



# Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components

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Essential oils are aromatic and volatile liquids extracted from plants. The chemicals in essential oils are secondary metabolites, which play an important role in plant defense as they often possess antimicrobial properties. The interest in essential oils and their application in food preservation has been amplified in recent years by an increasingly negative consumer perception of synthetic preservatives. Furthermore, food-borne diseases are a growing public health problem worldwide, calling for more effective preservation strategies. The antibacterial properties of essential oils and their constituents have been documented extensively. Pioneering work has also elucidated the mode of action of a few essential oil constituents, but detailed knowledge about most of the compounds' mode of action is still lacking. This knowledge is particularly important to predict their effect on different microorganisms, how they interact with food matrix components, and how they work in combination with other antimicrobial compounds. The main obstacle for using essential oil constituents as food preservatives is that they are most often not potent enough as single components, and they cause negative organoleptic effects when added in sufficient amounts to provide an antimicrobial effect. Exploiting synergies between several compounds has been suggested as a solution to this problem. However, little is known about which interactions lead to synergistic, additive, or antagonistic effects. Such knowledge could contribute to design of new and more potent antimicrobial blends, and to understand the interplay between the constituents of crude essential oils. The purpose of this review is to provide an overview of current knowledge about the antibacterial properties and antibacterial mode of action of essential oils and their constituents, and to identify research avenues that can facilitate implementation of essential oils as natural preservatives in foods.

**Keywords:** antimicrobial, mode of action, synergy, terpenes, terpenoids, phenylpropenes, carvacrol, thymol

## INTRODUCTION

Essential oils are aromatic and volatile liquids extracted from plant material, such as flowers, roots, bark, leaves, seeds, peel, fruits, wood, and whole plant (Deans and Ritchie, 1987; Hammer et al., 1999; Sánchez et al., 2010). Essential oils have been used for centuries in medicine, perfumery, cosmetic, and have been added to foods as part of spices or herbs. Their initial application was in medicine, but in the nineteenth century their use as aroma and flavor ingredients increased and became their major employment. Almost 3000 different essential oils are known, and 300 are used commercially in the flavor and fragrances market (Burt, 2004).

Essential oils are considered to be secondary metabolites and important for plant defense as they often possess antimicrobial properties (Fraenkel, 1959; Tajkarimi et al., 2010). The antibacterial properties of secondary metabolites were first evaluated using essential oil vapors by De la Croix in 1881 (Burt, 2004). Since then, essential oils or their components have been shown to not only possess broad-range antibacterial properties (Deans and Ritchie, 1987; Oussalah et al., 2007), but also antiparasitic (George et al., 2009), insecticidal (Essam, 2001; Kim et al., 2003), antiviral (Schnitzler et al., 2011), antifungal (Fitzgerald et al., 2003; Kalembe

and Kunicka, 2003; Silva et al., 2011; Tserennadmid et al., 2011), and antioxidant (Brenes and Roura, 2010) properties. Furthermore, they also function as growth enhancers for animals (Brenes and Roura, 2010; Ahmadifar et al., 2011).

Although the food industry primarily uses essential oils as flavorings, they represent an interesting source of natural antimicrobials for food preservation. However, application of essential oils as food preservatives requires detailed knowledge about their properties, i.e., the minimum inhibitory concentration (MIC), the range of target organisms, the mode of action, and the effect of food matrix components on their antimicrobial properties. The purpose of this review is to provide an overview of current knowledge about the antimicrobial mode of action of essential oil constituents, and to identify research avenues that can facilitate implementation of essential oil constituents as natural food preservatives in foods.

## ESSENTIAL OIL CONSTITUENT CLASSES: THEIR ANTIMICROBIAL ACTIVITY AND MODE OF ACTION

Plants produce a variety of compounds with antimicrobial activity. Some are always present while others are produced in response

to microbial invasion or physical injury (Roller, 2003). Identifying the most active antimicrobial compounds of essential oils is cumbersome because essential oils are complex mixtures of up to 45 different constituents (Delaquis et al., 2002; Djenane et al., 2011; Espina et al., 2011), and the composition of a particular essential oil may vary depending on the season of harvest, and the methods used to extract the oil (Nannapaneni et al., 2009; Pereira and Meireles, 2010; Sánchez et al., 2010; Demuner et al., 2011; Djenane et al., 2011; Paibon et al., 2011). Essential oil constituents are a diverse family of low molecular weight organic compounds with large differences in antimicrobial activity. The active compounds can be divided into four groups according to their chemical structure: terpenes, terpenoids, phenylpropenes, and “others.” This section will provide an overview of what is currently known about the antimicrobial properties and the mode of action of selected essential oil constituents. Although studies have been performed on the mode of action of some essential oils (Table 1), analyzing the mode of action behind each constituent in the oils can reveal details of its antimicrobial properties that might be concealed when studied in a mixture with many other compounds. We will thus focus this review on the individual constituents of essential oils.

Most studies concerning the antimicrobial mode of action of essential oil constituents have been performed on bacteria, while less is known about their action on yeast and molds. Gram-negative bacteria are generally less susceptible than Gram-positive bacteria (Trombetta et al., 2005). The outer membrane of Gram-negative bacteria contain hydrophilic lipopolysaccharides (LPS), which create a barrier toward macromolecules and hydrophobic compounds, providing Gram-negative bacteria with higher tolerance toward hydrophobic antimicrobial compounds like those found in essential oils (Nikaido, 1994, 2003). Most essential oil constituents have several targets (Table 2). It is therefore difficult to predict how susceptible a microorganism is and why the susceptibility varies from strain to strain. Predictions about the mode of action of crude essential oils require thorough investigations of their constituents' target site, their mode of action, and their interactions with the surrounding environment. In this context, the following is known about the mode of action of some selected essential oil constituents.

## TERPENES

Terpenes are hydrocarbons produced from combination of several isoprene units ( $C_5H_8$ ). Terpenes are synthesized in the cytoplasm of plant cells, and the synthesis proceeds via the mevalonic acid pathway starting from acetyl-CoA. Terpenes have a hydrocarbon backbone which can be rearranged into cyclic structures by cyclases, thus forming monocyclic or bicyclic structures (Caballero et al., 2003). The main terpenes are monoterpenes ( $C_{10}H_{16}$ ) and sesquiterpene ( $C_{15}H_{24}$ ), but longer chains such as diterpenes ( $C_{20}H_{32}$ ), triterpenes ( $C_{30}H_{40}$ ), etc., also exist. Examples of terpenes include *p*-cymene, limonene, terpinene, sabinene, and pinene (Figure 1).

Terpenes do not represent a group of constituents with high inherent antimicrobial activity. For example, *p*-cymene, one of the major constituents in thyme, had no antimicrobial activity against several Gram-negative pathogens even at 85700  $\mu\text{g/mL}$  concentration (Bagamboula et al., 2004). In a large scale experiment,

limonene,  $\alpha$ -pinene,  $\beta$ -pinene,  $\delta$ -3-carene, (+)-sabinene, and  $\alpha$ -terpinene showed no or low antimicrobial activity against 25 different genera of bacteria that pose problems in animals, plants, and food products (Dorman and Deans, 2000). Koutsoudaki et al. (2005) compared the effect of  $\alpha$ -pinene,  $\beta$ -pinene, *p*-cymene,  $\beta$ -myrcene,  $\beta$ -caryophyllene, limonene, and  $\gamma$ -terpinene against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, and their antimicrobial activity were low or absent. *p*-Cymene and  $\gamma$ -terpinene were ineffective as fungicides against *Saccharomyces cerevisiae* (Rao et al., 2010). These *in vitro* tests indicate that terpenes are inefficient as antimicrobials when applied as single compounds.

## *p*-Cymene

The carvacrol precursor *p*-cymene is a monoterpene that has a benzene ring without any functional groups on its side chains. *p*-Cymene is not an efficient antimicrobial compound when used alone (Juven et al., 1994; Mann et al., 2000; Aligiannis et al., 2001; Bagamboula et al., 2004), but it potentiates the activity of compounds like carvacrol (Ultee et al., 2002; Rattanachaikunsopon and Phumkhachorn, 2010) and polymyxin B nonapeptide (Mann et al., 2000).

Several studies indicate that *p*-cymene is likely to act as a substitutional impurity in the membrane, which partly perturbs the membrane of microorganisms. *p*-Cymene has a high affinity for membranes and causes membrane expansion and affect the membrane potential of intact cells (Ultee et al., 2002). Investigations on cell and vesicle systems confirm that *p*-cymene has no effect on the membrane permeability, but cause a decrease in the enthalpy and melting temperature of membranes (Cristani et al., 2007), supporting the hypothesis that *p*-cymene acts as a substitutional impurity in the membrane.

Even though the action of *p*-cymene on the cell membrane is well established, its effect on protein synthesis and cell motility has also been investigated. *p*-Cymene had a negligible effect on the protein synthesis of *E. coli* cells (Burt et al., 2007), while its effect on the membrane potential resulted in decreased cell motility, as a proton motive force is needed for flagellar movement (Gabel and Berg, 2003; Burt et al., 2007).

## TERPENOIDS

Terpenoids are terpenes that undergo biochemical modifications via enzymes that add oxygen molecules and move or remove methyl groups (Caballero et al., 2003). Terpenoids can be subdivided into alcohols, esters, aldehydes, ketones, ethers, phenols, and epoxides. Examples of terpenoids are: thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol, and geraniol (Figure 1).

The antimicrobial activity of most terpenoids is linked to their functional groups, and it has been shown that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons are important for antimicrobial activity. For example, the antimicrobial activity of the carvacrol derivatives carvacrol methyl ether and *p*-cymene were much lower than carvacrol (Dorman and Deans, 2000; Ultee et al., 2002; Ben Arfa et al., 2006). Exchanging the hydroxyl group of carvacrol with methyl ether affects its hydrophobicity, antimicrobial activity, and changes how

Table 1 | Overview of crude essential oils and their identified target sites and modes of action.

