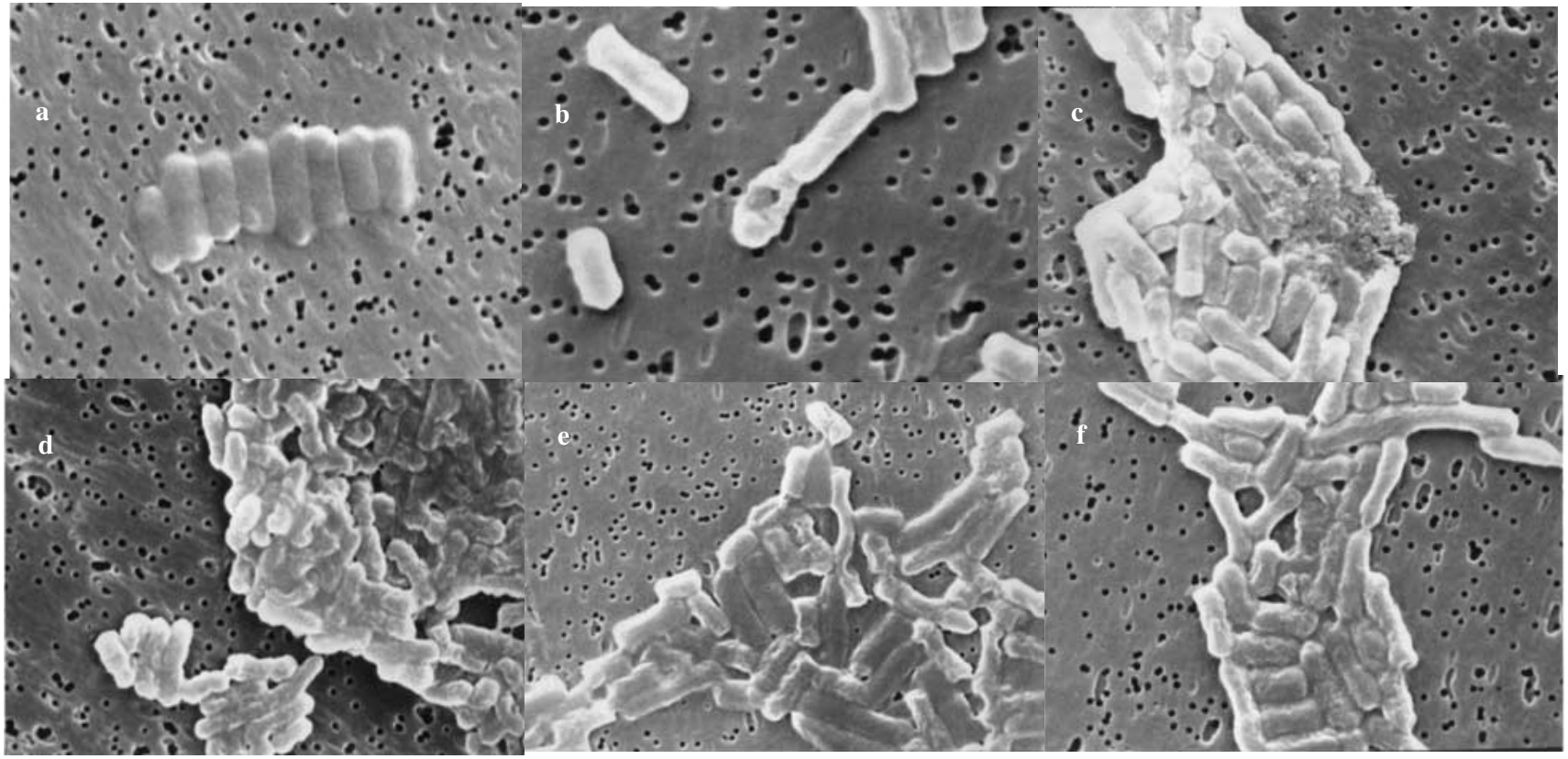

Fig. 1 Scanning electron microscope images of *E. coli* O157:H7 cells after treatment with natural antimicrobial compounds. Untreated cells (control) (a); cells damaged after treatment with carvacrol (b), eugenol (c), cinnamaldehyde (d), limonene (e) and thymol (f)

