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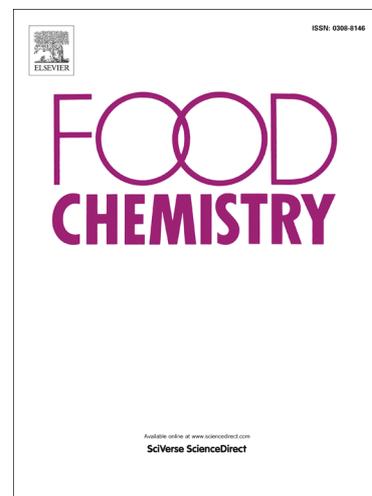
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Effects of sub-lethal concentrations of thyme and oregano essential oils, carvacrol, thymol, citral and *trans*-2-hexenal on membrane fatty acid composition and volatile molecule profile of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella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

Lorenzo Siroli¹, Francesca Patrignani¹, Fausto Gardini^{1,2}, Rosalba Lanciotti^{1,2*}

¹ Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Campus of Food Science, Piazza Goidanich 60, 47521 Cesena, Italy

² Interdepartmental Center for Industrial Agri-food Research, University of Bologna, Piazza Goidanich 60, 47521 Cesena (FC), Italy

*Rosalba Lanciotti

e-mail: rosalba.lanciotti@unibo.it

University of Bologna

p.zza Goidanich 60, 47521 Cesena (FC), Italy

phone 0039 0547 338132

running title: antimicrobials and pathogen membrane fatty acid composition

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.

Keywords: essential oils, natural antimicrobials, membrane fatty acids, pathogens, volatile molecule profile

Highlights

Natural antimicrobials induced marked changes in pathogen membrane fatty acid profiles

Natural antimicrobials induced marked changes in pathogen volatile molecule profiles

The data contributed to the comprehension of the pathogen stress response mechanisms

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 recognized 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 et al., 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 et al., 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 antimicrobials 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 et al., 2013). The presence of the hydroxyl group has a key role in the inactivation of microbial enzymes by thymol and carvacrol (Lambert et al., 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 (Aiemsard et al., 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 et al., 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 et al., 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 et al., 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 *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia 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 $-80\text{ }^{\circ}\text{C}$ before experiments, the strains were cultured twice in BHI broth at $37\text{ }^{\circ}\text{C}$ 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 μ L of BHI broth inoculated at three different levels (2, 4 or 6 log cfu/mL) with the tested pathogens were added to 200- μ L microtitre wells (Corning Incorporated, New York, NY). Natural antimicrobials were properly diluted in ethanol 96% (PROLABO; VWR International) and 50 μ L 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- μ L 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 μ m, 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 600nm (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 et al., (1990), in order to evaluate the cell growth parameters. Three replicates for each strain and each condition were performed.

2.3 Fatty acid analyses

Late exponential phase cells were collected by centrifugation (8000 $g \times 15$ min) and washed in physiological water. Lipid extraction and membrane fatty acid analyses were performed according to Suutari et al., (1990) while gas-chromatography analyses were performed according to Patrignani et al. (2008). FAs were identified by comparing their retention times and mass fragmentation profiles with those of the standards mix, BAME (Sigma-Aldrich, Milano, Italy). The data were expressed as a relative percentage of each FA compared to the total FA area. For each strain and each condition, three repetitions of three independent experiments were considered.

2.4 Volatile molecule profile analyses through solid phase microextraction-GC/MS

Late exponential phase cells, for each experimental condition, were collected by centrifugation and then 5 mL of the supernatant were sterilely taken and placed in a 10-mL vial sealed with a PTFE/silicon septum. For each strain and each condition, three repetitions of three independent experiments were considered and the samples were stored at -40 °C until analyses.

For the analysis and the gas-chromatographic conditions, the method reported by Patrignani et al., (2008) was used.

2.5 Data analysis

Principal component analysis (PCA) was performed using Statistica software (version 8.0; StatSoft., Tulsa, OK) to obtain a visual overview of FA composition of cell membranes and of the volatile molecule profile of the different strains in relation to the antimicrobials used.