Common name of plant from which essential oil is derived	Species of plant from which essential oil is derived	Major constituents of essential oil	Model organisms and measured MIC	Mechanism	Reference
African basil	<i>Ocimum gratissimum</i>	Thymol (53.2%), $\gamma$ -terpinene (25.7%), eugenol (12.7%), <i>p</i> -cymene (73%)	<i>E. coli</i> (6 $\mu$ g/mL) <i>Klebsiella</i> sp. (6 $\mu$ g/mL) <i>L. innocua</i> <i>L. monocytogenes</i> <i>Proteus mirabilis</i> (12 $\mu$ g/mL) <i>Pseudomonas aeruginosa</i> ( $\geq 24$ $\mu$ g/mL) <i>Salmonella enteritidis</i> (3 $\mu$ g/mL) <i>Shigella flexneri</i> (3 $\mu$ g/mL) <i>Staphylococcus aureus</i> (0.75 $\mu$ g/mL)	Permeabilized membrane	Nakamura et al. (1999), Cimanga et al. (2002), Nguetack et al. (2004b)
Bishop's weed	<i>Trachyspermum ammi</i> L.	Thymol (49.6%), $\beta$ -cymene (16.3%), eugenol (3%), $\beta$ -pinene (2.5%)	Three Gram-positive strains (12.5–175 $\mu$ g/mL) Six Gram-negative strains (12.5–462.5 $\mu$ g/mL)	Potassium and ATP leakage; cell lysis	Paul (2011)
Cinnamon	<i>Cinnamomum verum</i>	E-cinnamaldehyde (73.35%), $\beta$ -caryophyllene (4.09%), linalool (3.55%), cinnamyl acetate (2.96%), eugenol (2.68%)	<i>Campylobacter jejuni</i> (0.05% v/v) <i>Enterobacter aerogenes</i> <i>E. coli</i> (0.05% v/v) <i>Listeria monocytogenes</i> (0.03% v/v) <i>P. aeruginosa</i> (0.125% v/v) <i>S. enteritidis</i> (0.05% v/v) <i>S. aureus</i> (0.04–0.125% v/v) <i>B. cereus</i> (339 $\mu$ g/mL) <i>E. coli</i> (2640 $\mu$ g/mL) <i>L. monocytogenes</i> (2640 $\mu$ g/mL) <i>Salmonella infantis</i> (2640 $\mu$ g/mL) <i>S. aureus</i> (1320 $\mu$ g/mL)	Inhibited histidine decarboxylase <i>P. aeruginosa</i> : depolarized and permeabilized membranes; leakage and coagulation of cytoplasmic content; inhibited respiration activity <i>S. aureus</i> : entered a viable but non-cultivable state, and lost membrane integrity	Wendakoon and Morihiko (1995), Smith-Palmer et al. (1998), Bouhddid et al. (2010)
	<i>Cinnamomum cassia</i>	Cinnamaldehyde (75.3%), coumarin (10.6%), cinnamic alcohol (3%)		Released cellular content; reduced intracellular pH; affected membrane integrity	Alzoreky and Nakahara (2003), Oussalah et al. (2006)
Coriander	<i>Coriandrum sativum</i>	Linalool (25.9–64.4%), (E)-2-decenal (0–20.2%), decanol (0.14–8.4%), (E)-2-decen-1-ol (0–79%)	<i>Candida</i> species (0.05–0.4% v/v) <i>L. monocytogenes</i> (0.018–0.074% v/v)	Damage cytoplasmic membrane: released cellular content	De et al. (1999), Gill et al. (2002), Silva et al. (2011)
Cloves	<i>Syzygium aromaticum</i>	Eugenol (64%), eugenyl acetate (16.3%), caryophyllene (14.5%)	<i>C. jejuni</i> (0.05% v/v) <i>E. aerogenes</i> <i>E. coli</i> (0.04% v/v) <i>L. monocytogenes</i> (0.03% v/v) <i>S. enteritidis</i> (0.04% v/v) <i>S. aureus</i> (0.04% v/v)	Inhibited histidine decarboxylase ( <i>E. aerogenes</i> )	Wendakoon and Morihiko (1995), Smith-Palmer et al. (1998)

(Continued)

Table 1 | Continued

Common name of plant from which essential oil is derived	Species of plant from which essential oil is derived	Major constituents of essential oil	Model organisms and measured MIC	Mechanism	Reference
Cumin	<i>Cuminum cyminum</i> L. (seed)	Cumin aldehyde (29%), $\alpha$ -terpinen-7-ol (20.7%), $\gamma$ -terpinene (12.9%), <i>p</i> -cymene (8.6%)	<i>B. cereus</i> (0.05 $\mu$ L/mL) <i>B. subtilis</i> (0.05 $\mu$ L/mL or 1000 $\mu$ g/mL)	Mild changes in cytoplasm; cell envelope intact	De et al. (1999), Pajohi (2011)
Garlic	<i>Allium sativum</i>	Allicin (70%)	<i>Candida albicans</i> (470–940 $\mu$ g/mL) <i>E. coli</i> (15–15000 $\mu$ g/mL or 3.95% v/v) <i>L. monocytogenes</i> (8.8% v/v) <i>Salmonella typhi</i> (7% v/v) <i>S. aureus</i> (12–15000 $\mu$ g/mL or 5% v/v)	Induced leakage from <i>E. coli</i> cells	Hughes and Lawson (1991), Kumar and Berwal (1998), Ankri and Mirelman (1999), Perry et al. (2009)
Kaffir lime	<i>Citrus hystrix</i>	Citronellol (10.7%), limonene (73%), linalool (5.8%)	<i>Aspergillus flavus</i> (560 $\mu$ g/mL) <i>Aspergillus parasiticus</i> (1130 $\mu$ g/mL)	Reduced aflatoxin production	Rammanee and Hong-pattarakere (2011)
Lemon grass	<i>Cymbopogon citratus</i>	Geraniol (45.7%), myrcene (3.9%), 6-methylhept-5-en-2-one (2.7%)	<i>L. innocua</i> <i>L. monocytogenes</i>	Permeabilized membrane	Baratta et al. (1998), Nguefack et al. (2004b)
Lime	<i>Citrus aurantifolia</i>	Limonene (69.1%), <i>p</i> -cymene (12.8%)	<i>A. flavus</i> (560 $\mu$ g/mL) <i>A. parasiticus</i> (1130 $\mu$ g/mL) <i>E. coli</i> (0.2% v/v) <i>S. typhi</i> (0.2% v/v)	Reduced aflatoxin production; extra- and intracellular damages to cells Affected membrane integrity, released cell content; decreased intracellular ATP and pH, while external ATP increased	Rammanee and Hong-pattarakere (2011) Turgis et al. (2009)
Mustard oil					
Menthol	<i>Mentha longifolia</i>	Menthol (32.5%), menthone (20.7%), pulegone (17.8%), 1,8-cineole (5.6%), terpineol-4 (4.9%)	<i>Salmonella typhimurium</i> (1560 $\mu$ g/mL) <i>E. coli</i> (780 $\mu$ g/mL) <i>Micrococcus luteus</i> (190 $\mu$ g/mL) <i>S. aureus</i> (780 $\mu$ g/mL)	Damaged cell wall	Hafedh et al. (2010)
Oregano	<i>Origanum compactum</i>	Carvacrol (36.5%), thymol (29.7%), <i>p</i> -cymene (24.3%), $\gamma$ -terpinene (1.1%)	<i>P. aeruginosa</i> (1% v/v) <i>S. aureus</i> (0.031% v/v)	Dissipated potassium gradient; depolarized membranes; permeabilized membranes; inhibited cell respiration; affected cell structure: coagulated cytoplasmic material; liberation of membrane vesicles; mesosome-like structures	Bouhdid et al. (2009), Babili et al. (2011)
	<i>Origanum vulgare</i>	Carvacrol (68.1%), <i>p</i> -cymene (15.9%), $\alpha$ -pinene (2.6%), myrcene (2%)	<i>E. coli</i> (0.625 $\mu$ L/mL) <i>P. aeruginosa</i> (1648 $\mu$ g/mL) <i>S. aureus</i> (0.6 $\mu$ L/mL or 575 $\mu$ g/mL) Six bacteria (20–40 $\mu$ L/mL) Three <i>Candida</i> spp. (10–20 $\mu$ L/mL)	Suppressed enterotoxin production; released cellular content; cell morphological changes; permeabilized membranes; leaked potassium and phosphate; dissipated pH gradients	Lambert et al. (2001), Burt and Reinders (2003), De Souza et al. (2008, 2010)

Rosemary	<i>Rosmarinus officinalis</i>	Carnosic acid, carnosol, rosmadial, genkwanin, rosmarinic acid	Model membranes <i>C. albicans</i> (10 µg/mL) <i>Saccharomyces cerevisiae</i> (5 µg/mL) <i>B. subtilis</i> (10 µg/mL) <i>E. coli</i> (0.1% v/v or 40 µg/mL) <i>S. aureus</i> (0.1% v/v or 20 µg/mL)	Membrane-rigidifying effects; affected lipid polymorphism	Panizzi et al. (1993), Smith-Palmer et al. (1998), Pérez-Fons et al. (2006)
Savory	<i>Satureja montana</i>	Thymol (29%), <i>p</i> -cymene (12%), linalool (11%), carvacrol (10.7%)	<i>C. albicans</i> (5 µg/mL) <i>S. cerevisiae</i> (5 µg/mL) <i>E. coli</i> (0.05% v/v or 40 µg/mL) <i>L. monocytogenes</i> (0.05% v/v) <i>S. aureus</i> (0.013% v/v or 5 µg/mL) <i>S. Typhimurium</i> (0.05% v/v) <i>Clostridium perfringens</i> (1.56% v/v) <i>E. coli</i> (0.025% v/v) <i>L. monocytogenes</i> (0.025% v/v) <i>S. aureus</i> (0.013% v/v) <i>S. Typhimurium</i> (0.025% v/v) <i>L. monocytogenes</i> (125 µg/mL)	Increased extracellular ATP; reduced intracellular pH; affected membrane integrity; structural damages; and cell lysis	Panizzi et al. (1993), Oussalah et al. (2006, 2007), De Oliveira et al. (2011)
Spanish oregano	<i>Coridothymus capitatus</i>			Increased extracellular ATP; released cellular content; reduced intracellular pH; affected membrane integrity	Oussalah et al. (2006, 2007)
Thyme	<i>Thymus eriocalyx</i>	Thymol (63.8%), α-phellandrene (13.3%), cis-sabinene hydroxide (8.1%)		Damaged cell envelope	Rasooli et al. (2006)
	<i>Thymus vulgaris</i>	Thymol (31.4%), <i>p</i> -cymene (17%), carvacrol (12.4%), γ-terpinene (11.1%)	<i>C. albicans</i> (1 µg/mL) <i>S. cerevisiae</i> (2 µg/mL) <i>B. subtilis</i> (2 µg/mL) <i>C. jejuni</i> (0.04% v/v) <i>Erwinia amylovora</i> (1600 µg/mL) <i>Erwinia carotovora</i> (1600 µg/mL) <i>E. coli</i> (0.05% v/v or 2 µg/mL) <i>L. innocua</i> <i>L. monocytogenes</i> (0.02% v/v) <i>S. aureus</i> (0.02% v/v or 5 µg/mL) <i>S. enteritidis</i> (0.04% v/v) <i>L. monocytogenes</i> (125 µg/mL)	Permeabilized membrane; caused changes in outer membrane protein profile of <i>Erwinia</i> strains	Panizzi et al. (1993), Smith-Palmer et al. (1998), Nguefack et al. (2004b), Horváth et al. (2009)
	<i>Thymus x-porlock</i>	α-Phellandrene (38.7%), thymol (31.7%), cis-sabinene hydroxide (9.6%)		Damaged cell envelope; clumping of intracellular material	Rasooli et al. (2006)
	<i>Gnaphalium affine</i>	Eugenol (18.2%), linalool (10.6%), <i>trans</i> -caryophyllene (8.9%), α-terpineol (6%), <i>p</i> -cymene (5.8%)	Six bacteria (0.39–1.56 µg/mL) Five fungi (0.2 µg/mL)	Disrupted cell walls and membranes	Zeng et al. (2011)
	<i>Sphallerocarpus gracilis</i>	α-Asarone (33.1%), γ-terpinene (25.6%), <i>p</i> -cymene (17.4%)	Eight Gram-positive bacteria (160–640 µg/mL) Four Gram-negative bacteria (80–320 µg/mL) One fungus (no MIC)	Altered cell morphology	Gao et al. (2011)

Table 2 | Overview of essential oil components and their identified target sites and modes of action.

