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Effects of sub-lethal concentrations of thyme and oregano essential oils, carvacrol, thymol, citral and *trans*-2-hexenal on membrane fatty acid composition and volatile molecule profile of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteritidis*

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ABSTRACT

The aim of this work was to investigate the modifications of cell membrane fatty acid composition and volatile molecule profiles of *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli*, during growth in the presence of different sub-lethal concentrations of thyme and oregano essential oils as well as carvacrol, thymol, *trans*-2-hexenal and citral. The results evidenced that the tested molecules induced noticeable modifications of membrane fatty acid profiles and volatile compounds produced during the growth. Although specific differences in relation to the species considered were identified, the tested compounds induced a marked increase of some membrane associated fatty acids, particularly unsaturated fatty acids, *trans*-isomers, and specific released free fatty acids. These findings can contribute to the comprehension of the stress response mechanisms used by different pathogenic microorganisms often involved in food-borne diseases in relation to the exposure to sub-lethal concentrations of natural antimicrobials.

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1. Introduction

The interest of the food industry and consumers in natural antimicrobials as alternatives to traditional chemical additives, to prevent spoilage and pathogenic microorganisms, has increased significantly. Essential oils (EOs) and some of their components have proved to be a source of natural alternatives to improve food safety and shelf-life. EOs are characterised by a wide range of volatile compounds, some of which are important to food flavour quality, and they are generally recognised as safe (GRAS) (Belletti et al., 2004). For this reason the number of publications regarding their application as natural preservatives in different food matrices, such as meat, dairy products, minimally processed fruits and vegetables, and beverages is increasing (Belletti, Sado Kamdem, Tabanelli, Lanciotti, & Gardini, 2010).

In general, the antimicrobial effects of EOs have been mainly explained through the presence of C10 and C15 terpenes with aromatic rings and a phenolic hydroxylic group able to form hydrogen bonds with active sites of target enzymes (Picone et al., 2013). Nevertheless, other active terpenes and alcohols, aldehydes, as well as esters can contribute to the overall antimicrobial effects of the EOs. Some EOs, such as thyme and oregano, and some components of EOs such as carvacrol, thymol, citral (a mixture of monoterpene aldehydes: geranial and neral), hexanal and *trans*-2-hexenal, are promising natural alternatives to traditional preservatives, since their antimicrobial activity is well documented both in model and in real foods (Ivanovic, Misic, Zizovic, & Ristic, 2012). Although the antimicrobial properties of EOs and their major components are well known, their mechanisms of action have not been fully understood (Picone et al., 2013).

Given the structural differences and the presence of different functional groups it is most likely that the antibacterial activity of essential oil components is not attributable to one specific mechanism but to the action towards several specific cell targets (Burt, 2004). Generally, it is accepted that the principal target of EOs and their components are the cell wall, the cytoplasm membrane and membrane proteins. In addition, these natural antimicrobial can promote the leakage of contents out of the cell, the coagulation of cytoplasm, and the depletion of the microbial cell proton motive force (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). The presence of the hydroxyl group has a key role

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in the inactivation of microbial enzymes by thymol and carvacrol (Lambert, Skandamis, Coote, & Nychas, 2001).

The α,β-unsaturated aldehydes, after penetration into the cells, can react with biologically important nucleophilic groups. Moreover, the aldehydes may cross-link amino groups in the cell wall and cytoplasm and inhibit enzymes with a thiol group at the cytoplasmic membrane causing coagulation and precipitation of cytoplasmic constituents (Aiemsaard, Aiumlamai, Aromdee, Taweechaisupapong, & Khunkitti, 2011). In addition Patrignani et al. (2008) demonstrated that sub-lethal concentrations of *trans*-2-hexenal were able to induce noticeable modifications of the composition of cell membrane and the volatile compounds produced during the growth of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli*. In general, the hydrophobicity of EOs and their components allows them to interact with cell membrane phospholipid bilayer, interfering with its integrity and functionality. It is well known that when microbial cells are exposed to a sub-lethal stress, the cell membrane is able to change in order to cope with the new environment (Wouters, Alvarez, & Raso, 2001). In fact, the modification of membrane fatty acid composition is fundamental in maintaining both membrane integrity and functionality when exposed to external stresses (Tabanelli et al., 2013). The adaptive strategies depend on the species and on the stress considered and include alterations of saturation degree, carbon chain length, branching position, *cis*/*trans* isomerisation and conversion of unsaturated fatty acids (UFAs) into cyclopropanes (Guerzoni, Lanciotti, & Cocconcelli, 2001). In addition some authors showed that the modification of fatty acid composition of the cell membrane in response to stress exposure can modify cell gene expression and the premature production of quorum sensing signalling compounds (Chatterjee, Seunath, Khalawan, & Curran, 2000).