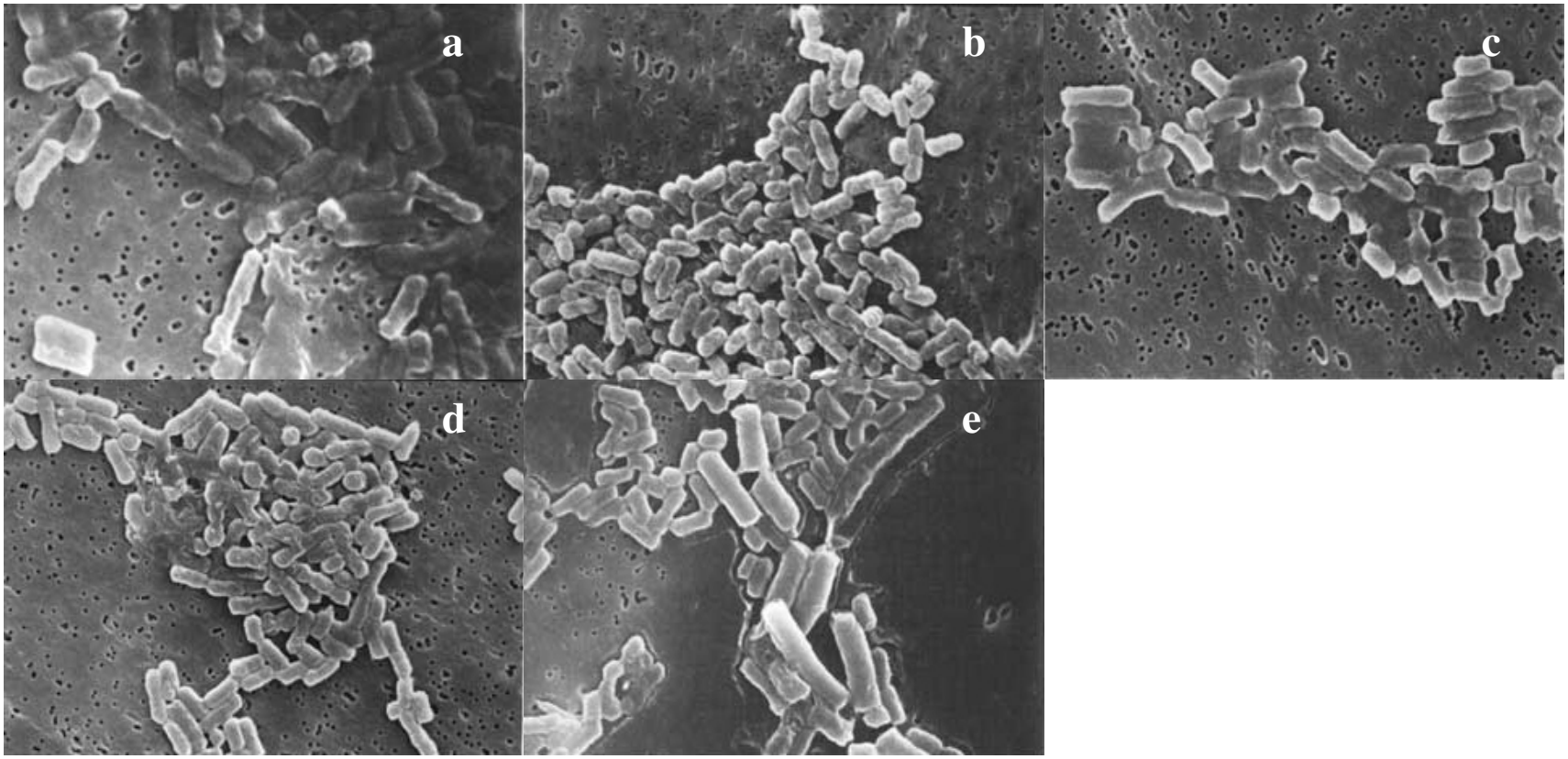

Fig. 2 Scanning electron microscope images of *Salmonella enterica* serovar Typhimurium cells after treatment with natural antimicrobial compounds. Untreated cells (control) (a); cells damaged after treatment with cinnamaldehyde (b), limonene (c), carvacrol (d) and thymol (e)

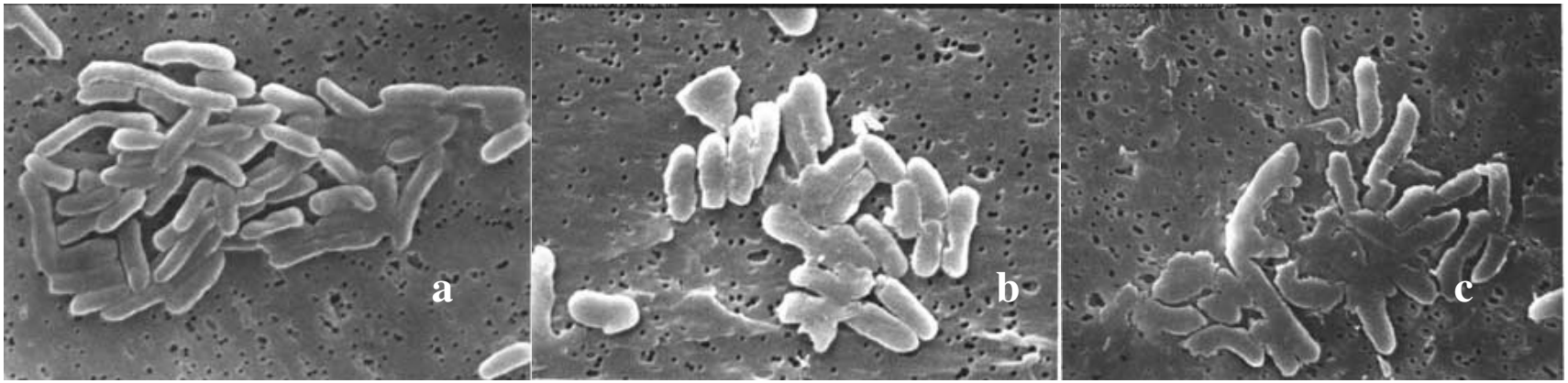

Fig. 3 Scanning electron microscope images of *Pseudomonas fluorescens* cells after treatment with natural antimicrobial compounds. Untreated cells (control) (a); cells damaged after treatment with limonene (b) and cinnamaldehyde (c)

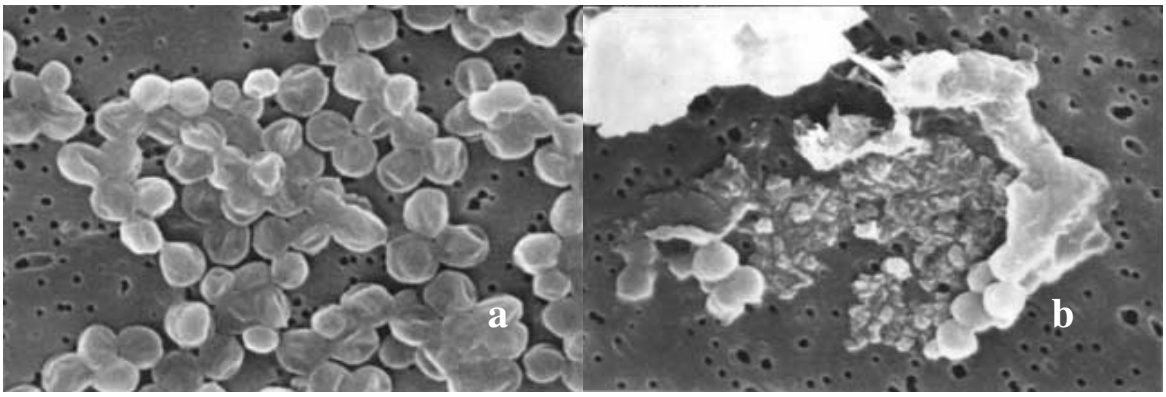

Fig. 4 Scanning electron microscope images of *Staphylococcus aureus* cells after treatment with natural antimicrobial compounds. Untreated cells (control) (a); cells damaged after treatment with cinnamaldehyde (b)