Compound (plant origin)	Chemical classification	MIC	Mechanism	Reference
Carvacrol (oregano and thyme)	Monoterpenoid phenol	<i>S. cerevisiae</i> (79.8–112.5 µg/mL)	Yeast: Depolarized and permeabilized membranes	Kim et al. (1995), Helander et al. (1998), Cosentino et al. (1999), Ultee et al. (1999, 2000, 2002), Lambert et al. (2001), Ultee and Smid (2001), Di Pasqua et al. (2006, 2007), Gill and Holley (2006a,b), Burt et al. (2007), Cristani et al. (2007), Xu et al. (2008), Horváth et al. (2009), Rao et al. (2010), Ahmad et al. (2011), La Storia et al. (2011)
		<i>Candida</i> strains (mean MIC 75–100 µg/mL)	Transient Ca <sup>2+</sup> surge	
		<i>B. cereus</i> (900 µg/mL)	Might act on specific signaling pathways rather than non-specific membrane damages	
		<i>Enterococcus faecalis</i> (225 µg/mL)	Upregulated genes involved in drug efflux, alternative metabolism, stress response, and autophagy	
		<i>E. amylovora</i> (800 µg/mL)	Down-regulated genes involved in RNA metabolism and ribosome biogenesis	
		<i>E. carotovora</i> (1600 µg/mL)	Impaired ergosterol biosynthesis	
		<i>E. coli</i> (225–2500 µg/mL)	Bacteria:	
		<i>L. monocytogenes</i> (450–1500 µg/mL)	Permeabilized cell membranes, and vesicles	
		<i>S. aureus</i> (450–1250 µg/mL)	Affected fatty acid and phospholipid head-group composition	
		<i>Staphylococcus epidermidis</i> (450 µg/mL)	Decreased melting temperature and transition enthalpy thus decreased membrane fluidity	
		<i>S. typhimurium</i> (150–250 µg/mL)	Dissipated pH gradient and membrane potential	
		<i>P. fluorescens</i> (1.84 µg/mL)	Depleted intracellular ATP; with no leakage of ATP (Gill and Holley, 2006a; claim release of ATP)	
		<i>Vibrio vulnificus</i> (250 µg/mL)	ATPase inhibition	
		<i>Yersinia enterocolitica</i> (225 µg/mL)	Incubated at sub-lethal concentration cells increased GroEL and decreased flagellin protein	
			Caused changes in outer membrane protein profile	
			Inhibited toxin production by <i>B. cereus</i>	
Eugenol (clove)	Phenylpropene phenol		Damaged cell morphology	Zemek et al. (1979, 1987), Thoroski (1989), Kim et al. (1995), Wendakoon and Morihiko (1995), Chang et al. (2001), Walsh et al. (2003), Bennis et al. (2004), Gill and Holley (2004, 2006a,b), Yamazaki et al. (2004), Rao et al. (2010)
		<i>Aspergillus niger</i> (3000 µg/mL)	Yeast:	
		<i>C. albicans</i> (3000 µg/mL)	Cells lysis and damaged cell surface	
		<i>S. cerevisiae</i> (490–3000 µg/mL)	Small Ca <sup>2+</sup> burst	
		<i>B. thermosphacta</i> (2.12 µg/mL)	Bacteria:	
		<i>Bacillus licheniformis</i> (250 µg/mL)	Inhibited: ATPase, histidine decarboxylase, and extracellular enzyme production (at sub-lethal concentrations)	
		<i>E. coli</i> (800–3000 µg/mL)	Permeabilize membranes	
		<i>L. monocytogenes</i> (800 to above 1000 µg/mL)	Leaked ATP and potassium ions	
		<i>L. sakei</i> (985 µg/mL for kill)		
		<i>M. luteus</i> (250 µg/mL)		
		<i>P. aeruginosa</i> (1500–3000 µg/mL)		
		<i>P. fluorescens</i> (2.12 µg/mL)		
		<i>S. aureus</i> (2.12–750 µg/mL)		
		<i>S. enterica</i> serovar Thyphimurium (3.18–500 µg/mL)		
		<i>V. vulnificus</i> (500 µg/mL)		

Thymol (thyme)	Monoterpenoid phenol	<i>S. cerevisiae</i> (112.5–270 µg/mL) <i>Candida</i> strains (mean MIC 100–150 µg/mL) <i>B. cereus</i> (450 µg/mL) <i>B. thermosphacta</i> (0.58 µg/mL) <i>E. amylovora</i> (1600 µg/mL) <i>E. carotovora</i> (1600 µg/mL) <i>E. coli</i> (225–5000 mg/mL)  <i>E. faecalis</i> (225 µg/mL) <i>L. monocytogenes</i> (450 µg/mL) <i>P. fluorescens</i> (2.88 µg/mL) <i>S. aureus</i> (225–310 µg/mL) <i>S. enterica</i> serovar Thyphimurium (0.96 µg/mL) <i>S. epidermidis</i> (225 µg/mL) <i>S. typhimurium</i> (56.25–150 µg/mL) <i>Y. enterocolitica</i> (225 µg/mL)	Yeast: Disrupted cell membrane Impaired ergosterol biosynthesis Lysed cells Damaged cell surface Ca <sup>2+</sup> bursts Similar up- and down-regulation of genes as carvacrol, except of repression of genes involved in vitamin B1 biosynthesis and sulfur metabolism Bacteria: Interacted with phospholipid membranes causing fluidifying effect Affected lipid composition Permeabilized cell membranes and vesicles Decreased melting temperature and transition enthalpy of membranes Leaked H <sup>+</sup> and K <sup>+</sup> ions and ATP Depolarized cells Affected cell morphology Impaired citrate metabolic pathway Inhibited enzymes involved in ATP synthesis Upregulated GroEL, DnaK, and outer membrane proteins Caused changes in outer membrane protein profile Altered gene response Decreased membrane melting temperature and transition enthalpy Decreased membrane potential Reduced cell motility Incorporate and expand membranes Might perturb membrane of microorganisms Decreased membrane melting temperature and transition enthalpy Might perturb the membrane of microorganisms	Helander et al. (1998), Cosentino et al. (1999), Lambert et al. (2001), Walsh et al. (2003), Bennis et al. (2004), Trombetta et al. (2005), Di Pasqua et al. (2006, 2007, 2010), Cristani et al. (2007), Shapira and Mimran (2007), Xu et al. (2008), Horváth et al. (2009), Rao et al. (2010), Ahmad et al. (2011)
<i>p</i> -Cymene (oregano and thyme)	Monoterpene	<i>E. coli</i> (2500 µg/mL) <i>S. aureus</i> (1250 µg/mL)	Decreased membrane melting temperature and transition enthalpy Decreased membrane potential Reduced cell motility Incorporate and expand membranes Might perturb membrane of microorganisms	Ultee et al. (2002), Burt et al. (2007), Cristani et al. (2007)
$\gamma$ -Terpinene (oregano and thyme)	Monoterpene	<i>E. coli</i> (5000 µg/mL) <i>S. aureus</i> (2500–34000 µg/mL)	Decreased membrane melting temperature and transition enthalpy Might perturb the membrane of microorganisms	Carson and Riley (1995), Cristani et al. (2007)
Cinnamaldehyde (cinnamon)	Phenylpropene aldehyde	<i>B. cereus</i> (0.3 µg/mL) <i>B. thermosphacta</i> (0.84 µg/mL) <i>E. aerogenes</i> <i>E. faecalis</i> (250 µg/mL) <i>E. coli</i> (397–1322 µg/mL) <i>L. monocytogenes</i> (3965 µg/mL) <i>P. aeruginosa</i> (750–1500 µg/mL) <i>P. fluorescens</i> (3.15 µg/mL) <i>S. aureus</i> (2.1–750 µg/mL) <i>S. typhimurium</i> (397 µg/mL)	Yeast: Inhibited cell wall synthesizing enzymes, and cytokinesis Bacteria: Concentration dependent ATPase inhibition Lost cell motility Studies argued both for and against membrane disintegration properties Inhibited: histidine decarboxylase and cytokinesis No significant protein leakage No effect on cell morphology (an effect was observed by Di Pasqua et al. (2007)	Zemek et al. (1987), Wendakoon and Morihiko (1995), Helander et al. (1998), Bang et al. (2000), Chang et al. (2001), Kwon et al. (2003), Gill and Holley (2004, 2006a,b), Yamazaki et al. (2004), Di Pasqua et al. (2007), Domadia et al. (2007), Hemaiswarya et al. (2011)

(Continued)

Table 2 | Continued

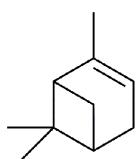
Compound (plant origin)	Chemical classification	MIC	Mechanism	Reference
Linalool (basil and citrus oils)	Monoterpenoid	<i>C. albicans</i> (2145 µg/mL)	Permeabilized membranes	Carson and Riley (1995), Kim et al. (1995), Bagamboula et al. (2004), Fisher and Phillips (2006, 2008), Ait-Ouazzou et al. (2011)
	alcohol	<i>B. cereus</i> (1073 µg/mL)		
		<i>C. jejuni</i> (515 µg/mL)		
		<i>E. coli</i> (515–2145 µg/mL)		
		<i>L. monocytogenes</i> (1000–2145 µg/mL)		
		<i>S. aureus</i> (1073–2145 µg/mL)		
Carvone (caraway and dill)	Monoterpenoid ketone	<i>S. typhimurium</i> (1000 µg/mL)	No effect on outer membrane and ATP pool	Helander et al. (1998), Ceylan and Fung (2004)
		<i>V. vulnificus</i> (1000 µg/mL)		
		<i>E. coli</i> (1500 µg/mL)		
		<i>S. typhimurium</i> (1500 µg/mL)		
		<i>E. coli</i> (5000 µg/mL)		
		<i>S. aureus</i> (1250 µg/mL)		
Linalyl acetate (bergamot)	Monoterpenoid acetate ester	<i>E. coli</i> (2282 µg/mL)	Perturbed membrane permeability Released cellular content Might interact with intracellular components Inhibited cell respiration Permeabilized cell membranes Dissipated potassium and pH gradients Stimulated ATP production in some cells and had no effect on remaining cells	Trombetta et al. (2005), Schipilliti et al. (2011)
		<i>S. cerevisiae</i> (3195 µg/mL)		
		<i>Z. bailii</i> (3043 µg/mL)		
		<i>Z. rouxii</i> (1978 µg/mL)		
		<i>E. coli</i> (2282 µg/mL)		
		<i>Lactobacillus plantarum</i> (11411 µg/mL)		
Vanillin (vanilla)	Phenylpropene phenolic aldehyde	<i>Listeria innocua</i> (5325 µg/mL)	Changed membrane fatty acid composition Damaged cell morphology	Di Pasqua et al. (2006, 2007), Espina et al. (2011)
		18 yeasts and molds (456–1460 µg/mL)		
		<i>B. thermosphacta</i> (168 µg/mL)		
		<i>E. coli</i> (8.4 µg/mL)		
		<i>P. fluorescens</i> (8.4 µg/mL)		
		<i>S. aureus</i> (1.68 µg/mL)		
Limonene (orange, lemon, and mandarin)	Monoterpenoid	<i>S. enterica</i> serovar Thyphimurium (8.4 µg/mL)	Perturbed membrane permeability Released cellular content Might interact with intracellular components	Işcan et al. (2002), Trombetta et al. (2005), Bassolé et al. (2010)
		<i>C. albicans</i> (2500 µg/mL)		
		<i>B. cereus</i> (1250 µg/mL)		
		<i>Enterobacter aerogenes</i> (1250 µg/mL)		
		<i>E. coli</i> (1250–2500 µg/mL)		
		<i>Klebsiella pneumoniae</i> (2500 µg/mL)		
Menthol (peppermint)	phenol	<i>L. monocytogenes</i> (1250 µg/mL)		
		<i>Proteus vulgaris</i> (1250 µg/mL)		
		<i>P. aeruginosa</i> (2500 µg/mL)		
		<i>S. typhimurium</i> (625 µg/mL)		
		<i>S. aureus</i> (620 µg/mL)		
		<i>S. epidermidis</i> (625 µg/mL)		
		<i>Yersinia enterocolitica</i> (2500 µg/mL)		