3. Results

3.1 Effects of the selected compounds on the growth kinetics of Listeria monocytogenes, Escherichia coli and Salmonella enteritidis

The growth in the presence of the tested antimicrobials caused a reduction of A value (maximum cell loads attained in stationary phase) of μ_{\max} (growth rate in exponential phase) and an increase of λ (duration of lag phase), compared to the controls and the control samples added with 1% of ethanol, independently of strain considered (data not shown). No significant differences were observed among the control samples and samples added with 1% ethanol. The inhibition of growth kinetics was dependent on the strain, natural antimicrobial employed and its concentration, resulting in different times necessary to reach late exponential phase (Table 1). However to evaluate the effect of the natural antimicrobials on membrane fatty acid and volatile molecules profiles, late exponential phase cells were considered.

3.2 Cell fatty acid changes induced by sub-lethal concentrations of *trans*-2-hexenal, citral, carvacrol, thymol, thyme EO and oregano EO

As shown in Table 2, for *L. monocytogenes* the main membrane FAs detected in the control cells (with or without ethanol) were C15ante, C15iso, C17ante, C16:0 and C18:0. Carvacrol supplementation induced the decrease of C17iso and C17ante, associated with an increase of C14:0, C16:0 and C18:1cis9 causing an increase of the unsaturation level (UL). The growth in the presence of citral mainly induced the increase of UL due to the increment of C14:1cis11, C16:1cis9, C18:2 and C18:1cis9 independently of the concentration used. The supplementation with increasing oregano EO concentrations induced a relevant increase of unsaturated fatty acids (UFAs), chain length (CL) and the level of some saturated FAs, such as C16:0 and C18:0, compared to control cells. Also thyme EO induced an increase of CL.

The data of the free fatty acids (FFAs) showed that, in *L. monocytogenes*, ethanol supplementation increased C15iso, C15ante, C16iso and C17ante levels, as well as decreased C16:0 and C18:0 relative percentages compared to the control cells (data not shown). The supplementation with carvacrol and citral increased the relative percentages of medium-chain FAs. At the highest concentrations these

antimicrobials effected a marked increase of unsaturated FAs such as C18:1*cis*9 and C18:2. Oregano EO induced a marked increase of the relative percentages of UFAs and C15iso, associated with a decrease of saturated FAs such as C16:0, C18:0, C15ante and C17ante. An increase of the relative percentages of C10:0, C12:0 and C14:0 was observed, with few exceptions, at increasing thymol concentrations. A similar trend was observed for C15iso, C15ante and C16iso. Thyme EO induced an increase of C10:0, C12:0, C14:1, C15iso, C16iso, C16:1, C17iso, C17ante, C18:2 and C18:1*cis*9 relative percentages, accompanied by a marked decrease of C16:0 and C18:0 compared to control cells (data not shown).

The fatty acid composition of *E. coli* 555 is reported in Table 3. The main membrane FAs detected in the control cells were C12:0, C14:0, C12cyc, C16:0, C17cyc, C18:1*cis*9, C18:0 and C19cyc. The growth in the presence of the tested antimicrobials caused an increase of the UL, with the exception of the samples added with 40 mg/kg of thymol, mainly due to the decrease of C12:0 and C14:0, compared to the control cells. In addition, the growth in the presence of the chosen natural antimicrobials resulted, with few exceptions, in an increase of specific saturated FAs, in an increase of C18:1*trans*9 levels and in a decrease of cyclopropanic FA relative percentages. Concerning FFA percentages, the tested natural antimicrobials caused an increase of the UL, with the exception of the samples treated with 50 mg/kg of oregano, and the lowest concentrations of *trans*-2-hexenal (100 and 170 mg/kg) employed. Only the samples supplemented with thymol or *trans*-2-hexenal showed an increase of the CL with respect to the controls. Also C16:0, the main FFA, showed an increase independently of antimicrobials and their concentrations compared to the control cells, with the exception of the samples supplemented with thymol, and carvacrol at the lowest concentration. In all samples supplemented with natural antimicrobials an increase of UFAs, (with few exceptions) was observed. Only the samples added with thymol or *trans*-2-hexenal at 170 ppm showed a slight increase of C19cyc.