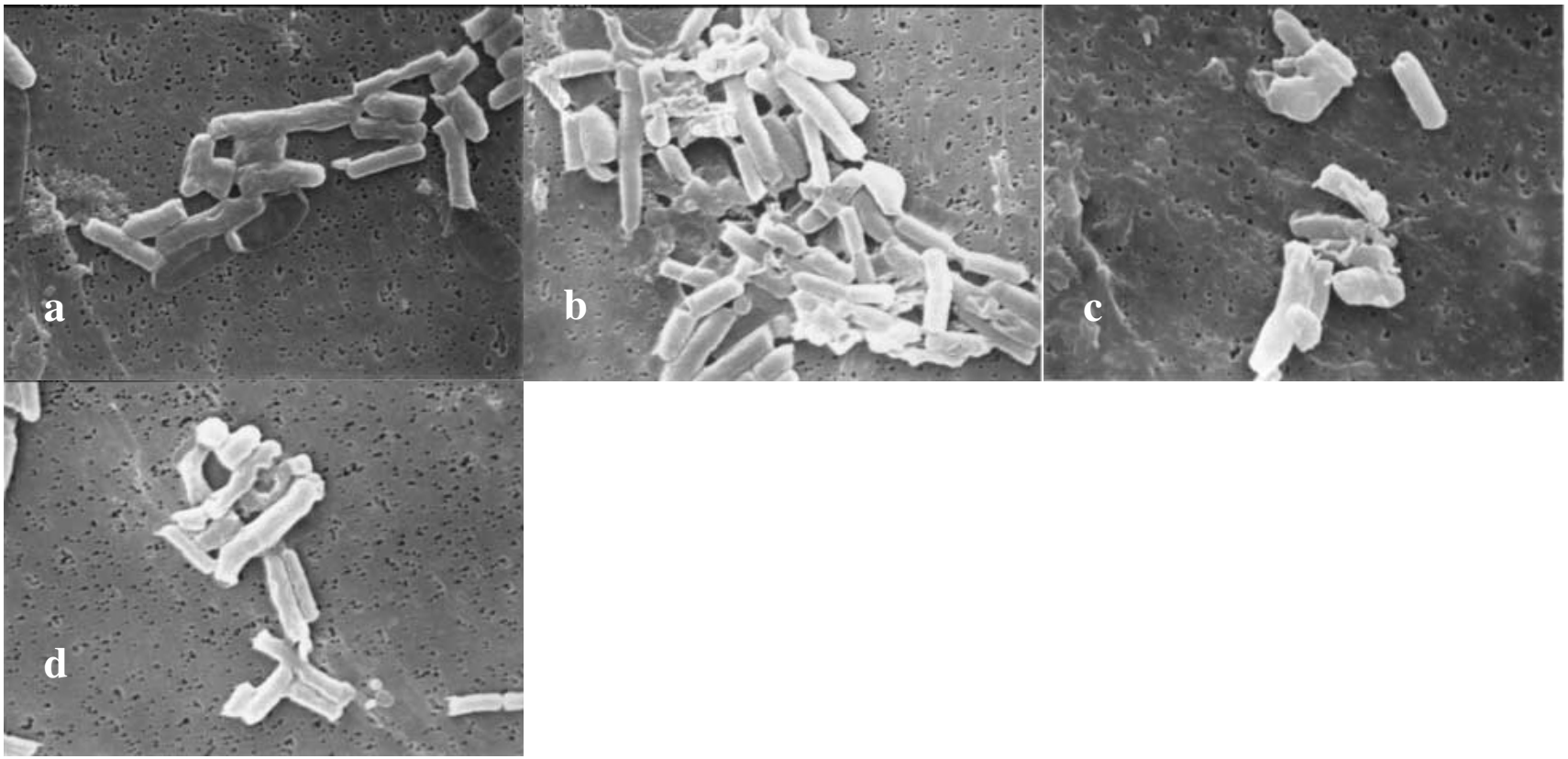

Fig. 5 Scanning electron microscope images of *Brochothrix thermosphacta* cells after treatment with natural antimicrobial compounds. Untreated cells (control) (a); cells damaged after treatment with eugenol (b), cinnamaldehyde (c) and limonene (d)

CHAPTER 5

5.1 OVERALL CONCLUSIONS

Over the years many authors dealt with the antimicrobial activity of essential oils and their compounds. Most of them made known the antimicrobial action of new compounds, or essential oils, like presumed. Later other authors clarified some of the mode of action of these compounds.

While research has provided knowledge to link membrane structure and function, there are still many areas where information is lacking In particular, those linking alterations in fatty acid composition with membrane fluidity changes. The subtleties of the lipid compositional changes provide for the multitude of interactions between lipids and proteins, which act to control the membrane embedded protein molecules.

We already said that these compounds interact with the cell membrane causing leakage of cellular components, change fatty acid and phospholipid constituents, impair energy metabolism, alter nutrient uptake and electron transport, and influence genetic material synthesis in addition to the inhibition of phosphorylation and other enzymatic reactions. It is clear that this area of research is very extensive and require as much extension of experience. Work on the interactions of lipophilic compounds with phospholipid membranes has not been restricted to microbiological research but is also being performed in other disciplines, ranging from detailed molecular studies of modified acyl-acyl interactions (physical chemistry), which provide a thermodynamic basis for predicting the membrane-directed action of anesthetics and drugs (anesthesiology and pharmacology).

Lipids are not the only molecules responsive to disturbance. Little information is available in the literature about membrane proteins and lipid–protein interactions due to

the difficulty in crystallizing a membrane protein and in resolving the membrane lipids that surround them.

The study of stress adaptation in microorganisms has shown that complex changes in cell composition and regulation take place as a result of exposure to stressful environments. Such changes enable microorganisms to maintain the physiology of the cell and thus potentially survive and grow after exposure to stress conditions in food products. It has also been shown that there are many molecular mechanisms that microorganisms use to adapt and survive. Adaptive changes to environmental stresses require large amounts of energy, and during the adaptation stages, all normal cell division is stopped. This has several important consequences for microbial food poisoning or spoilage; as the lag phase before growth extends, the growth rate decreases and the final cell numbers decrease. The nutritional requirements and the enzymatic and chemical composition of cells are also affected (Herbert 1986, 1989).

Under stress conditions, the increased production of low molecular weight stress proteins can result (Margesin and Schinner, 1994; Thieringer *et al.*, 1998). These proteins can be produced in response to temperature (i.e. heat and cold shock proteins) or to other stressors.

In this work we have focussed our attention just on the fatty acids, as site of action of the tested compounds, demonstrating remarkable that the fatty acid profile undergoes a noteworthy change in presence of both sublethal amounts of lipophilic compounds and lethal concentrations. In this last case the cells present alterations of the external structure well visible after electron scanning microscopy analysis.

Of course these represent just a side of the possible approach we could improve to study the mechanism of action of EO compounds. The antimicrobial action on the protein

profile, membrane-embedded enzymes, metabolic activities and genic regulation, determining structural-physiological changes could be investigated, in further studies, by the Atomic Force Microscopy (AFM). In fact, AFM can be used to probe the physical properties of the specimen, such as molecular interactions, surface hydrophobicity, surface charges, and mechanical properties. These measurements provide new insight into the structure-function relationships of microbial surfaces. Remarkably, conformational changes induced by a physiological signal can be monitored in single membrane proteins, providing novel insight into structure-function relationships. For living cells, high-resolution imaging remains challenging, but a wealth of novel structural information can be obtained, such as visualizations of surface ultra structure in native conditions and monitoring of physiological changes in real time. Knowledge of the mechanism of the inhibitory action of essential oils and their compounds is helpful in technical applications of microorganisms (fermentation, environmental biotechnology) and in selection of novel antibiotics and food preservatives. However, it remains to be seen whether the modifications in membrane composition and other changes demonstrated in this research really lead to an increased resistance or are just part of a general adaptive response.

Of course this kind of research should be carried out applying methods useful to evaluate the relation between the alterations of the cellular structure and metabolic pathways and the gene expression after stress exposure of the cells.

REFERENCES

Akgül A. and M. Kivanç 1988. Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. Int. J. Food Microiol., **6**: 263– 268.

Akgül A., M. Kivanç and S. Sert 1991. Effect of carvacrol on growth and toxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. Sci. Aliment, **11**: 361–370.