Since the practical exploitation of antimicrobials in foods has to be supported by a full comprehension of the mechanisms of action and the cell membrane is the primary target of natural antimicrobials, the main aim of this work was the study of the changes in cell membrane fatty acid composition and volatile molecule profiles of *L. monocytogenes*, *S. enteritidis*, *E. coli*, when grown in the presence of different sub-lethal concentrations of thyme and oregano EOs, carvacrol, thymol, *trans*-2-hexenal and citral.

2. Materials and methods

2.1. Natural antimicrobials and microbial strains

Oregano and thyme EOs were obtained from Flora s.r.l. (Pisa, Italy) while *trans*-2-hexenal, citral, carvacrol and thymol were purchased from Sigma–Aldrich (Milano, Italy).

The strains used in this study, i.e. *L. monocytogenes* Scott A, *E. coli* 555 and *S. enteritidis* E5, belonged to the Department of Agricultural and Food Sciences of Bologna University. The strains were maintained at −80 °C before experiments, the strains were cultured twice in BHI broth at 37 °C for 24 h.

2.2. Cell treatments and determination of growth kinetics in relation to natural antimicrobial concentrations

The inoculation of the tested strains was performed in 100-mL conical flasks, containing 50 mL of BHI broth (Oxoid, Milano, Italy) at a level of about 2.5 log cfu/mL. Immediately after the inoculum, the samples were supplemented with selected concentrations of each natural antimicrobial. The concentrations used corresponded to 1/2, 1/3, 1/5 of the minimal inhibition concentration (*MIC*) values determined according to the method reported by Siroli et al. (2014). Briefly, for the *MIC* determination, 150 μL of BHI broth inoculated at three different levels (2, 4 or 6 log cfu/mL) with the tested pathogens were added to 200-μL microtitre wells (Corning Incorporated, New York, NY). Natural antimicrobials were properly diluted in ethanol 96% (PROLABO; VWR International) and 50 μL of the different dilutions were added to the microtitre wells, in order to obtain concentrations ranging between 50 and 400 mg/L. Microtitre plates were incubated at 37 °C and checked after 48 h. The MBC were determined by spotting 10-μL of each well after 48 h, onto BHI agar plates.

In particular, the compounds and the concentrations added to the growth medium of the different target microorganisms are reported in Table 1.

The EOs or their components used were conveyed through ethanol used at 1% in the final solution (*v*/*v*). The different antimicrobials and ethanol were previously sterilised through filtration (0.22 μm, Millex-GS-Millipore, Molsheim France). For each strain and condition three flasks were considered on three different days. Inoculated samples added just with 1% of ethanol and samples without any addition were considered as controls. The incubation was performed overnight at 37 °C. The growth kinetics in the presence of the different compounds were evaluated on the basis of the optical density at 600 nm (OD_{600}) using a spectrophotometer UV-1204 (Shimadzu, Kyoto, Japan), and periodically (every hour during exponential growth phase) a plate count onto plate count agar (Oxoid, Basingstoke, UK) incubated at 37 °C for 48 h was performed. The *OD* data were modelled with Statistica software (Version 8.0; StatSoft., Tulsa, OK), by using the Gompertz equation as modified by Zwietering, Jongenburger, Rombouts, and Van 'T Riet (1990), in order to evaluate the cell growth parameters. Three replicates for each strain and each condition were performed.

Table 1
Molecules and relative concentrations (mg/kg) used on *E. coli*, *L. monocytogenes* and *S. enteritidis* for the determination of the growth kinetics and subsequently to study the modifications in fatty acid composition of cell membranes.

	Oregano EO (mg/kg)	Thyme EO (mg/kg)	Thymol (mg/kg)	Carvacrol (mg/kg)	Citral (mg/kg)	*trans*-2-Hexenal (mg/kg)
E. coli	50 (10.5)[a]	70 (11)[a]	40 (11)[a]	40 (11)[a]		100 (13)[a]
	80 (11)[a]	120 (11)[a]	70 (11)[a]	70 (11)[a]		170 (22)[a]
	120 (14)[a]	170 (12)[a]	100 (11.5)[a]	100 (11)[a]		250 (44)[a]
L. monocytogenes	30 (30)[a]	40 (38)[a]	40 (24)[a]	20 (23)[a]	50 (24)[a]	
	40 (34)[a]	70 (50)[a]	70 (27)[a]	35 (24)[a]	85 (27)[a]	
	50 (37)[a]	100 (55)[a]	100 (29)[a]	50 (24)[a]	125 (31)[a]	
S. enteritidis	60 (10)[a]	50 (10)[a]	40 (9.5)[a]	40 (9.5)[a]		
	100 (10.5)[a]	90 (10.5)[a]	70 (10)[a]	70 (10)[a]		
	150 (13)[a]	135 (11)[a]	100 (10)[a]	100 (10)[a]		

The time of collection was established on the basis of Gompertz equation modified according to Zwietering et al. (1990).
[a] Within the brackets, the times (in hours) at which the cells were collected for fatty acid analyses.

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