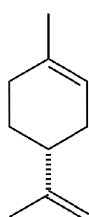
Allyl isothiocyanate (mustard oil)	Organosulfur	<i>Aeromonas hydrophilia</i> (200 µg/mL) <i>E. coli</i> (51–200 µg/mL) <i>Lactobacillus brevis</i> (1000 µg/mL) <i>Leuconostoc mesenteroides</i> (500 µg/mL) <i>Pediococcus pentosaceus</i> (1000 µg/mL)  <i>P. fluorescens</i> (200 µg/mL) <i>S. aureus</i> (200 µg/mL) <i>B. subtilis</i> (200 µg/mL) <i>C. albicans</i> (7 µg/mL) <i>E. coli</i> (28 µg/mL)  <i>S. aureus</i> (28 µg/mL)	AITC readily cross membranes Caused leakage of metabolites No cell lysis React with SH- and amino-group containing compounds Inhibited: oxygen uptake, acetate kinase, thioredoxin reductase, and cytochrome c oxidase activity (yeast) Function as a non-specific inhibitor of intracellular enzymes and alter proteins by oxidative cleavage of disulfide bonds and attack of free amino groups in lysine and arginine Readily permeates phospholipid membranes Inhibited: thiol-containing enzymes, RNA, DNA, and protein synthesis, acetyl-CoA synthetase (yeast), and the bacterial acetyl-CoA-forming system Might react with non-SH amino acids	Kojima (1971), Kawakishi and Namiki (1982), Kawakishi and Kaneko (1985, 1987), Delaquis (1995), Shofran et al. (1998), Lin et al. (2000), Ahn (2001), Luciano et al. (2008), Luciano and Holley (2009), Feldberg et al. (1988), Focke et al. (1990), Hughes and Lawson (1991), Rabinov et al. (1998), Miron et al. (2000), Kyung (2011)  Onawunmi (1989), Kim et al. (1995), Nazer et al. (2005), Fisher and Phillips (2008), Somolinos et al. (2010)
Allicin (garlic)	Organosulfur			
Citral (citrus fruits)	Monoterpene aldehyde	<i>Aspergillus fumigatus</i> (714 µg/mL) <i>C. albicans</i> (447 µg/mL) <i>E. coli</i> (447–500 µg/mL) <i>L. monocytogenes</i> (500 µg/mL) <i>Microsporium gypseum</i> (447 µg/mL) <i>S. typhimurium</i> (500 µg/mL) <i>S. aureus</i> (447 µg/mL) <i>Trichophyton mentagrophytes</i> (714 µg/mL) <i>V. vulnificus</i> (100 µg/mL) <i>S. aureus</i> (24 µg/mL) <i>E. coli</i> (no inhibition) <i>Trichophyton mentagrophytes</i> (2.3 µg/mL) <i>Candida parapsilosis</i> (no inhibition)	The sigma factor RpoS increase resistance of <i>E. coli</i> cells against citral, damaged cell membranes	
T-cadinol (myrrh)	Sesquiterpene alcohol		Lysed cells	Claeson et al. (1992)

## Terpenes

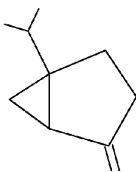
### Monoterpenes



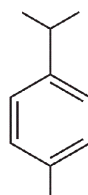
$\alpha$ -Pinene



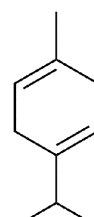
Limonene



Sabinene

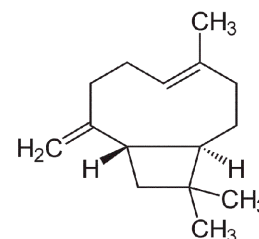


p-Cymene



$\gamma$ -Terpinene

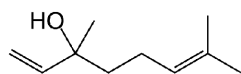
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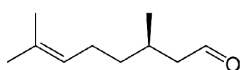
$\beta$ -Caryophyllene

## Terpenoids

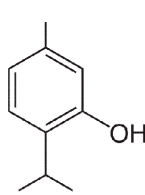
### Monoterpenoids



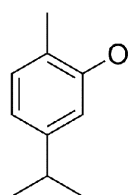
Linalool



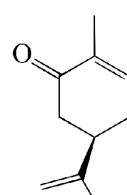
Citronellal



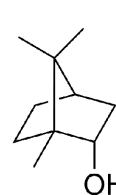
Thymol



Carvacrol

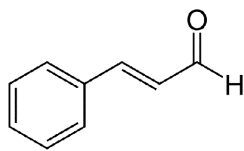


Carvone

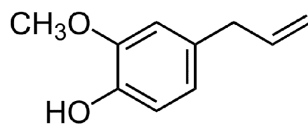


Borneol

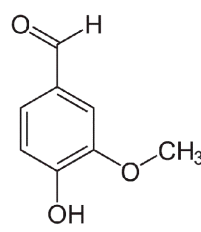
## Phenylpropanoids



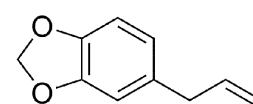
Cinnamaldehyde



Eugenol

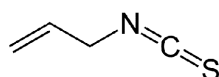


Vanillin

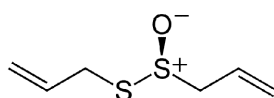


Safrole

## Others



Allyl-isothiocyanate



Allicin

FIGURE 1 | Chemical structures of selected essential oil constituents.

the molecule interacts with the membrane (Veldhuizen et al., 2006). Carvacrol's antimicrobial activity is comparable to that of 2-amino-*p*-cymene, which indicates that the hydroxyl group is important, but not essential for carvacrol's activity (Veldhuizen et al., 2006). The antimicrobial activity of essential oils can often be correlated to its content of phenolic constituents (Aligiannis et al., 2001; Kalembe and Kunicka, 2003; Rhayour et al., 2003).

The terpenoids are a large group of antimicrobial compounds that are active against a broad spectrum of microorganisms, with the most active monoterpenoids identified so far being carvacrol

and thymol. Dorman and Deans (2000) investigated the effect of many terpenoids against 25 different bacterial strains, and showed that all terpenoid compounds, except borneol and carvacrol methyl ester, exhibited a broad antimicrobial activity. The antimicrobial activity of carvacrol, thymol, linalool, and menthol were evaluated against *Listeria monocytogenes*, *Enterobacter aerogenes*, *E. coli*, and *Pseudomonas aeruginosa*. The most active compound was carvacrol followed by thymol with their highest MIC being 300 and 800  $\mu$ g/mL, respectively (Bassolé et al., 2010). These results confirm the high antimicrobial activity of

a broad collection of terpenoids, and because their chemical structures are closely related to that of terpenes, the increased activity compared to terpenes can be attributed to the functional moieties.

### Thymol

The mode of action of thymol, a phenolic monoterpene and one of the major constituents of thyme oil, has received much attention from researchers. Thymol is structurally very similar to carvacrol, having the hydroxyl group at a different position on the phenolic ring (**Figure 1**). The antimicrobial action of phenolic compounds, such as thymol and carvacrol, are expected to cause structural and functional damages to the cytoplasmic membrane (Sikkema et al., 1995). The primary mode of antibacterial action of thymol is not fully known, but is believed to involve outer- and inner membrane disruption, and interaction with membrane proteins and intracellular targets.

Studies have shown that thymol interacts with cell membranes. The interaction affects membrane permeability, and this has been documented by loss of membrane potential, cellular uptake of ethidium bromide, and leakage of potassium ions, ATP, and carboxyfluorescein (Helander et al., 1998; Lambert et al., 2001; Walsh et al., 2003; Xu et al., 2008). Although the protective properties of lipopolysaccharide (LPS) against thymol had been confirmed using random transposon-insertion mutants, treatment of *E. coli* cells with thymol caused release of LPS and disruption of the outer membrane (Helander et al., 1998; Shapira and Mimran, 2007). The outer membrane disruption could not be prevented by addition of magnesium, suggesting that thymol did not disrupt the membrane by chelating cations (Helander et al., 1998). Thymol integrates at the polar head-group region of a lipid bilayer causing alterations to the cell membrane, which at low concentrations induce adaptational changes in the membrane lipid profile in order to compensate for thymol's fluidifying effects and to maintain the membrane function and structure (Turina et al., 2006; Di Pasqua et al., 2007).

In addition to interacting with membrane phospholipids, evidence has accumulated that documents thymol's interaction with membrane proteins and intracellular targets, which hinder cell recovery after temporary exposure. The ability of thymol to interact with proteins was examined using the protein bovine serum albumin (BSA) and the organic compound deferoxamine, which is also rich in amine groups but otherwise known for its  $\text{Fe}^{3+}$ -chelating properties. These compounds react similarly to that of amine groups in bacterial membrane proteins (Juven et al., 1994). Based on the antimicrobial activity of thymol in the absence and presence of the thymol-inhibiting deferoxamine or BSA, Juven et al. (1994) hypothesized that thymol forms a complex with membrane-bound or periplasmic proteins by means of hydrogen bonds and hydrophobic interactions. Interaction with membrane proteins was further supported by Di Pasqua et al. (2010) who exposed *Salmonella enterica* to sub-lethal concentrations of thymol, and observed accumulation of misfolded outer membrane proteins and upregulation of genes involved in synthesis of outer membrane proteins. Contrarily, down-regulation of outer membrane proteins was shown in *Erwinia* spp. (Horváth et al., 2009). Upon exposure to thymol, *S. enterica* upregulated production of

the chaperon proteins Heat Shock Protein 60 (GroEL), and Heat Shock Protein 70 (DnaK), which are key proteins in the protection against thermal stress and misfolding of proteins (Di Pasqua et al., 2010; Hartl et al., 2011). Thymol also impaired the citrate metabolic pathway and affected many enzymes directly or indirectly involved in the synthesis of ATP (Di Pasqua et al., 2010). Thymol's intracellular action indicates that it affects important energy-generating processes, which lower a cell's ability to recover after exposure to thymol.

The mode of action of thymol against yeast and fungi has been sparsely investigated, but studies point to interactions with the cell envelope and intracellular targets. Thymol disrupted vesicles and cell membranes, and impaired ergosterol biosynthesis in *Candida* strains, which consequently affected cell membrane integrity because ergosterol regulates membrane fluidity and asymmetry similarly to cholesterol in animal cells (Ghannoum and Rice, 1999; Cristani et al., 2007; Ahmad et al., 2011). Interestingly, thymol induced cell lysis and only altered the cell structure of proliferating *S. cerevisiae* cells, indicating the effect of thymol depends on cell proliferation (Bennis et al., 2004). Contrary to this, Rao et al. (2010) proposed that thymol activates specific signaling pathways in yeast, rather than causing non-specific lesion of membranes. This proposal was based on the observation that thymol caused cytosolic  $\text{Ca}^{2+}$  bursts and transcription responses similar to  $\text{Ca}^{2+}$  stress and nutrient starvation (Rao et al., 2010).

### Carvacrol

Carvacrol is a phenolic monoterpene and a major constituent of oregano. Together with its closely related isomer thymol, it is one of the most extensively studied essential oil constituents. The antimicrobial effect of carvacrol is expected to be similar to that of thymol, causing structural and functional damages to the cell membrane (Sikkema et al., 1995). The primary mode of action of carvacrol is its ability to position in the membrane where it increase permeability, however, other more specific actions may be important and will be discussed.

Carvacrol has been demonstrated to affect the outer membrane of Gram-negative bacteria (La Storia et al., 2011). Disintegration of the outer membrane caused release of LPS from Gram-negative bacteria (Helander et al., 1998). Although carvacrol affects the outer membrane, its site of action is thought to be the cytoplasmic membrane, resulting in passive transport of ions across the membrane. Carvacrol has a hydroxyl group that has been proposed to function as a transmembrane carrier of monovalent cations across the membrane, carrying  $\text{H}^+$  into the cell cytoplasm and transporting  $\text{K}^+$  back out (Ultee et al., 2002; Ben Arfa et al., 2006). However, Veldhuizen et al. (2006) found the hydroxyl group of carvacrol not to be essential for antimicrobial activity, and proposed that although the transmembrane proton carrier mechanism plays a role in the antimicrobial activity, the relatively high activity of a non-hydroxyl compound ruled it out as the main mode of action of carvacrol.