Arras G. and G.E. Grella 1992. Wild thyme, *Thymus capitatus*, essential oil seasonal changes and antimycotic activity. J. Hort. Sci., **67** (2): 197–202.

Aureli P., A. Costantini and S. Zolea 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. J. Food Prot., **55** (5): 344–348.

Azzouz M.A. and L.B. Bullerman 1982. Comparative antimycotic effects of selected herbs, spices, plant components and commercial antifungal agents. J. Food Prot., **45** (14): 1298–1301.

Bagamboula C.F., M. Uyttendaele and J. Debevere 2003. Antimicrobial effect of spices and herbs on *Shigella sonnei* and *S. flexneri*. J. Food Prot., **66**: 668-673.

Baratta M.T., H.J.D. Dorman, S.G. Deans, A.C. Figueiredo, J.G. Baroso and G. Ruberto 1998. Antimicrobial and antioxidant properties of some commercial essential oils. Flavour Fragrance J., **13**: 235-244.

Barry A.L. 1976. The Antimicrobic Susceptibility Test: Principles and Practices. Lea and Febiger, Philadelphia.

Bassett I.B., D.L. Pannowitz and R.S.C. Barnetson 1990. A comparative study of tea-tree oil versus benzoylperoxide in the treatment of acne. Med. J. Australia, 153: 455–458.

Bassole I.H.N., A.S. Ouattara, R. Nebie, C.A.T. Ouattara, Z.I. Kabore, S.A. Traore 2003. Chemical composition and antibacterial activities of the essential oils of *Lippia chevalieri* and *Lippia multiflora* from Burkina Faso. Phytochem., **62**: 209–212.

Bauer K. and D. Garbe 1985. Common Fragrance and Flavor Materials. Preparation, Properties and Uses. VCH Verlagsgesellschaft, Weinheim, p. 213.

Bauer K., D. Garbe and H. Surburg 2001. Common Fragrance and Flavor Materials: Preparation, Properties and Uses. Wiley-VCH, Weinheim, p. 293.

Beuchat L.R., 1976. Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids. J. Food Sci., **41**: 899–902.

Biavati B., M. Ozcan and R. Piccaglia 2004. Composition and antimicrobial properties of *Satureja cuneifolia* Ten. and *Thymbra sintenisii* Bornm. et Aznav. subsp. *isaurica* P.H. Davis essential oils. Ann. Microbiol., **54**: 393-401.

Bishop C.D. 1995. Antiviral activity of the essential oil of *Melaleuca alternifolia* (Maiden and Betche) Cheel (tea tree) against tobacco mosaic virus. J. Essent. Oil Res.,**7**: 641– 644.

Bleasel N., B. Tate and M. Rademaker 2002. Allergic contact dermatitis following exposure to essential oils. Australasian J. Dermatol., **43** (3): 211 – 213.

Board R.G. and G.W. Gould 1991. Future prospects. In: Gould N.J.R.a.G.W. (Ed.), Food Preservatives. Blackie, Glasgow, pp. 267–284.

Boyle W. 1955. Spices and essential oils as preservatives. American Perfum. Essent. Oil Review, **66**: 25– 28.

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. J. App. Bacteriol., **78**: 609– 615.

Burt S.A. and R.D. Reinders 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Lett. Appl. Microbiol., **36**: 162-167.

Canillac N. and A. Mourey 2001. Antibacterial activity of the essential oil of *Picea excelsa* on *Listeria*, *Staphylococcus aureus* and coliform bacteria. Food Microbiol., **18**: 261– 268.

Carson C.F. and T.V. Riley 1993. Antimicrobial activity of the essential oil of *Melaleuca alternifolia*. Lett Appl. Microbiol., **16**: 49– 55.

Carson C.F., and T.V. Riley 1995. Antimicrobial activity of major components of the essential oil of *Melaleuca alternifolia.* J. Appl. Bacteriol., **78**: 264-269.

Carson C.F. and T.V. Riley 2001. Safety, efficacy and provenance of tea tree (*Melaleuca alternifolia*) oil. Contact Dermatitis, **45**: 65– 67.

Carson C.F., B.D. Cookson, H.D. Farrelly and T.V. Riley 1995a. Susceptibility of methicillin-resistant Staphylococcus aureus to the essential oil of *Melaleuca alternifolia*. J. Antimicrob. Chemoth., **35**: 421–424.

Carson C.F., K.A. Hammer and T.V. Riley 1995b. Broth microdilution 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.

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. Antimicrob. Agent Chemoth., **46** (6): 1914–1920.

Ceylan E. and D.Y.C. Fung 2004. Antimicrobial activity of spices. J Rap. Meth. Automat. Microbiol., **12**: 1-55

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 Microbiol., **14**: 161–174.

Chiou R.Y.-Y., R.D. Phillips, P. Zaho, M.P.Doyle and L.R. Beuchat. 2004. Ethanol-mediated variations in cellular fatty acids composition and protein profile of two genotypically different strains of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. **70**: 2204-2210.

Cimanga, K., K. Kambu, L. Tona, S. Apers, T. De Bruyne, N. Hermans, J. Totte, 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. J. Ethnopharmacol., **79**: 213– 220.

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, pp. 1– 137.

Commission 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, pp. 1 –160.

Commission 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, pp. 8 – 16.

Commission 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, pp. 10–11.

Conner D.E. and L.R. Beuchat1984. Effects of essential oils from plants on growth of food spoilage yeasts. J. . Food Sci., **49**: 429– 434.

Cosentino S., C.I.G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arredi and F. Palmas 1999. In vitro antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Lett. Appl Microbiol., **29**: 130– 135.

Cosentino S., A. Barra, B. Pisano, M. Canizza, F.M. Pirisi and F. Palmas 2003. Composition and antimicrobial properties of Sardinian *Juniperus* essential oil against foodborne pathogens and spoilage microrganisms. J. Food Prot., **66**: 1288-1291.

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). J. Appl. Microbiol., **88**: 170–175.

Cremieux A., H. Guirard-Dauriac and G. Dumenil 1981. Neutralisation des antiseptiques et désinfectants. J. . Pharmacie de Belgique, **36:** (4), 223– 226.

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. J. Food Prot., **66:** (3) 390– 395.

Crosthwaite D., 1998. UK trade within the flavour and fragrance industry. International Federation of Essential Oils and Aroma Trades—21st International Conference on Essential Oils and Aroma's. IFEAT, London, pp. 6 –12.

Daferera D.J., B.N. Biogas and M.G. Polissiou 2000. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. J. Agric. Food Chem., **48:** 2576–2581.

Daferera D.J., B.N. Biogas and M.G. Polissiou 2003. The effectiveness of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. Crop Prot., **22**: 39-44.

Davidson P.M. 1997. Chemical preservatives and natural antimicrobial compounds. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Food Microbiology: Fundamentals and Frontiers. ASM, Washington, pp. 520–556.