S. enteritidis modified the membrane FA composition in response to the stress conditions applied during the growth mainly increasing the relative percentages of unsaturated FAs, such as C16:1*trans*9 and C18:1*trans*9 (Table 4). The cyclopropanic FAs were subjected to specific changes in relation to the natural antimicrobial supplemented in the growth medium. In fact, the addition of ethanol and thyme caused a remarkable decrease of C12cyc associated with an increase of C17cyc and C19cyc. A decrease of cycFAs was observed with the addition of carvacrol. The supplementation of thymol at different concentrations provoked a decrease of C12cyc and C17cyc, while an increase of C19cyc was detected in the samples supplemented with 40 and 70 mg/kg of thymol. The UL level increased also in the FFAs of the cells grown in the presence of the natural antimicrobials (data not shown), principally due to 18:1 *trans*9 and C16:1*cis*9. By contrast a decrease of cyclopropanic FAs was observed.

3.3 Principal component analysis of cell fatty acid data

To better show the relationships between membrane FAs composition and chemical stress applied during the growth, principal component analyses (PCA) of the FA percentages detected in the cells grown in the presence of ethanol (1%) or supplemented with the natural antimicrobials were performed.

In Figure 1, the PCA loading plots of FAs composition of late exponential phase cells of *S. enteritidis* (1a) and *E. coli* (1b) in relation to the stress condition applied are reported. All the samples of *S. enteritidis* were mapped in the space spanned by the first two principal components PC1 versus PC2. PC1 accounted for 45.27% of the variability, and PC2 for 38.92%. The samples were grouped mainly on the basis of the added antimicrobials independently of their concentrations. Three clusters were evident: cluster 1 included all the samples added with carvacrol and thymol at the highest concentrations used; in cluster 2 were present the samples treated with thyme EO independently of its concentration and the other samples added with thyme EO; cluster 3 grouped all the samples added

with oregano EO. The samples added with 1% ethanol were not included in any clusters. Also for *L. monocytogenes* samples were grouped mainly on the basis of the supplemented compounds independently of the concentrations added (data not shown) while five clusters were evident for *E. coli* (Figure 1b) even if the clusters 4 and 5 showed marked scatterings among the samples along the PC2 accounting for 21.20% of the variability. In this case the projection of the variables on the factor plane for the first two factors showed that the main negative effects on factor 2 were determined by C14:0, C16:0 and C12cyc, while the main positive effects were related with C17cyc, C19cyc and the chain length (CL) (data not shown). In addition, factor 1 was highly positively related with C16:0, C14:0, C17cyc and C19cyc, and negatively related with UFAs such as C16:1*cis*9, C18:1*cis*9 and C18:1*trans*9. Also for *S. enteritidis* and *L. monocytogenes* UFAs such as C16:1*cis*9 and C18:1*trans*9 were fundamental for the sample clustering (data not shown).

3.4 Volatile molecule profile changes induced by sub-lethal concentrations of *trans*-2-hexenal, citral, carvacrol, thymol, thyme and oregano EOs