The evidences for the membrane as carvacrol's site of action are many, and the results suggest that the mode of action of carvacrol is to increase fluidity and permeability of membranes. It has been proposed that cells exposed to carvacrol change the fatty acid composition of the membrane as an adaptation mechanism

to maintain optimal membrane structure and function because of carvacrol's effect on fluidity (Ultee et al., 2000; Di Pasqua et al., 2006, 2007). It is well established that increased membrane fluidity enhances the permeability of membranes (Nikaido, 1994). Membrane permeabilization by carvacrol has been confirmed by monitoring the efflux of  $H^+$ ,  $K^+$ , carboxyfluorescein, and ATP, and the influx of nucleic acid stains (Helander et al., 1998; Ultee et al., 1999; Lambert et al., 2001; Cristani et al., 2007; Xu et al., 2008).

Besides the interaction with membranes, carvacrol has been proposed to interact with membrane proteins and periplasmic enzymes (Juven et al., 1994), but the evidence for this is limited. The only example used isolated bacterial membranes with ATPase activity as the indicator for direct molecular binding of carvacrol in an assay with excess amounts of ATP added (Gill and Holley, 2006b). Carvacrol has also been proposed to have intracellular targets, but the studies documenting this are few and do not identify the possible intracellular targets. Inhibitory concentrations of carvacrol caused over-expression of outer membrane proteins in *Erwinia amylovora* cells (Horváth et al., 2009), indicating that carvacrol possibly affect outer membrane protein folding or insertion. In another study, *E. coli* cells grown in the presence of sub-lethal concentration of carvacrol produced significant amounts of GroEL, indicating that protein folding was affected. Furthermore, it inhibited the synthesis of flagellin, which caused new cells to be without flagella (Burt et al., 2007). Cells that had flagella exhibited decreased motility at increasing carvacrol concentration, indicating that carvacrol disrupts the membrane potential and thereby the proton motive force needed to drive flagellar movement (Gabel and Berg, 2003; Burt et al., 2007).

The mechanism of antifungal activity of carvacrol resembles that of thymol, showing disruption of  $Ca^{2+}$  and  $H^+$  homeostasis, up- and down-regulation of gene transcription similar to  $Ca^{2+}$ -stress and nutrient starvation (Rao et al., 2010), disruption of membrane integrity and impairment of ergosterol biosynthesis in *Candida* strains (Ahmad et al., 2011).

## PHENYLPROPENES

Phenylpropenes constitute a subfamily among the various groups of organic compounds called phenylpropanoids that are synthesized from the amino acid precursor phenylalanine in plants. Phenylpropanoids have their name from the six-carbon aromatic phenol group and the three-carbon propene tail of cinnamic acid, produced in the first step of phenylpropanoid biosynthesis. The phenylpropenes constitute a relatively small part of essential oils, and those that have been most thoroughly studied are eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde (Figure 1).

Comparison of molecules that are chemically similar to eugenol and isoeugenol indicated that the free hydroxyl groups are important for their activity against bacteria, but not yeast (Laekeman et al., 1990). Some of isoeugenol's activity might be attributed to the double bond in the  $\alpha$ ,  $\beta$  positions of the side chain, and a methyl group in the  $\gamma$  position (Jung and Fahey, 1983). Furthermore, the antimicrobial activity of phenylpropenes depends on the kind and number of substituents on the aromatic ring, selected microbial strains, and the experimental test parameters such as choice of growth medium, temperature, etc. (Pauli and Kubeczka, 2010).

The antibacterial activity of eugenol was evaluated against 25 different bacterial strains of which only one strain was not inhibited (Dorman and Deans, 2000). Isoeugenol and eugenol showed pronounced inhibition activity against yeasts and 6 out of 10 Gram-positive and Gram-negative bacteria at 1000  $\mu\text{g/mL}$  (Laekeman et al., 1990). The antimicrobial properties of isoeugenol appear more potent than eugenol, as lower MIC values are found against a variety of bacteria, yeast, and molds (Zemek et al., 1979, 1987). Interestingly, isoeugenol and eugenol have higher antimicrobial activity against Gram-negative bacteria, yeasts, and molds than Gram-positive bacteria (Mygind, unpublished). This is unusual for essential oil constituents because they normally are more effective against Gram-positive bacteria.

Cinnamaldehyde appears less potent than eugenol. In a study of *L. monocytogenes* and *Lactobacillus sakei*, 3965 and 66080  $\mu\text{g/mL}$  of cinnamaldehyde, but only 821 and 985  $\mu\text{g/mL}$  of eugenol were required to obtain a bactericidal effect (Gill and Holley, 2004). However, when tested against *E. coli* and *Salmonella typhimurium*, the antimicrobial activity of cinnamaldehyde equals that of the potent monoterpenoids thymol and carvacrol (Helander et al., 1998). Another phenylpropene, vanillin, inhibits a range of yeasts, molds, and bacteria (Fitzgerald et al., 2003, 2004, 2005; Rupasinghe et al., 2006). It should be noted that some yeasts were able to convert sub-lethal concentrations of vanillin into non-inhibitory compounds (Fitzgerald et al., 2003).

## Eugenol

Eugenol is a major constituent in clove essential oil, and its antimicrobial activity is linked to its ability to permeabilize the cell membrane and interact with proteins. Eugenol's action on membranes occurs mainly by a non-specific permeabilization. The non-specific permeabilization of the cytoplasmic membrane by eugenol has been demonstrated in various studies as increased transport of potassium and ATP out of the cells (Walsh et al., 2003; Gill and Holley, 2006a; Hemaiswarya and Doble, 2009). Eugenol induced minor changes in the fatty acid profile of *Pseudomonas fluorescens*, *E. coli*, *Brochetrix thermosphacta*, *S. enterica*, and *S. aureus*, and cell damages to *E. coli* and *B. thermosphacta* cells (Di Pasqua et al., 2006, 2007).

The hydroxyl group of eugenol is thought to bind to and affect the properties of proteins, thereby contributing to eugenol's inhibitory effect at sub-lethal concentrations. Consistent with this, eugenol has proven to inhibit the activity of the following enzymes: ATPase, histidine decarboxylase, amylase, and protease (Thoroski, 1989; Wendakoon and Morihiko, 1995; Gill and Holley, 2006b). Inhibition of the ATPase may be important for cell killing at high eugenol concentrations because energy generation needed for cell recovery is impaired (Gill and Holley, 2006b).

The antifungal mode of action of eugenol needs further investigation, but it is known to depend on cell proliferation. Eugenol treatment altered cell membrane and cell wall structures of proliferating *S. cerevisiae* cells resulting in the release of cellular content (Bennis et al., 2004).

## Cinnamaldehyde

Aldehyde groups are reactive and have the ability to cross-link covalently with DNA and proteins through amine groups, thereby

interfering with their normal function (Feron et al., 1991). However, the mode of action of cinnamaldehyde, a phenylpropene aldehyde, is inconclusive. At least three things are believed to occur: At low concentrations, cinnamaldehyde inhibits different enzymes involved in cytokinesis or less important cell functions. At higher but sub-lethal concentrations, it acts as an ATPase inhibitor, and at lethal concentrations it perturbs cell membrane. Cinnamaldehyde was suggested to inhibit cytokinesis as a mode of action on *B. cereus* because cells could not separate although septa were present after division (Kwon et al., 2003). It has been established that cinnamaldehyde binds to the FtsZ protein, inhibiting its GTP dependent polymerization and thereby preventing cell division (Domadia et al., 2007; Hemaiswarya et al., 2011). The FtsZ protein is an attractive target for antimicrobial therapies as it is evolutionary distant from eukaryotic tubulin, and the predicted interaction of H2 and H3 of cinnamaldehyde with G295 and V208 of FtsZ, respectively, is conserved among FtsZ proteins from several species (Domadia et al., 2007; Hemaiswarya et al., 2011). Other enzymes, e.g., the histidine decarboxylase, is also inhibited by cinnamaldehyde (Wendakoon and Morihiko, 1995).

At sub-lethal concentrations, cinnamaldehyde gains access to the periplasm and inhibits the activity of transmembrane ATPase. Sub-lethal concentrations of cinnamaldehyde did not affect the integrity of the outer membrane of *E. coli*, but it inhibited growth and bioluminescence of *Photobacterium leiognathi*, indicating that cinnamaldehyde does gain access to the periplasm and possibly also the cytoplasm (Helander et al., 1998). The ability of cinnamaldehyde to access the periplasm was confirmed by demonstrating a decrease in ATPase activity of isolated cell membranes at increasing concentrations of cinnamaldehyde (13.6–1362 µg/mL; Gill and Holley, 2006a,b). ATPase inhibition was, however, suggested not to be the primary cause of cell death because the concentration required to inhibit the ATPase also resulted in membrane disruption of *E. coli* cells (681–1362 µg/mL; Gill and Holley, 2006a).

Many studies have demonstrated that cinnamaldehyde interacts with the cell membrane, but it is not yet clear how it perturbs membranes. It is not a general mode of action of cinnamaldehyde to disrupt membranes as illustrated by Di Pasqua et al. (2007). Cinnamaldehyde altered the membrane lipid profile with large increases in saturated fatty acids, yielding a more rigid membrane probably compensating for a fluidifying effect of cinnamaldehyde, and cell structure of *E. coli*, *S. enterica*, *P. fluorescens*, and *B. thermosphacta*, while only *S. aureus* demonstrated disintegration of the cell envelope (Di Pasqua et al., 2006, 2007). *Cinnamomum verum* essential oil (73% cinnamaldehyde) caused membrane depolarization, loss of membrane integrity, reduced respiratory activity, and coagulation of cytoplasmic material of *P. aeruginosa*, while exposure of *S. aureus* cells caused them to enter a viable but non-cultivable state (Bouhdid et al., 2010).

Among fungi, the primary mode of action of cinnamaldehyde has also been proposed to be inhibition of cell division. This was proposed because cinnamaldehyde inhibited the cell wall synthesizing enzymes in *S. cerevisiae* by functioning as a non-competitive inhibitor of  $\beta$ -(1,3)-glucan synthase and a mixed inhibitor of chitin synthase isozymes (Bang et al., 2000).

## Vanillin

The mode of action of the phenylpropene phenolic aldehyde vanillin is not well understood, but it has been proposed to function as a membrane active compound that might have intracellular targets.

The proposed membrane and protein interactions of vanillin are based on one study. Vanillin inhibited respiration of *E. coli* and *Listeria innocua* cells, and disrupt the potassium and pH homeostasis of *Lactobacillus plantarum* cells (Fitzgerald et al., 2004). Propidium iodide staining demonstrated that treatment with vanillin disrupted membrane integrity of only a sub-population of cells and it was proposed that although vanillin primarily is a membrane active compound, it may also have intracellular target sites (Fitzgerald et al., 2005).

Not much is known about vanillin's mechanism of antifungal activity, but it has been suggested that the aldehyde moiety of vanillin plays an important role in its antifungal activity. The rationale for this is that *S. cerevisiae* convert vanillin into vanillic acid and vanillyl alcohol, which possess no antimicrobial activity, confirming the key-role of the aldehyde moiety (Feron et al., 1991; Fitzgerald et al., 2005).

## OTHER ESSENTIAL OIL CONSTITUENTS

Essential oils contain a number of different degradation products originating from unsaturated fatty acids, lactones, terpenes, glycosides, and sulfur- and nitrogen-containing compounds (Caballero et al., 2003). Two examples of sulfur- and nitrogen-containing compounds with known antimicrobial activity are allicin and allyl isothiocyanate (AITC).