Davidson P.M. and M.E. Parish 1989. Methods for testing the efficacy of food antimicrobials. Food Technol., **43**: 148–155.

Deans S.G. and G. Ritchie 1987. Antibacterial properties of plant essential oils. Int. J. Food Microbiol., **5:** 165– 180.

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.

de Mendoza D. and J.E. Cronan. 1983. Thermal regulation of membrane lipid fluidity in bacteria. Trends Biochem. Sci. **8**: 49– 52

Delaquis P.J., K. Stanich, B. Girare and G. **Mazza** 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. Int. J. Food Microbiol., **74:** 101–109.

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. J. Ess. Oil Res., **13**: 460– 462.

Denich T.J., L.A. Beaudette, H. Lee and J.T. Trevors. 2003. Effect of selected environmental and physico-chemical facters on bacterial cytoplasmic membranes. J. Microbiol. Met. **52**: 149-182.

Denyer S.P. and W.B. Hugo 1991a. Biocide-induced damage to the bacterial cytoplasmic membrane. In: Denyer, S.P., Hugo, W.B. (Eds.), Mechanisms of Action of Chemical Biocides. The Society for Applied Bacteriology, Technical Series No 27. Oxford Blackwell Scientific Publication, Oxford, pp. 171– 188.

Denyer S.P. and W.B. Hugo 1991b. Mechanisms of antibacterial action—A summary. In: Denyer, S.P., Hugo, W.B. (Eds.), Mechanisms of Action of Chemical Biocides. Blackwell, Oxford, pp. 331– 334.

Dhar P., U. Ayala, E. Andarge, S. Morisseau and T. Snyder-Leiby 2004. Study of the structural changes on the antimicrobial activity of [3.1.1]-bicyclics. J. Ess. Oil Res.,

Di Pasqua R., V. De Feo F. Villani and G. Mauriello 2005. *In vitro* antimicrobial activity of essential oils from Mediterranean *Apiaceae*, *Verbenaceae* and *Lamiaceae* against pathogens and spoilage bacteria. Ann. Microbiol. **55**: 139-143.

Dorman H.J.D. and S.G. Deans 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol., **88**: 308– 316.

Elgayyar M., F.A. Draughon, D.A. Golden, J.R. Mount 2001. Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. J. Food Prot., **64** (7): 1019– 1024.

European Pharmacopoeia, Maisonneuve SA, Sainte-Ruffine 1975 , Vol. 3, p. 68.

Evans R.I., P.J. McClure, G.W. Gould and N.J. Russell. 1998. The effect of growth temperature on the phospholipid and fatty acyl compositions of non-proteolytic *Clostridium botulinum*. Int. J. Food Microbiol. **40**: 159-167.

Faleiro M.L., M.G. Miguel, F. Ladeiro, F. Venâncio, R. Tavares, J.C. Brito, A.C. Figueiredo J.G., Barroso and L.G. Pedro 2003. Antimicrobial activity of essential oils isolated fro Portuguese endemic species of *Thymus*. Lett. Appl. Microbiol., **36**: 35-40.

Farag R.S., Z.Y. Daw, F.M. Hewedi and G.S.A. El-Baroty 1989. Antimicrobial activity of some Egyptian spice essential oils. J. Food Prot., **52** (9): 665– 667.

Firouzi R., M. Azadbakht and A. Nabinedjad 1998. Anti-listerial activity of essential oils of some plants. J. Appl. Anim. Res., **14**: 75– 80.

Fitzgerald D.J., M. Strattford, M.J. Gasson, J. Uecker, A. Bios and A. Narbad 2004. Mode of action of vanillin against *Escherichia coli, Lactobacillus Plantarum and Listeria innocua*. J. Appl. Microbiol., **97**: 104-113

Fozo E.M., J.K. Kajafasz and R.G. Quivey Jr. 2004. Low pH-induced membrane fatty acids alterations in oral bacteria. FEMS Microbiol. Letters. **238**: 291-295.

Fozo E.M. and R.G. Quivey Jr. 2004. The fabM gene product of *Streptococcus* mutants is responsible for the synthesis of monounsaturated fatty acids and is necessary for survival at low pH. J. Bacteriol. **186**: 4152–4158.

Friedman M., P.R. Henika and R.E. Mandrell 2002. bactericidal activities of plant essential oils and some of their constituents against *Campylobacter jejuni, Listeria monocytogenes,* and *Salmonella enterica*. J. Food Prot., **65**: 1545-1560

Gill A.O., P. Delaquis, P. Russo and R.A. Holley 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. Int. J. Food Microbiol., **73**: 83– 92.

Gill A.O. and R. A. Holley 2004. Mechanisms of Bactericidal Action of Cinnamaldehyde against *Listeria monocytogenes* and of Eugenol against *L. monocytogenes* and *Lactobacillus sakei.* Appl. Environ. Microbiol., **70**: 5750-5755.

Gould G.W., 1996. Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. J. . Food Prot., 82–86.

Guenther E. 1948. The Essential Oils. D. Van Nostrand, New York.

Guerzoni M.E., R. Lanciotti and S. Cocconcelli. 2001. Alteration in cellular fatty acid composition as response to salt, acid, oxidative and thermal stress in *Lactobacillus helveticus.* Microbiol. **147**: 2255-2264.

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.* Lett. Appl. Microbiol., **26**: 194–198.

Hammer K.A., C.F. Carson and T.V. Riley 1999. Antimicrobial activity of essential oils and other plant extract. J. Appl. Microbiol., **86**: 985-990.

Hao Y.Y., R.E. Brackett and M.P. Doyle 1998. Efficacy of plant extract in inhibiting *Aeromonas hydrophila* and *Listeria monocytogenes* in refrigerated, cooked poultry. Food Microbiol., **15**: 367-378.

Hao Y.Y., R.E. Brackett and M.P. Doyle 1998b. Inhibition of *Listeria monocytogenes* and *Aeromonas hydrophila* by plant extracts in refrigerated cooked beef. J. Food Prot., **61** (3): 307–312.

Harpaz S., L. Glatman, V. Drabkin and A. Gelman 2003. Effects of herbal essential oils used to extend the shelf life of freshwaterreared Asian sea bass fish (*Lates calcarifer*). J. Food Prot., **66** (3): 410–417.

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. Industr. Crop. Prod., **4**: 3 –13.

Heipieper H.J., F. Meinhardt and A. Segura. 2003. The *cis-trans* isomerase of unsaturated fatty acids in *Pseudomonas* and *Vibrio*: biochemistry, molecular biology and physiological function of a unique stress adaptive mechanism. FEMS Microbiol. Lett. **229**: 1-7.4.

Helander I.M., H.L. Alakomi, K. Latva-Kala T., Mattila-Sandholm I., Pol E.J., Smid L.G.M., Gorri and A. von Wright 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. J. Agric. Food Chem., **46**: 3590-3595.

Herbert RA. 1986. The ecology and physiology of psychrophilic microorganisms In: Herbert RA, Codd GA, editors. Microbes in extreme environments. London: The Society for General Microbiology, Academic Press. p 1–24.