The supplementation of the growth media with the considered natural antimicrobials provoked marked modifications of the SPME/GC-MS profiles of the tested strains. As expected, the volatile profiles of the strains differed according to the species. However, aldehydes, hydrocarbons, pyrazines and alcohols were the principal families of molecules independently of species and natural antimicrobials supplemented in the growth medium. The chemical stress applied significantly affected the volatile molecule profiles of the species that showed the presence of the supplemented antimicrobials and their detoxification compounds. The occurrence of the *trans*-2-hexenal detoxification products, such as *trans*-2-hexen-1-ol, *cis*-2-hexen-1-ol, 2-hexenoic acid, ethyl ester and 1,1-diethoxybutane, characterised the SPME-GC profiles of cells subjected to the unsaturated aldehyde exposure; while the presence of citral detoxification molecules such as nerol, geraniol, β -citronellol, 6-methyl-5-hepten-2-

one, and 2,3-dimethyl-1-pentene, characterised the volatilome of cells subjected to citral exposure. The volatilomes of the cells exposed to oregano and thyme EOs was characterised by thymol, carvacrol, caryophyllene, *p*-cymene, γ -terpinene, 1-terpinen-4-ol and linalool, the main components of these essential oils (Daferera et al., 2000).

To better understand the effects of the tested antimicrobials on the volatilome of *L. monocytogenes*, *E. coli* and *S. enteritidis*, principal component analyses (PCA) were performed on the volatile molecule data obtained from cells grown in the presence of different sub-lethal concentrations of natural antimicrobials. In the analysis, data regarding the added antimicrobials and their detoxification products were excluded. As controls, data from cell grown in presence of ethanol were used. Although the volatile molecule profiles differed with the species, the PCA results showed clearly that the antimicrobials used induced specific changes in the compound release independently of their concentrations. In fact, the samples grouped generally in relation to antimicrobial used independently of the species considered. In particular, the PCA loading plot of volatile molecules of *L. monocytogenes* in relation to the stress condition applied showed that the samples were mapped in the space spanned by the first two principal components, PC1 versus PC2 accounting for 37.81% and 20.69% of the variability (Figure 2a). The same behaviour was evident in PCA loading plots of *E. coli* (not shown) and *S. enteritidis* (Figure 2b). However, for *E. coli*, PC1 accounted for 34.40% of the variability, and PC2 for 23.89% while for *S. enteritidis* PC1 and PC2 accounted for 50.10% and 20.80% of the variability, respectively.

The projection of the variables on the factor planes showed that molecules belonging to pyrazine class, saturated and unsaturated aldehydes, and alcohols were the molecules significantly affecting the clustering of the *Listeria monocytogenes* samples along PC1 and PC2 (data not shown). The same class of molecules affected the clustering of the *E. coli* samples along PC1 and PC2 (data not shown). By contrast, the clustering of *S. enteritidis* was mainly affected by esters and alcohols (data not shown).

4. Discussion

Microbial cells have adopted proficient defence systems to survive a variety of physicochemical adverse conditions and to adapt to environmental stresses. Particularly essential for bacterial cells is to retain integrity and functionality of the membrane in response to environmental stresses. In the presence of stresses, microbial cells can respond by modulating the ratio of saturated to unsaturated FA, *cis* to *trans* unsaturation, branched to unbranched structure and type of branching and acyl chain length. The modulation mechanisms are different depending on the species, the strains and the physiological state of the cells (Patrignani et al., 2008).

In the considered microorganisms a uniform response was not recognised in membrane fatty acid composition modulation in response to the chemical stresses applied. On the other hand the literature reports species or strain-dependent response mechanisms (Tabanelli et al., 2013). However, a general increase of the UL in the presence of the tested antimicrobials was observed independently of the species. The increase of UL was determined mainly by the increase of C16:1*cis*, C18:1*cis*9 and C18:1*trans*9. and sometimes C18:2.

The crucial role of unsaturated FAs has been reported by several works and in response to several different stresses, including low or high growth temperatures, oxidative stress, acid stress and ethanol and salt addition stress and high pressure homogenization (Wu et al., 2012). Patrignani et al. (2008) demonstrated the key role of C18:1 and C18:2 in the resistance of some pathogenic species to antimicrobials, such as hexanal and *trans*-2-hexenal. Di Pasqua et al., (2006) detected an increase of some UFAs and of the membrane fluidity in *E. coli* and *B. thermosphacta* grown in the presence of sub-lethal concentrations of thymol, limonene, carvacrol, eugenol and cinnamaldehyde.