Alliin (diallyl thiosulfinate) is found in garlic and plays an important role in plant defense (Ankri and Mirelman, 1999). Inside the garlic cloves, the amino acid cysteine is converted to alliin (*S*-allyl-L-cystein-*S*-oxide), a known sulfoxide with no antimicrobial activity (Block, 1992; Ankri and Mirelman, 1999). Conversion of alliin to the antimicrobial allicin requires the enzyme alliinase. Studies suggest that alliin and alliinase are located in two different compartments (Ankri and Mirelman, 1999), and when garlic cloves are crushed, alliinase comes into contact with alliin and produces allicin (Ankri and Mirelman, 1999). Allicin has a pungent smell of garlic and exhibits antibacterial, antifungal, antiparasitic, and antiviral properties (Kyung, 2011). Allicin is equally effective against Gram-negative and Gram-positive bacteria (Cavallito and Bailey, 1944). Allicin is bactericidal with LD<sub>50</sub> values against different bacteria ranging between 3 and more than 100 µg/mL, while a bacteriostatic effect was observed at much lower concentrations reflected in MIC values for fungal pathogens were in the range 0.15–1.5 µg/mL (Ankri and Mirelman, 1999). Although allicin shows great potential as a food preservative when evaluated *in vitro*, conflicting results have been obtained in food matrixes (Kyung, 2011).

Isothiocyanates, also known as mustard oils, are common essential oil constituents from plants belonging to the mustard family (Brassicaceae), such as mustard, broccoli, horseradish, and turnips (Nielsen and Rios, 2000). Isothiocyanates arise in plants as a result of enzymatic cleavage of released glucosinolates from intracellular compartments by membrane-bound myrosinase upon damage to the plant (Delaquis, 1995). Myrosinase

promotes hydrolysis and intramolecular rearrangement of intermediates, resulting in the three main groups of substances: nitriles, thiocyanates, and isothiocyanates (Zhang and Talalay, 1994). The latter group includes the non-phenolic volatile AITC which can constitute close to 90% of the oil composition (Ward et al., 1998). Allyl isothiocyanate in vapor and liquid forms has demonstrated high bactericidal activity against various food spoilage microorganisms and food pathogens, including *E. coli* O157:H7 (Luciano and Holley, 2009), *S. typhimurium*, *L. monocytogenes*, and other aerobic Gram-negative spoilage bacteria (Delaquis, 1997), and a broad spectrum of fungi (Delaquis, 1997; Nielsen and Rios, 2000).

### Allicin

The antimicrobial activity of allicin has been known since it was first isolated and studied by Cavallito and Bailey (1944), since then the mode of action of allicin have been elucidated in great detail. Allicin is shown to target intracellular enzymes by interacting with their free SH groups.

Allicin is readily transported across the cell membrane into the cytoplasm where it can exert its antimicrobial action. Garlic extract, with allicin as major component, induced no significant changes in *E. coli* and *S. aureus* cell morphology, supporting the hypothesis that allicin acts intracellularly (Perry et al., 2009). In another study, Miron et al. (2000) showed that allicin freely permeated phospholipid vesicles and reacted with encapsulated SH-containing molecules.

The antimicrobial activity of allicin is ascribed to its reactive chemical group that binds to and inhibits a broad-range of intracellular targets. Allicin's -S(O)-S- group reacts with SH groups of enzymes (Rabinkov et al., 1998). Allicin irreversibly inhibited the thiol-protease papain, NADP<sup>+</sup>-dependent alcohol dehydrogenase from *Thermoanaerobium brockii*, and a NAD<sup>+</sup>-dependent alcohol dehydrogenase from horse liver (Rabinkov et al., 1998). Interestingly, all three enzymes could be reactivated with thiol-containing components like dithiothreitol, 2-mercaptoethanol, and glutathione (Rabinkov et al., 1998), demonstrating that the interaction leading to inhibition is reversible. Focke et al. (1990) also demonstrated reversible inhibition by specific binding of allicin to the enzymes involved in acetyl-CoA synthesis in bacteria, plants, yeasts, and mammals. Allicin only partially and transiently inhibited the DNA replication and protein synthesis in *S. typhimurium*, while RNA synthesis was reduced by more than 90% for at least 30 min, suggesting RNA synthesis as the primary target of allicin (Feldberg et al., 1988). Collectively, these studies indicate that allicin is a non-specific inhibitor of many enzymes. Allicin could potentially be used in combination with other antimicrobials because it has inhibiting effects on RNA synthesis and thereby reduce or hinder cell protection mechanisms induced by other antimicrobials.

### Allyl isothiocyanate

The mode of action behind AITC's antimicrobial activity is not yet fully understood, but since it might penetrate membranes and no single site of action has been described, it is generally regarded as a non-specific inhibitor of periplasmic or intracellular targets.

It is not yet clear if AITC rapidly crosses membranes and enters the cytoplasm of prokaryotic and eukaryotic cells, or if it has an

effect on cell membranes. Ahn (2001) visualized the AITC-treated cells by transmission electron microscopy (TEM) and showed that AITC altered the internal cell structures without causing ATP leakage or cell wall damages to *L. monocytogenes*. However, it did reduce the internal levels of ATP, indicating that cellular energy-generating processes were affected. In contrast, another study showed that AITC caused cell membrane damages to *E. coli* and *Salmonella* Montevideo leading to leakage of cellular metabolites, but not cell lysis (Lin et al., 2000).

The mode of action of AITC is due to its chemical group. The central carbon atom of isothiocyanate ( $R-N=C=S$ ) is highly electrophile and reacts readily, and under mild conditions with oxygen-, sulfur-, or nitrogen-centered nucleophiles resulting in carbamates, thiocarbamates, or thiourea derivatives, respectively (Zhang and Talalay, 1994; Verma, 2003). Inside a cell, AITC can react with glutathione, sulfites, amino acids, oligopeptides, proteins, and water (Kawakishi and Namiki, 1982; Kawakishi and Kaneko, 1985, 1987; Cejpek et al., 2000). AITC cleaves the cysteine disulfide bond in proteins through an oxidative process (Kawakishi and Namiki, 1982), but also attacks free amino groups and arginine residues (Kawakishi and Kaneko, 1987). The antimicrobial mode of action of AITC is thus related to its general inhibition of enzymes and alteration of proteins by oxidative cleavage of disulfide bonds (Delaquis, 1995; Luciano and Holley, 2009).

The action of AITC on yeast is not well understood and warrants more investigation. Allyl isothiocyanate stalls oxygen uptake of yeasts, and uncouples the oxidative phosphorylation through the inhibition of cytochrome c oxidase in the electron transport chain (Kojima, 1971). Due to its very general mode of action in prokaryotes, similar effects are likely observed in eukaryotes also.

## EXPERIMENTAL APPROACHES TO INVESTIGATE THE ANTIMICROBIAL MODE OF ACTION

The diversity of essential oil constituents is enormous and presents a wide range of compounds. Some have low or no efficiency against microorganisms while others are potent antimicrobials. The majority of antimicrobial compounds found in essential oils are terpenoids and phenylpropenes with the most active being phenols, although some aldehydes and non-phenolic substances also present promising antimicrobial activity. The target site and mode of action of most essential oil components is still not well understood, especially in yeast. Commercial applications of essential oils would benefit from deeper insight into the mode of action behind individual compounds, as this could facilitate the exploitation of, e.g., synergistic combinations with more powerful antimicrobial properties.

Many different techniques have been applied to elucidate the mode of action of essential oils and their constituents. Here we will present and discuss the most common experimental approaches. After establishing the killing or inhibition activity of a compound, an array of experiments can be performed to identify how a compound interacts with the cell to cause the observed effects. In this context, it is important to distinguish between experiments that identify the target site from those that elucidate the mode of action. The site of action refers to the part of the cell which interacts with the compound, e.g., the cell membrane, cell wall, or intracellular proteins, enzymes, nucleic acids, or metabolites. The mode

of action, however, yields more elaborate knowledge about the molecular mechanisms or intermolecular interactions behind the inhibition or killing effects. An overview of methods addressing the site or mode of action of antimicrobial compounds is provided in **Table 3**.

### LOCATING THE SITE OF ACTION

High-resolution microscopy, such as electron microscopy or atomic force microscopy (AFM), can reveal the most extreme consequences of exposure to an antimicrobial compound, i.e., deformation of cells occurring from lysis or from damages to the cell wall. An advantage of TEM is that ultra-thin cross sections can reveal ultrastructural changes in the interior of the cell. Scanning electron microscopy (SEM) and AFM only image the cell surface. AFM has one important advantage over electron microscopy, in that it allows measurements in liquid under physiological conditions, avoiding difficult sample preparation and the artifacts associated herewith (Alessandrini and Facci, 2005). A limitation of both AFM and electron microscopy is, however, that specific cellular structures must be identified according to morphology unless some form of labeling can be applied. While antibodies conjugated to metal nanoparticles have been used with TEM in a few studies (Romero et al., 2010), no labeling techniques have been reported for SEM and AFM. It is, however, possible to combine AFM with optical microscopy and thus take advantage of the numerous options for fluorescent labeling of biomolecules.

An important site of action is the cell membrane, and indeed, many essential oil constituents have been proposed to act on the bacterial membrane. Interaction of antimicrobial compounds with the membrane can affect the transport of nutrients and ions, the membrane potential, and the overall permeability of the cell. These effects are investigated by measuring the efflux of intracellular ions like  $K^+$  and  $H^+$  (Ultee et al., 1999; Lambert et al., 2001). Efflux of small ions is not necessarily indicative of complete loss of membrane function, and can be observed in viable cells where growth is inhibited because the cell uses energy for repair or survival rather than cell proliferation (Bouhdid et al., 2010). Effects on the cell membrane that lead to cell death is more accurately predicted by detecting the efflux of larger molecules like ATP or carboxyfluorescein diacetate (cFDA) after esterase reaction (Xu et al., 2008), or by influx of large polar organic DNA-binding stains like ethidium bromide (Lambert et al., 2001) and propidium iodide (Bouhdid et al., 2010). It should be pointed out that it is always good practice to validate the observed effects by combining several techniques. Monitoring the release of calcein encapsulated in membrane vesicles can for example be used as a complimentary technique to confirm the membrane as the site of action (Miron et al., 2000).

If no effects are observed on cell structure and membrane functionality, it is assumed that the site of action is intracellular. The target can be proteins and enzymes in general, or it can be essential cellular processes involved in biosynthesis or energy generation. An intracellular site of action can for example be determined by incorporation of radioactively labeled substrates used in particular biosynthesis pathways (Schneider et al., 2010). Lack of or decreased incorporation is then taken as an indication of the process being affected by the antimicrobial compound. For

example, radiolabeled nucleotides or amino acids can be used to detect if DNA replication or protein synthesis takes place, respectively (Schneider et al., 2010).

Some compounds have multiple sites of action, and in that case it can be difficult to pinpoint which one is ultimately responsible for cell death. For example, a compound that affects membrane permeability will also affect the membrane potential and thereby energy generation by respiration. It is thus difficult to distinguish direct effects on energy-generating processes from the indirect effect a permeable membrane has on these processes. At sub-lethal concentrations, changes to the transcriptome and proteome during exposure can reveal how the cell responds to the compound, and upregulation of genes involved in certain metabolic or biosynthesis pathways can be indicative of which cell structures or processes that are affected (Burt et al., 2007; Rao et al., 2010).

### ELUCIDATING THE MODE OF ACTION

The probably most comprehensive approach to investigate the mode of action of a particular compound is to perform random transposon mutagenesis in order to search for mutations that compensate for the antimicrobial effect of a particular compound. In this way, it is possible to identify the mode of action of compounds that interact very specifically with, e.g., a single enzyme or with particular proteins or lipids in the membrane (Shapira and Mimran, 2007; Van Hoang et al., 2011). The approach is, however, not suited for investigating antimicrobial compounds that act simultaneously on several components in the cell, as a single mutation is unlikely to facilitate compensation for the antimicrobial effect on the cell as a whole.