Herbert RA. 1989. Microbial growth at low temperature. In: Gould GW, editor. Mechanisms of action of food preservation procedures. London: Elsevier Applied Science. p 71–96.

Hodges N.A. and G.W. Hanlon 1991. Detection and measurement of combined biocide action. In: Denyer, S.P., Hugo, W.B. (Eds.), Mechanisms of Action of Chemical Biocides. Blackwell, Oxford, pp. 297– 310.

Ingram L.O. 1976. Adaptation of membrane lipids to alcohols. J.Bacteriol. **125**: 670-678.

Ingram, L. O. 1977. Changes in lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. Appl. Environ. Microbiol. **33:** 1233–1236.

Ilsley S., H. Miller, H. Greathead and C. **Kamel** 2002. Herbal sow diets boost pre-weaning growth. Pig Progress, **18** (4): 8 –10.

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. J. Food Prot., **53** (11): 958– 960.

Janssen A.M., 1989. Antimicrobial Activities of Essential Oils—A Pharmacological Study. University of Leiden, Leiden, p. 182.

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 Med., **53**: 396–398.

Jayashree T. and C. Subramanyam 1999. Antiaflatoxigenic activity of eugenol is due to inhibition of lipid peroxidation. Lett. Appl. Microbiol., **28**: 179– 183.

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 J. Food Sci. Technol., **36**: 649– 654.

Juglal S., R. Govinden and B. Odhav 2002. Spice oils for the control of co-occurring mycotoxin-producing fungi. J. Food Prot., **65** (4): 683–687.

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. J. Ess. Oil Res., **12:** 516–522.

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. J. App. Bacteriol., **76**: 626– 631.

Karapinar M. and S.E. Aktug 1987. Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole. Intern. J. Food Microbiol., **4:** 161–166.

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. J. Appl. Microbiol., **89**: 296– 301.

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. J. Appl. Microbiol., **90**: 463– 469.

Karpouhtsis I., E. Pardali, E. Feggou, S. Kokkini, Z.G. Scouras and P. Mavragani-Tsipidou, 1998. Insecticidal and genotoxic activities of oregano essential oils. J. Agric. Food Chem., **46**: 1111– 1115.

Keweloh H. and H. J. Heipieper. 1996. Trans unsaturated fatty acids in bacteria. Lipids **31**: 129-136.

Kim J.M., M.R. Marshall, J.A. Cornell, J.F. Preston and C.I. Wei 1995b. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* on culture medium and on fish cubes. J. Food Sci., **60** (6): 1364–1374.

Knobloch K., H. Weigand, N. Weis, H.-M. Schwarm and H. Vigenschow 1986. Action of terpenoids on energy metabolism. In: Brunke, E.J. (Ed.), Progress in Essential Oil Research: 16th International Symposium on Essential Oils. De Gruyter, Berlin, pp. 429– 445.

Knobloch K., A. Pauli and B. Iberl 1989. Antibacterial and antifungal properties of essential oils components. J. Essent. Oil Res., **1**: 119-128.

Koedam A. 1977a. Antimikrobielle Wirksamkeit ätherischer Öle: Eine Literaturarbeit 1960 – 1976—Fortsetzung und Schluss. Riechs. Arom. Kosmet., **27** (2): 36– 41.

Koedam A., 1977b. Antimikrobielle Wirksamkeit ätherischer Öle: Eine Literaturarbeit 1960–1976—Teil I. Riechs. Arom. Kosmet., **27** (1): 8– 11.

Koga T., N. Hirota and K. Takumi 1999. Bactericidal activities of essential oils of basil and sage against a range of bacteria and the effect of these essential oils on *Vibrio parahaemolyticus*. Microbiol. Res., **154**: 267-273.

Kokkini S., R. Karousou, A. Dardioti, N. Krigas and T. Lanaras 1997. Autumn essential oils of Greek oregano. Phytochem., **44** (5): 883–886.

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** (6): 616– 619.

Lambert R.J.W. and J. Pearson 2000. Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. J. Appl. Microbiol., **88**: 784–790.

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. J. Appl. Microbiol., **91**: 453–462.

Lancaster M.V. and R.D. Fields 1996. Antibiotic and cytotoxic drug susceptibility assays using resazurin and poising agents. US Patent and Trademark Office Patent nr. 5,501,959, U.S.A., Alamar Biosciences Laboratory, Inc. (Chicago,IL).

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.

Leistner L., 1978. Hurdle effect and energy saving. In: Downey, W.K. (Ed.), Food Quality and Nutrition. Applied Science Publ., London, p. 553.

Lennarz W.J. 1966. Lipid metabolism in the bacteria. Adv. Lipid Res. **4**: 175-225

Lis-Balchin M., S. Hart, S.G. Deans and E. Eaglesham 1996. Comparison of the pharmacological and antimicrobial action of commercial plant essential oils. J. Herb., Spice. Med. Plant., **4** (2): 69– 86.

Lis-Balchin M., R.J. Ochoka, S.G. Deans, M. Asztemborska and S. Hart 1999. Differences in bioactivity between the Enantiomers of α-pinene. J. Ess. Oil Res., **11**: 393– 397.

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. J. Ethnopharmacol., **81**: 1– 4.

Mahmoud S.S. and R.B. Croteau 2002. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trend. Plant Sci., **7** (8): 366– 373.

Manabe A., S. Nakayama and K. Sakamoto 1987. Effects of essential oils on erythrocytes and hepatocytes from rats and dipalitoyl phophatidylcholine-liposomes. Japan. J. Pharmacol., **44**: 77–84.

Mann C.M. and J.L. Markham 1998. A new method for determining the minimum inhibitory concentration of essential oils. J. Appl. Microbiol., **84**: 538– 544.

Mann C.M., S.D. Cox and J.L. Markham 2000 The outer membrane of Pseudomonas aeruginosa NCTC 6749 contributes to its tolerance to the essential oil of *Melaleuca alternifolia* (tea tree oil). Lett. Appl. Microbiol., **30**: 294-297.

Margesin R. and F. Schinner 1994. Properties of cold-adapted microorganisms and their potential role in biotechnology. J. Biotechnol., **33**: 1 –14.

Mari M., P. Bertolini and G.C. Pratella 2003. Non-conventional methods for the control of post-harvest pear diseases. J. Appl. Microbiol., **94**: 761– 766.

Marino M., C. Bersani and G. Comi, , 1999. Antimicrobial activity of the essential oils of *Thymus vulgaris L.* measured using a bioimpedometric method. J. Food Prot., **62** (9): 1017– 1023.

Marino M., C. Bersani and G. Comi 2001. Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceae* and *Compositae*. Int. J. Food Microbiol., **67**: 187-195.

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. Flav. Frag. J., **9**: 125– 129.