Also the increase of *trans* isomers, observed both in the Gram positive and Gram negative strains considered, is reported to play a key role in the microbial resistance to chemical stresses (Härtig et al., 2005). Also Patrignani et al., (2008) showed the increase of C18:1*trans*9 both in *E. coli* and *S.*

enteritidis cells as a response to ethanol, hexanal and *trans*-2-hexenal supplemented in the growth media. In fact, the isomerisation of double bonds is reported to confer chemical stability and protection to the membrane against toxic compounds (Härtig et al., 2005). Isomerisation of membrane FA, in addition to cyclisation, was shown to be the main response mechanism of *Escherichia coli* to acidic growth conditions (Gianotti et al., 2009).

The data obtained showed that also cyclopropanic fatty acids (CFA) were subjected to significant changes in the two Gram-negative bacteria in relation to the added antimicrobials. In the major part of the samples supplemented with natural antimicrobials the reduction of CFA relative percentages was observed with the exception of *E. coli* cells grown in the presence of thyme (showing a slight increase of C17cyc and C19 cyc relative percentages). In fact, also the marked increase of C12cyc observed in *S. enteritidis* and *E. coli* grown in the presence of sub-lethal concentration of oregano was counteracted by the marked reductions of C17cyc and C19cyc. Also Patrignani et al., (2008) showed a general reduction of CFA in *E. coli* and *S. enteritidis*, grown in the presence of sub-lethal concentrations of hexanal and *trans*-2-hexenal. It is well documented in the literature that the modulation of the synthesis of CFA is one of the main responses of Gram-negative bacteria to adverse environmental conditions (Yuk & Marshall 2004). The literature data show that they play a key role also in the stress response mechanisms of several lactic acid bacteria (Montanari et al., 2010). Other authors (Di Pasqua et al., 2006) have reported that increased fatty acid length is another important membrane modification for survival in adverse environments, such as acidic conditions or in the presence of antimicrobial compounds. Also under our experimental conditions, an increase of the CL in all the treated samples of *E. coli*, *S. enteritidis* and *L. monocytogenes* strains was observed. These FA modifications probably compensated and overcame the fluidising effect of the increase of unsaturation level. It is well known that the ability to maintain the proper membrane fluidity in response to changing environmental conditions is fundamental for cell survival and adaptation (Di Pasqua et al., 2006). The only exceptions

to the general increase of CL were represented by the cells of *S. enteritidis* supplemented with oregano, and *L. monocytogenes* supplemented with ethanol or thymol at different concentrations. However, *Salmonella enteritidis* cells supplemented with oregano showed, compared to the other treated samples, the lowest concentration of *trans*-isomers associated with the highest unsaturation level. In this case the fluidising effects of the increase of UL was mainly compensated by the *trans*-UFA increase. In fact, *trans*-UFA with their long linear structure behave more like saturated FA, taking up less volume and creating a more ordered membrane (Guerzoni et al., 2001). These samples evidenced also high levels of C12 cyc. The literature data concerning the role of cyclic acid in membrane fluidity are quite contrasting. In fact, some authors attributed to the presence of a cyclopropane ring within membrane FA an increase of stability of the structural and dynamic properties of biological membrane and a decrease of fluidity. On the contrary, other authors reported that cyclopropane fatty acids confer fluidity upon the cell membrane and assist in tolerance towards disturbance factors (Gianotti et al., 2009).

L. monocytogenes supplemented with ethanol or with different concentration of thymol probably guaranteed the proper membrane fluidity also reducing CL. Also concerning the FFA, the decrease of chain length associated with the increase of UL was pinpointed, with few exceptions, as a common mechanism independent of the species and natural antimicrobials. These data were in agreement with previous literature reports that attributed these phenomena with the accumulation of the precursors of some membrane-associated FAs, due to interruption of the corresponding biosynthetic pathways in the cells exposed to several stress conditions (Montanari et al., 2010).