Antimicrobial compounds that act on the membrane can cause depolarization or increased permeability through various mechanisms. For example, some antimicrobial peptides form pores (Cotter et al., 2005; Fantner et al., 2010) while other compounds, such as certain essential oil constituents, have a fluidifying effect on the membrane (Trombetta et al., 2005; Cristani et al., 2007). Membrane properties like lipid packing can be investigated in membrane vesicles by LAURDAN staining combined with spectrofluorometry (Nielsen and Otzen, 2010), and membrane fluidity can be investigated directly in bacteria by differential scanning calorimetry (Trombetta et al., 2005) or fluorescence anisotropy measurements of DPH using a spectrofluorometer (Liao et al., 2010). AFM imaging has also in recent years allowed high-resolution visualization of native membranes on a solid support. Structural changes resulting from integration of an antimicrobial compound into the membrane can thus be visualized directly (Brasseur et al., 2008), and the effect on membrane rigidity can be quantified by AFM force spectroscopy (Sullan et al., 2010). Functionalizing the AFM tip with the antimicrobial compound of interest furthermore allows investigation of interaction forces between the compound and its target. This approach was for example used to map binding events of vancomycin on the surface of bacteria and confirmed that binding occurred at the site of cell wall synthesis in dividing cells (Gilbert et al., 2007).

### ESSENTIAL OILS IN FOOD PRESERVATION

Food-borne diseases are a growing public health problem worldwide. It is estimated that each year in the United States, 31 species of



**Table 3 | Overview of experimental approaches used to identify target sites and modes of action of antimicrobial compounds.**

Target site or mode of action	Experiment	Reference
Changes in cell morphology	Scanning electron microscopy (SEM) visualizes the cell structure with sub-micron resolution.	Burt and Reinders (2003), Kwon et al. (2003), Bennis et al. (2004), Di Pasqua et al. (2007), Turgis et al. (2009), De Souza et al. (2010), Gao et al. (2011), Lv et al. (2011), Paul (2011)
	Transmission electron microscopy (TEM) can visualize changes in cell morphology, damages to cell wall and cell membrane, and coagulation of intracellular content.	Claeson et al. (1992), Gustafson et al. (1998), Ahn (2001), Carson et al. (2002), Rasooli et al. (2006), Bouhdid et al. (2009, 2010), Fisher and Phillips (2009), De Oliveira et al. (2011), Pajohi (2011), Rammanee and Hongpattarakere (2011), Zeng et al. (2011)
	Atomic force microscopy (AFM) visualizes cells at nanometer resolution in liquid under physiological conditions, and can provide information about changes in cell topography and elasticity.	Perry et al. (2009), Hafedh et al. (2010), La Storia et al. (2011)
Disruption of cytoplasmic membrane	Cell lysis or release of cellular content can be detected spectrophotometrically at 260 nm.	Carson et al. (2002), Bennis et al. (2004), Turgis et al. (2009), De Souza et al. (2010), Lv et al. (2011), Paul (2011)
	Measurement of potassium or phosphate leakage from the cells using ion-selective electrodes, atomic absorption spectroscopy, or flame photometry.	Lambert and Hammond (1973), Ultee et al. (1999), Cox et al. (2000), Lambert et al. (2001), Walsh et al. (2003), Fitzgerald et al. (2004), Shapira and Mimran (2007), Bouhdid et al. (2009, 2010)
	Measurement of ATP leakage from the cells using an assay based on luciferase activity quantified by bioluminescence.	Helander et al. (1998), Ultee et al. (1999, 2002), Ahn (2001), Fitzgerald et al. (2004), Gill and Holley (2004, 2006b), Fisher and Phillips (2009), Turgis et al. (2009), Sánchez et al. (2010), Paul (2011)
	Uptake of fluorescent DNA-binding stains, such as propidium iodide (PI), SYTO9, ethidium bromide (EB), and carboxyfluorescein diacetate (cFDA), using fluorescence microscopy or flow cytometry.	Cox et al. (2000), Lambert et al. (2001), Fitzgerald et al. (2004), Nguefack et al. (2004a), Gill and Holley (2006a), Paparella et al. (2008), Bouhdid et al. (2009, 2010), Somolinos et al. (2010), Ahmad et al. (2011), Ait-Ouazzou et al. (2011)
	Leakage of the self-quenching dyes calcein or carboxyfluorescein encapsulated in phospholipid vesicles is as an increase in fluorescence intensity as the intravesicular concentration decreases.	Cox et al. (2000), Miron et al. (2000), Trombetta et al. (2005), Cristani et al. (2007)
Disruption of outer membrane in Gram-negative bacteria	Changes in concentration gradients of ions across a cell membrane can be detected by fluorometry using bis-oxonol or DiSC <sub>3</sub> (5), or by flow cytometry using bis-oxonol, DiOC <sub>2</sub> (3), or BOX.	Ultee et al. (1999, 2002), Veldhuizen et al. (2006), Xu et al. (2008), Bouhdid et al. (2009, 2010), Fisher and Phillips (2009), Sánchez et al. (2010), Silva et al. (2011)
	Damages to the outer membrane is detected by monitoring the uptake of the hydrophobic fluorescent probe.	Helander et al. (1998), Fisher and Phillips (2009)
	1- <i>N</i> -phenyl-1-naphthylamine (NPN) into the membrane using fluorescence microscopy.	
	Release of phospholipid and LPS from the outer membrane is detected by capillary gas chromatography and compared with an internal fatty acid standard. The release of proteins from the outer membrane is detected by a electrophoresis (SDS-PAGE) in which the protein profiles of cell-free supernatants of treated and untreated cells are compared.	Helander et al. (1998)
	Changes in the protein profile of the outer membrane can be measured by separating the proteins according to mass and labeling for detection by laser induced fluorescence.	Horváth et al. (2009)
Changes in membrane properties	Membrane expansion can be detected by relief of fluorescence self-quenching by the liposome probe octadecyl rhodamine $\beta$ chloride.	Ultee et al. (2002)
	Effects on membrane melting temperature, fluidity, and phase separation can be detected using differential scanning calorimetry, Fourier-transform infrared spectrometer, nuclear magnetic resonance, or small-angle X-ray diffraction.	Ultee et al. (2000), Trombetta et al. (2005), Pérez-Fons et al. (2006), Cristani et al. (2007)

(Continued)



Table 3 | Continued

Target site or mode of action	Experiment	Reference
	Changes in yeast cell's ergosterol biosynthesis can be evaluated by comparing the intracellular content of ergosterols of cells grown in the absence or presence of antimicrobials. The content of ergosterols in an intracellular extract can be calculated using data obtained from a spectrophotometrically scan of the extract between 240 and 300 nm.	Ahmad et al. (2011)
Disruption of membrane potential	Changes in concentration gradients of ions across a cell membrane can be detected either with a spectrofluorometer using bis-oxonol or DiSC <sub>3</sub> (5), or by flow cytometry using bis-oxonol, DiOC <sub>2</sub> (3), or BOX.	Ultee et al. (1999, 2002), Veldhuizen et al. (2006), Xu et al. (2008), Bouhdid et al. (2009, 2010), Fisher and Phillips (2009), Sánchez et al. (2010), Silva et al. (2011)
Disruption of intracellular pH homeostasis	5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) is readily taken up by bacteria and hydrolyzed by esterases to 5-(and 6)-carboxyfluorescein succinimidyl ester (cFSE). The intracellular pH can then be determined from the ratio of the fluorescent signal of cFSE at the pH-sensitive 490 nm and the pH-insensitive 440 nm. Intracellular pH is measured with pH-sensitive fluorescent probes pHluorin (cytoplasmic) and BCECF AM (vacuoles).	Breeuwer et al. (1996), Ultee et al. (1999, 2002), Fitzgerald et al. (2004), Fisher and Phillips (2009), Turgis et al. (2009), Sánchez et al. (2010)
Disruption of intracellular Ca <sup>2+</sup> homeostasis	Intracellular Ca <sup>2+</sup> concentration is measured after transformation with a plasmid containing the gene for aequorin. Aequorin emits light upon binding Ca <sup>2+</sup> , and Ca <sup>2+</sup> is thus quantified by measuring luminescence.	Rao et al. (2010)
Disruption of cellular respiration	Disruption of the cellular respiration can be detected by oxygen consumption measured with oxygen electrodes or by reduction of the stain 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which is reduced by the electron transport chain to the insoluble and fluorescent formazan.	Rao et al. (2010)
Complex reaction mechanism	Reaction with thiol groups in a variety of targets can be monitored by complex formation with cysteine or glutathione as free SH-group compounds. Complex formation is then detected with high performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectroscopy (LC-MS), or NMR. Competitive binding of thiol groups can also be tested by adding thiol-containing compounds, e.g., the protein bovine serum albumin (BSA) or the organic compound desferal to the growth medium and test if the antimicrobial effect can be relieved.	Cox et al. (2000), Fitzgerald et al. (2004), Bouhdid et al. (2009, 2010)
		Rabinkov et al. (1998), Miron et al. (2000), Luciano et al. (2008), Luciano and Holley (2009)
		Juven et al. (1994)
Inhibition of particular enzymes	Inhibition of the cell wall synthesizing enzymes $\beta$ -(1,3)-glucan synthase and chitin synthase have been monitored using the radioactive substrates UDP[ <sup>14</sup> C]-Glu and UDP[U- <sup>14</sup> C]-GlcNAc by a liquid scintillation counter.	Bang et al. (2000)
	Changes in enzyme activity of proteases, alcohol dehydrogenases, thioredoxin reductase, acetate kinase, decarboxylases, $\alpha$ -amylase, subtilisin, acetyl-CoA-forming enzyme systems, and ATPase has been investigated, using techniques such as spectrophotometer, luminometer, HPLC, pH monitoring, liquid scintillation counter, and zone of proteolysis.	Thoroski (1989), Focke et al. (1990), Wendakoon and Morihiko (1995), Rabinkov et al. (1998), Gill and Holley (2006b), Luciano and Holley (2009)
Inhibition of cell division	The effect on FtsZ assembly and hence on the cell division apparatus can be investigated using light scattering assay, GTP hydrolysis, TEM, isothermal titration calorimetry, saturation transfer difference NMR spectroscopy (STD NMR), and <i>in silico</i> molecular modeling.	Domadia et al. (2007), Hemaiswarya et al. (2011)
Changes in transcriptome	Random mutation can be used to identify the role of a particular genes in resistance or susceptibility mechanisms. Transcriptional up- and down-regulation can subsequently be detected for genes of interest using RT-qPCR, or for a large number of genes simultaneously using microarrays.	Somolinos et al. (2010), Shapira and Mimran (2007)

(Continued)

Table 3 | Continued

Target site or mode of action	Experiment	Reference
	Changes in RNA, DNA, and protein biosynthesis can be detected by continuous incorporation of radioactive labeled uridine, thymidine, and leucine, respectively.	Feldberg et al. (1988)
Changes in proteome	Expression of specific proteins can be determined by SDS-PAGE gel electrophoresis and western blotting followed by identification of peptide fragments by mass spectrometry.	Burt et al. (2007), Liu et al. (2010)
	Changes in a proteome profile can be detected by 2D-PAGE electrophoresis separation followed by selection and excision of up- or down-regulated protein-spots, which are then identified by mass spectrometry.	Di Pasqua et al. (2010), Liu et al. (2010)
Changes in toxin production	The effect on excreted toxin production can be measured using enzyme linked immunosorbent assay (ELISA) and spectrophotometric quantification.	Ultee and Smid (2001), De Souza et al. (2010)

pathogens cause 9.4 million cases of food-borne illnesses (Scallan et al., 2011). Successful control of food-borne pathogens requires the use of multiple preservation techniques in the manufacturing and storage of food products. A recent consumer trend toward preference for products with lower salt and sugar content presents an increased need for efficient food preservatives, as lowering the salt and sugar content would otherwise compromise the product's shelf-life (Zink, 1997). A wide range of preservatives are used to extend the shelf-life of a product by inhibiting microbial growth. However, an increasingly negative consumer perception of synthetic food additives has spurred an interest in finding natural alternatives to the traditional solutions (Zink, 1997). Although originally added to change or improve taste, the antimicrobial activity of essential oils makes them an attractive choice for substituting synthetic preservatives.