Mazzanti, G., L. Battinelli and G. Salvatore 1998. Antimicrobial properties of the linalol-rich essential oil of *Hyssopus officinalis* L. var *decumbens* (Lamiaceae). Flavour Fragrance J., **13**: 289-294.

McCaskill D. and R. Croteau 1999. Strategies for bioengineering the development and metabolism of glandular tissues in plants. Nat. Biotechnol., **17**: 31– 36.

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. Flav. Frag. J., **9**: 347– 352.

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. Lett. Appl. Microbiol., **34**: 27– 31.

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. J. Food Saf., **17:** 47– 55.

Moleyar V. and P. Narasimham 1992. Antibacterial activity of essential oil components. Inter. J. Food Microbiol., **16**: 337– 342.

Mourey A. and N. Canillac 2002. Anti-*Listeria monocytogenes* activity of essential oils components of conifers. Food Contr., **13:** 289– 292.

Moyler D., 1998. CO2 extraction and other new technologies: an update on commercial adoption. International Federation of Essential Oils and Aroma Trades—21st International Conference on Essential Oils and Aroma's. IFEAT, London, pp. 33– 39.

Mrozik A., Z. Pietrovska-Seget and S. Labuzek. 2004. Changes in whole cell-derived fatty acids induced by naphthalene in bacteria from genus *Pseudomonas*. Microbiol. Res. **159**: 87-95.

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, PA 19087-1898. USA.

Negi P.S., G.K. Jayaprakasha, L. Jagan Rao Mohan and K.K. Sakariah 1999. Antibacterial activity of turmeric oil: a byproduct from *curcumin*. J. Agric. Food Chem., **47:** 4297– 4300.

Nychas G.J.E. 1995. Natural antimicrobials from plants. In: Gould, G.W. (Ed.), New Methods of Food Preservation. Blackie Academic and Professional, London, pp. 58– 89.

Onawunmi G.O. 1989. Evaluation of the antimicrobial activity of citral. Lett. Appl. Microbiol., **9**: 105– 108.

Ouattara B., R.E. Simard, R.A. Holley, G.J.-P. Piette and A. Be´gin 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Inter. J. Food Microbiol., **37**: 155– 162.

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.). Inter. J. Food Microbiol., **68**:1–9.

Packiyasothy E.V. and S. Kyle 2002. Antimicrobial properties of some herb essential oils. Food Australia, **54** (9): 384– 387.

Pandey R., A. Kalra, S. Tandon, , N. Mehrotra, H.N. Singh and S. Kumar 2000. Essential oil compounds as potent source of nematicidal compounds. J. Phytopathol., **148** (7–8): 501–502.

Pandit V.A. and L.A. Shelef 1994. Sensitivity of *Listeria monocytogenes* to rosemary (*Rosmarinus officinalis L.*). Food Microbiol., **11**: 57– 63.

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.

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. J. Food Prot., **58** (1): 81– 85.

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*. Int. J. Food Microbiol., **68:** 141– 148.

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 Sci. Technol. Int., **7** (6): 487– 492.

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*. Vet. Parasitol. **109** (1– 2): 59–63.

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. Flav. Fragr. J., **17**: 15– 19.

Pol I.E. and E.J. Smid 1999. Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*. Lett. Appl. Microbiol., **29**: 166– 170.

Pol I.E., J. Krommer and E.J. Smid 2002. Bioenergetic consequences of nisin combined with carvacrol towards *Bacillus cereus.* Inn. Food Sci. Emerg. Technol., **3**: 55– 61.

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. J. Food Prot., **64** (7): 1012– 1018.

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. J. Ess. Oil Res., **7**: 165– 173.

Ratledge C. and S.G. Wilkinson 1988. An overview of microbial lipids. In: Ratledge, C.,Wilkinson, S.G. (Eds.), Microbial Lipids, vol. 1. Academic Press, London, pp. 3– 22.

Remmal A., T. Bouchikhi, K. Rhayour and M. Ettayebi 1993a. Improved method for the determination of antimicrobial activity of essential oils in agar medium. Journal J. Ess. Oil Res., **5** (2): 179–184.

Remmal A., T. Bouchikhi, A. Tantaoui-Elaraki and M. Ettayebi 1993b. Inhibition of antibacterial activity of essential oils bij Tween 80 and ethanol in liquid medium. J. Pharm. Belg., **48** (5): 352– 356.

Renzini G., F. Scazzocchio, M. Lu, G. Mazzanti and G. Salvatore 1999. Antibacterial and cytotoxic activity of *Hyssopus officinalis L.* oils. J. Ess. Oil Res., **11**: 649– 654.

Rios J.L., M.C. Recio and A. Villar 1988. Screening methods for natural antimicrobial products with antimicrobial activity: a review of the literature. J. Ethnopharmacol., **23**: 127– 149.

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. Lett. Appl. Microbiol., **35**: 390– 394.

Roller S. 2003. Natural antimicrobials for the minimal processing of food. Ed S. Roller. Woodhead Publishing Ltd.

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. Plant. Med., **66**: 687– 693.

Russell N. J. 1984. Mechanism of thermal adaptation in bacteria: blueprints for survival. Trends Biochem. Sci. **9**: 108-112.

Russell N.J. and M. Fukanaga. 1990. A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. FEMS Microbiol Rev. **75**: 171-182.

Russell N.J. 1995. Psychrotrophy and adaptation to low temperatures: microbial membrane lipids. Proceedings of the 19th International Congress on Refrigeration. Workshop Refrigeration and Microbiology: Health, Food, Drinks and Flowers, vol. 1, pp. 359– 365.

Russell N.J. 1997. Psychrophilic bacteria—molecular adaptations of membrane lipids. Comp. Biochem. Physiol., Part A Physiol. **118**: 489–493.

Russo M., G.C. Galletti, P. Bocchini and A. Carnicini 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. J. Agric. Food Chem., **46:** 3741– 3746.

Sagadic O., A. Kuscu, M. Ozcan and S. Ozcelik 2002. Effect of Turkish spices extracts at various concentrations on the growth of *Escherichia coli* O157:H7. Food Microbiol., **19**: 473-480.

Salvat A., L. Antonnacci, R.H. Fortunato, E.Y. Suarez and H.M. Godoy 2001. Screening of some plants from Northern Argentina for their antimicrobial activity. Lett. Appl. Microbiol., **32**: 293– 297.

Salzer U.-J. 1977. The analysis of essential oils and extracts (oleoresins) from seasonings—a critical review. Crit. Rev. Food Sci. Nut., **9** (4): 345– 373.

Sampathkumar B., G.G. Khachatourians, and D.R. Korber. 2004. Treatment of *Salmonella enterica* serovar Enteritidis with a sublethal concentration of trisodium phosphate or alkaline pH induces thermotolerance. Appl. Environ. Microbiol. **70**: 4613-4620.

Scheffer J.J.C. and A. Baerheim Svendsen 1981. Zur Analytik ätherische Ö le: Zehn Jahre Ä therische-Ö le-Forschung 1969 bis 1979. Riech. Arom. Kosmetic., **31** (2): 39–41.