Also the SPME-GC-MS analyses showed that growth media supplementation with natural antimicrobials modified significantly the volatile molecule profiles of *L. monocytogenes*, *E. coli* and *S. enteritidis* with a high production of detoxification products deriving from the natural antimicrobial used. In fact, in cells grown in the presence of citral and *trans*-2-hexenal, an increase of nerol, geraniol,

trans-2-hexen-1-ol, and *cis*-2-hexen-1-ol were observed. These data are in agreement with those previously reported by other authors (Patrignani et al., 2013) that attributed the dramatic increase of the abovementioned alcohols to detoxification of the added antimicrobials as well as of aldehydes, that are regarded as end products of the breakdown of peroxidated unsaturated fatty acids formed after oxidative stress (Patrignani et al., 2008) induced by the adopted growth conditions. In fact, stress conditions are reported to result in an oxidative stress for the cell, due to an imbalance that occurs when the survival mechanisms are unable to deal adequately with the reactive oxygen species in the cells. On the other hand the PCA analysis performed on the volatile molecule profiles without the added antimicrobials and their detoxification products allowed the recognition of some common response mechanism. In fact, although specific volatile profiles were recorded in relation to chemical stress applied and target microorganism, an increase of aldehydes and alcohols was observed in the volatilome of all the considered strains grown in the presence of the test antimicrobials. *S. enteritidis* volatilome showed also the increase of esters of short-chain FAs in response to the chemical stresses applied. On the other hand, saturated and unsaturated aldehydes, lactones, alcohols, medium chain FAs and their esters, belonging to the oxylipin family, are regarded as UFA oxidation products, whose accumulation in plants, fungi and some bacteria is associated with stress conditions (Montanari et al., 2013).

The PCA analyses of *L. monocytogenes* and *E. coli* volatilome showed that several pyrazines had significant modification in relation to the chemical stresses applied. Pyrazines are heterocyclic nitrogen-containing compounds found mainly in processed food, where they are created chemically in a dry heating process. Some microorganisms are known to produce pyrazines during their primary or secondary metabolism. Some pyrazines exhibit bactericidal or chemoprotective activities and others are involved in microbial cell–cell communication in soil (Bechara et al., 2007). In addition Larroche et al., (1999) obtained increased alkylpyrazine synthesis in *B. subtilis* through abnormal cultivation

conditions. These authors attribute the pyrazine overproduction to the metabolic overflow resulting from the stress conditions applied.

In conclusion, the findings of this work can contribute to the comprehension of the stress response mechanisms used by different foodborne microorganisms in relation to their exposure to sub-lethal concentrations of the tested natural antimicrobials. Since the application of new antimicrobials is subordinate to the comprehension of their mechanisms of action, the data obtained can favour the exploitation at industrial level of these antimicrobials, as required by consumers interested in a reduction in traditional preservatives.

Figure captions

Figure 1. Principal component analysis loading plot of fatty acid composition of late exponential phase cells of *S. enteritidis* (a) and *E. coli* (b) in relation to the stress condition applied.

Figure 2. Principal component analysis loading plot of volatile molecules of *L. monocytogenes* (a) and *S. enteritidis* (b) in relation to the stress condition applied.