#### PERSPECTIVES AND LIMITATIONS IN APPLICATION OF ESSENTIAL OILS IN FOOD

A range of essential oil components have been accepted by the European Commission for their intended use as flavorings in food products. The registered flavorings are, e.g., linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene, all of which are considered to present no risk to the health of the consumer. The United States Food and Drug Administration (FDA) also classifies these substances as generally recognized as safe (GRAS). The crude essential oils classified as GRAS by FDA include amongst others clove, oregano, thyme, nutmeg, basil, mustard, and cinnamon. There are regulatory limitations on the accepted daily intake of essential oils or essential oil components, so before they can be used in food products, a daily intake survey should be available for evaluation by FDA.

Despite the demonstrated potential of essential oils and their constituents *in vitro*, their use as preservatives in food has been limited because high concentrations are needed to achieve sufficient antimicrobial activity. In many food products, the hydrophobic essential oil constituents are impaired by interactions with food matrix components, such as fat (Cava-Roda et al., 2010; Rattanachaiakunsopon and Phumkhachorn, 2010), starch (Gutierrez

et al., 2008), and proteins (Cerrutti and Alzamora, 1996; Kyung, 2011). Furthermore, the antimicrobial potency of essential oil constituents also depends on pH (Juven et al., 1994), temperature (Rattanachaiakunsopon and Phumkhachorn, 2010), and the level of microbial contamination (Somolinos et al., 2010). Extrapolation of results from *in vitro* tests to food products is thus difficult at best, and a lower performance of the antimicrobial compound must be expected. For example, Cilantro oil had significant antibacterial activity at 0.018% *in vitro*, but when applied to a ham model, even 6% cilantro oil had no antimicrobial activity (Gill et al., 2002). Before being added to food products, it is therefore useful to investigate how essential oils or their constituents interact with food components *in vitro*. Food matrix interactions with the essential oils or their constituents can be investigated by measuring the growth of microorganisms in culture medium containing a range of concentrations of fat, protein, or starch as well as the antimicrobial compound of interest. Such experiments have been performed using a so-called food model media (Gutierrez et al., 2009), and can be used to provide quick answers to which kind of food products the compound in question can be used in.

The intense aroma of essential oils, even low concentrations, can cause negative organoleptic effects exceeding the threshold acceptable to consumers (Lv et al., 2011). Having to increase the concentration of essential oils to compensate for their interactions with food matrix components is therefore highly unfortunate and limits their application to spicy foods where the acceptable sensory threshold is relatively high. Different strategies can be used to circumvent this problem. One option is to use essential oils in active packaging rather than as an ingredient in the product itself. Essential oils can be encapsulated in polymers of edible and biodegradable coatings or sachets that provide a slow release to the food surface or to the headspace of packages of, e.g., fruit, meat, and fish (Pelissari et al., 2009; Sánchez-González et al., 2011). Sachets that release volatile essential oils into the headspace environment are simply placed within an enclosed food package (Ahvenainen, 2003). The advantage of incorporating volatile components of essential oils in films or edible coatings is that the diffusion rate of the agents away from the food product can be reduced,

thereby maintaining the active compounds in the headspace or on the product surface for extended periods of time (Phillips and Laird, 2011; Sánchez-González et al., 2011). A way to minimize organoleptic effects of essential oils added to the matrix of a food product is to encapsulate essential oils into nanoemulsions. This approach increases the stability of volatile components, protecting them from interacting with the food matrix, and increases the antimicrobial activity due to increased passive cellular uptake (Donsí et al., 2011).

Lowering the concentration of essential oils without compromising their antimicrobial activity can also be obtained by applying them in combination with other antimicrobial compounds that provide a synergistic effect (Nguefack et al., 2012). Synergies are known to occur for essential oil combinations, and it is therefore a field with countless opportunities to find potent antimicrobial blends, which may be the key to implementing essential oils in food preservation without simultaneous organoleptic effects.

### SYNERGIES BETWEEN ESSENTIAL OIL COMPONENTS

The interaction between antimicrobials in a combination can have three different outcomes, synergistic, additive, or antagonistic. Synergy occurs when a blend of two antimicrobial compounds has an antimicrobial activity that is greater than the sum of the individual components. An additive effect is obtained when the combination of antimicrobials has a combined effect equal to the sum of the individual compounds. Antagonism occurs when a blend of antimicrobial compounds has a combined effect less than when applied separately (Davidson and Parish, 1989; Burt, 2004).

The combined effect of a blend is analyzed by using measurements of the MIC to calculate the fractional inhibition concentration index ( $FIC_{Index}$ ) according to the formulas defined by (Davidson and Parish, 1989):  $FIC_A = MIC_{A+B}/MIC_A$ ,  $FIC_B = MIC_{B+A}/MIC_B$ ,  $FIC_{Index} = FIC_A + FIC_B$ . The  $MIC_{A+B}$  value is the MIC of compound A in the presence of compound B, and vice versa for  $MIC_{B+A}$ . Calculating the FIC value for either substance A or B then requires determination of the MIC for the individual components. Theoretically, a  $FIC_{Index}$  near 1 indicates additive interactions, while below 1 implicates synergy, and above 1 antagonism (Davidson and Parish, 1989). However, this definition has been replaced by a more general one where the  $FIC_{Index}$  results are interpreted as synergistic if  $FIC_{Index} < 0.5$ , additive if  $0.5 < FIC_{Index} < 4$ , or antagonistic if  $FIC_{Index} > 4$  (Odds, 2003).

The antimicrobial activity of a given essential oil may depend on only one or two of the major constituents that make up the oil. However, increasing amounts of evidence indicate that the inherent activity of essential oils may not rely exclusively on the ratio in which the main active constituents are present, but also interactions between these and minor constituents in the oils. Various synergistic antimicrobial activities have been reported for constituents or fractions of essential oils when tested in binary or ternary combinations (Delaquis et al., 2002; Pei et al., 2009; García-García et al., 2011; Nguefack et al., 2012). For example, García-García et al. (2011) found the most synergistic binary combination against *L. innocua* to be carvacrol and thymol, and the most active ternary combination to be carvacrol, thymol, and eugenol. Reports on greater antimicrobial activity of crude essential oils compared to blends of their major individual

components suggests that trace components in the crude essential oils are critical to the activity and may have a synergistic effect (Marino et al., 2001; Delaquis et al., 2002; Burt, 2004; Koutsoudaki et al., 2005). In contrast to this, trace components may also cause antagonistic interactions, which were seen by comparing the antimicrobial effect of pure carvacrol to oregano oil where carvacrol is a major constituent. Pure carvacrol was 1500 times more effective than the crude essential oil (Rao et al., 2010). Among individual essential oil constituents, synergy has been observed for carvacrol and *p*-cymene on *B. cereus* (Ultee et al., 2002; Rattanachaiyapong and Phumkhaichorn, 2010). It appears that *p*-cymene swells bacterial cell membranes, probably enabling easier entrance of carvacrol into the cell membrane where it exerts its action (Ultee et al., 2002). Furthermore, Bassolé et al. (2010) showed that if linalool or menthol was combined with eugenol it showed the highest synergy, suggesting that a monoterpenoid phenol combined with a monoterpenoid alcohol is an effective combination.

Little is currently known about what governs synergy and antagonism among essential oil constituents. Four theoretical mechanisms of antimicrobial interactions produce synergy: (i) sequential inhibition several steps in a particular biochemical pathway, (ii) inhibition of enzymes that degrade or excrete antimicrobials, (iii) interaction of several antimicrobials with the cell wall, or (iv) interaction with the cell wall or membrane that leads to increased uptake of other antimicrobials (Davidson and Parish, 1989; Eliopoulos et al., 1996). Another possibility for synergistic effects could be that antimicrobials have different mode of actions, thereby attacking two different sites on or in the cell, which indirectly depend on each other. Even less is known about the cause antagonism, it is hypothesized to occur when: (i) combining bacteriostatic and bactericidal antimicrobials, (ii) antimicrobials have the same site of action, (iii) antimicrobials interact with each other (Davidson and Parish, 1989), Larson (1985) in Roller (2003). The hypothesized synergistic or antagonistic interactions are based on 15 year old results, and with the emergence of new techniques this field is likely to see some significant advances in our understanding of how antimicrobial compounds affect each other when acting in concert.

In practice, the knowledge needed to exploit synergistic combinations of essential oils in food products is (i) the site and mode of action of each essential oil constituent, and (ii) the mechanisms resulting in synergy or antagonism between several compounds, and (iii) how each compound interacts with food matrix components in a way that affects is antimicrobial properties. When the mechanistic details for synergistic interactions are better understood, it will be easier to exploit synergies using intelligent combinations of constituents to combat food spoilage microorganisms.

### OUTLOOK

An attractive application of essential oils and their constituents is in food products to prolong the shelf-life of foods by limiting growth or survival of microorganisms. The organoleptic impact of essential oils and their components in food products currently limits their usage to spicy foods normally associated with herbs, spices, or seasonings. Synergistic interactions should therefore be

exploited to lower the organoleptic impact and thereby facilitate the use in a broader range of products.

The lack of detailed knowledge about the mode of action of the individual essential oil constituents is also the underlying cause for our superficial understanding about what governs synergy and antagonism. Future research should thus explore the mode of action of individual essential oil constituents further, while also initiating systematic investigations into the mechanisms of synergy among different constituents. Many studies have investigated the site of action, while few proceed to reveal the mode of action. Furthermore, most work to date has focused on prokaryotes, and little is known about how essential oils interact with yeast and fungi. Regardless of the microorganism, future research into the mode of action will need a standardization of investigation methods, complementary experiments that validate results, and implementation of new techniques. Taking a systems biology approach to investigating the mode of action of antimicrobial compounds will no doubt further this field. Transcriptomic and proteomic analyses can identify pathways targeted by an antimicrobial, whereas nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, and computer modeling help identify key residues involved in the molecular interactions between target and the antimicrobial (Wang et al., 2006; Domadia et al., 2007; Liu et al., 2010; Schneider et al., 2010). Interactions with cell surface structures or cell membranes can be studied in detail by

AFM force spectroscopy (Brasseur et al., 2008; Dufrêne, 2008). These techniques provide valuable information about an antimicrobial's specific intracellular targets, the structural nature of interaction, and what governs susceptibility, adaptation, and resistance mechanisms (Schneider et al., 2010).

Synergistic blends that have commercial interest must be evaluated under the relevant environmental conditions which reflect the food matrixes to which they should be applied, as interactions with food matrix ingredients could decrease their activity. Investigating the molecular interactions behind the inhibition of food matrix ingredients opens an entirely different research direction, which focuses on formulating essential oils in foods with the aim to minimize organoleptic effects without compromising the antimicrobial properties. Encapsulation and controlled/sustained release of potent synergistic combinations could potentially reduce the organoleptic impact and simultaneously increase the antimicrobial potency if the encapsulation material facilitates close interaction with the microorganisms. New strategies for nanoencapsulation may thus provide an interesting platform for this research avenue in the future.

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