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). J. Agric. Food Chem., **44:** 1327– 1332.

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. Flav. Fragr. J., **15**: 186– 189.

Shelef L.A., 1983. Antimicrobial effects of spices. J. Food Saf., **6:** 29– 44.

Shelef L.A., E.K. Jyothi and M.A. Bulgarelli 1984. Growth of enteropathogenic and spoilage bacteria in sage-containing broth and foods. J. Food Sci., **49:** (737–740) 809.

Sikkema, J., F.J Weber., H.J. Heipieper and J.A.M. De Bont, 1994. Cellular toxicity of lipophilic compounds: mechanisms, implications, and adaptations. Biocatal., **10**: 113–122.

Sikkema J., J.A.M. De Bont, B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews **59**: 201– 222.

Sivropoulou A., S. Kokkini, T. Lanaras and M. Arsenakis 1995. Antimicrobial activity of mint essential oils. J. Agric. Food Chem., **43**: 2384– 2388.

Sivropoulou A., E. Papanikolaou, C. Nikolaou and S. Kokkini, , 1996. Antimicrobial and cytotoxic activities of *Origanum* essential oils. J. Agric. Food Chem., **44**: 1202–1205.

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. Appl. Environ. Microbiol., **66** (4): 1646–1653.

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. J. Appl. Microbiol., **91**: 1011 –1022.

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. W. J. Microbiol. Biotechnol., **16:** 31– 35.

Skandamis P., K. Koutsoumanis, K. Fasseas and G.-J.E. Nychas 2001. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. It. J. Food Sci., **13** (1): 65– 75.

Smid E.J. and L.G.M. Gorris 1999. Natural antimicrobials for food preservation. In: Rahman, M.S. (Ed.), Handbook of Food Preservation. Marcel Dekker, New York, pp. 285–308.

Smith-Palmer A., J. Stewart and L. Fyfe 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Lett. Food Microbiol., **26**: 118–122.

Smith-Palmer A., J. Stewart and L. Fyfe 2001. The potential application of plant essential oils as natural food preservatives in soft cheese. Food Microbiol., **18:** 463–470.

Stammati A., P. Bonsi, F. Zucco, R. Moezelaar, H.-l. Alatomi and A. Von Wright 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxicol., **37**: 813–823.

Stecchini M.L., I. Sarais and P. Giavedoni 1993. Effect of essential oils on *Aeromonas hydrophila* in a culture medium and in cooked pork. J. Food Prot., **56** (5): 406– 409.

Suppakul P., J. Miltz K., Sonneveld, and S.W. Bigger 2003. Antimicrobial properties of basil and its possible application in Food Packaging. J. Agr. Food Chem., **51**: 3197-3207.

Suutari M., K. Linkkonen and S. Laakso. 1990. Temperature adaptation in yeast: the role of fatty acids. J. Gen. Microbiol. **136**: 1469-1474.

Suutari M., and S. Laakso. 1994. Microbial fatty acid and thermal adaptation. Crit. Rev. Microbiol. **20**: 285-328.

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. J. Appl. Bacteriol., **78**: 593– 600.

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. J. Food Prot., **59** (1): 31– 34.

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 Res. Int., **33**: 273– 280.

Thieringer H.A., P.A. Jones and M. Inouye 1998. Cold shock and adaptation. Bio Essay., **20**: 49– 57.

Thoroski J., G. Blank and C. Biliaderis 1989. Eugenol induced inhibition of extracellular enzyme production by *Bacillus cereus*. J. Food Prot., **52** (6): 399– 403.

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. J. Appl. Microbiol., **89**: 901–909.

Tuley de Silva K. (Ed.), 1996. A Manual on the Essential Oil Industry. United Nations Industrial Development Organization, Vienna.

Ultee A. 2000. Bactericidal action of carvacrol towards the food pathogen *Bacillus cereus*. A Case Study of a Novel Approach to Mild Food Preservation. Wageningen University, Wageningen, The Netherlands. 97 pp.

Ultee A. and E.J. Smid 2001. Influence of carvacrol on growth and toxin production by *Bacillus cereus*. Intern. J. Food Microbiol. **64**, 373–378.

Ultee A., L.G. Gorris and E.J. Smit 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus.* J. Appl. Microbiol., **85:** 211 –218.

Ultee A., E.P.W. Kets and E.J. Smid 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Appl. Environ. Microbiol., **65** (10): 4606– 4610.

Ultee A., E.P.W. Kets, M. Alberda, F.A. Hoekstra and E.J. Smith. 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Arch. Microbiol. **174**: 233-238.

Ultee A., E.P.W. Kets, M. Alberda, F.A. Hoekstra and E.J. Smid 2000a. Adaptation of the food-borne pathogen Bacillus cereus to carvacrol. Archiv. Microbiol.,**174** (4): 233– 238.

Ultee A., R.A. Slump, G. Steging and E.J. Smid 2000b. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. J. Food Prot., **63** (5): 620–624.

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*. Appl. Environ. Microbiol., **68** (4): 1561–1568. PhD thesis, ISBN 90- 5808-219-9.

Vaara M. 1992. Agents that increase the permeability of the outer membrane. Microbiologic. Rev., **56** (3): 395– 411.

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, p. 116.

Van Krimpen, M.M., Binnendijk, G.P., 2001. RopadiarR as alternative for antimicrobial growth promoter in diets of weanling pigs. Lelystad, Praktijkonderzoek Veehouderij, May 2001. ISSN 0169-3689, p. 14.

Van Welie R.T.H. 1997. Alle cosmetica ingrediënten en hun functies. Nederlandse Cosmetica Vereniging, Nieuwegein, p. 126.

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*. J. Appl. Microbiol., **84:** 152–158.

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. J. Food Prot., **56** (5): 410– 413.

Wendakoon C.N. and M. Sakaguchi 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. J. Food Prot., **58** (3): 280– 283.

WHO, 2002. Food safety and foodborne illness. World Health Organization Fact sheet 237, revised January 2002. Geneva.

Wilkins C. and J.O. Madsen, , 1991. Oregano headspace constituents. Zeitschrift für Lebensmittel-Untersuchung und -Forschung **192**, 214– 219.

Wilkinson J.M., M. Hipwell, T. Ryan and H.M.A. Cavanagh 2003. Bioactivity of *Backhousia citriodora*: Antibacterial and antifungal activity. J. Agric. Food Chem., **51**: 76– 81.

Wimpenny J.W.T. and M.W.A. Lewis 1977. The growth and respiration of bacterial colonies. J. Gen. Microbiol., **103:** 9 – 18.

Yuk, H.-G. and D. L. Marshall. 2004. Adaptation of *Escherichia coli* O157:H7 to pH alters membrane lipid composition, verotoxin secretion, and resistance to simulated gastric fluid acid. Appl. Environ. Microbiol. **70**: 3500-3505.