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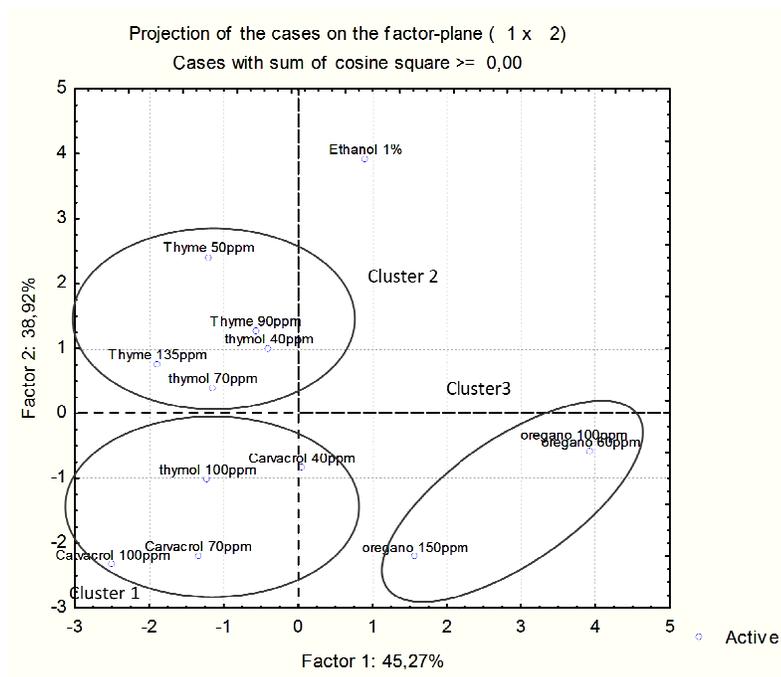


Figure 1a

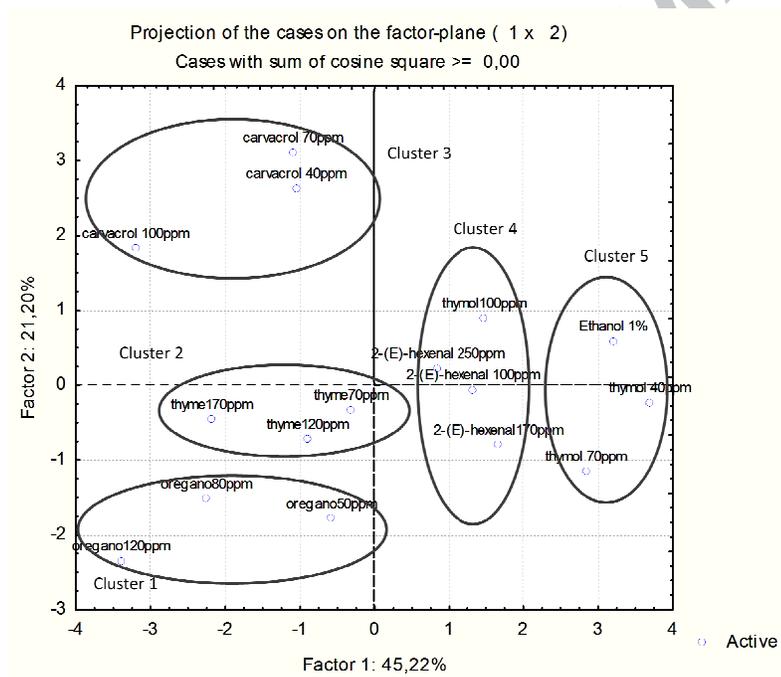


Figure 1b

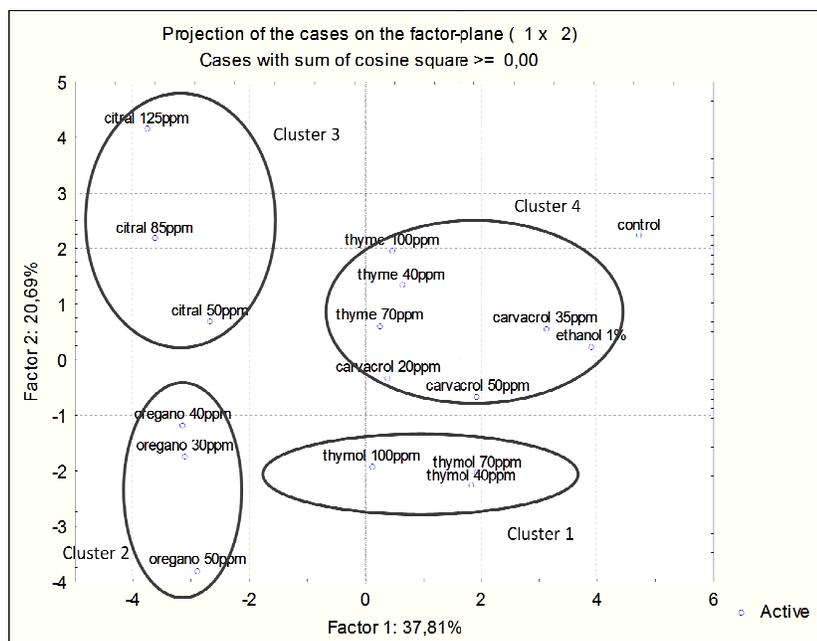


Figure 2a

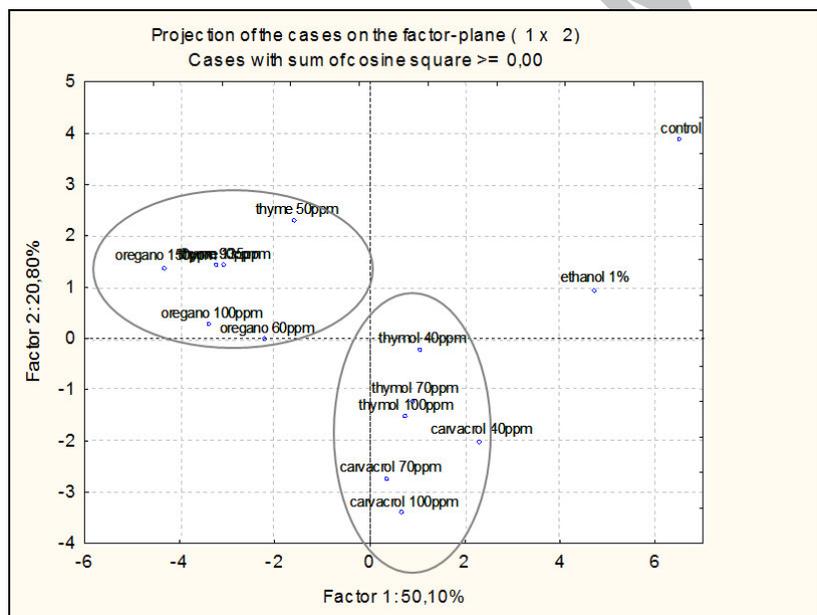


Figure 2b

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)	<i>trans</i> -2-hexenal (mg/kg)
<i>E. coli</i>	50 (10.5)* 80 (11)* 120 (14)*	70 (11)* 120 (11)* 170 (12)*	40 (11)* 70 (11)* 100 (11.5)*	40 (11)* 70 (11)* 100 (11)*		100 (13)* 170 (22)* 250 (44)*
<i>L. monocytogenes</i>	30 (30)* 40 (34)* 50 (37)*	40 (38)* 70 (50)* 100 (55)*	40 (24)* 70 (27)* 100 (29)*	20 (23)* 35 (24)* 50 (24)*	50 (24)* 85 (27)* 125 (31)*	
<i>S. enteritidis</i>	60 (10)* 100 (10.5)* 150 (13)*	50 (10)* 90 (10.5)* 135 (11)*	40 (9.5)* 70 (10)* 100 (10)*	40 (9.5)* 70 (10)* 100 (10)*		

*Within the brackets, the times (in hours) at which the cells were collected for fatty acid analyses.

The time of collection was established on the basis of Gompertz equation modified according to Zwietering et al. (1990).

Table 2. Membrane fatty acid composition of *L. monocytogenes* Scott A in relation to the stress condition applied

	Total Fatty Acids (%)													UL*	CL**	
	C10:0	C12:0	C14:1cis11	C14:0	C15 iso	C15 ante	C16 iso	C16:1cis9	C16:0	C17 iso	C17 ante	C18:2 (cis-cis)	C18:1trans9			C18:0
control	0.1	1.2	0.0	2.5	16.2	28.6	4.3	0.4	10.1	7.3	16.6	0.6	3.9	5.8	0.06	1587
ethanol 1%	0.1	0.7	0.2	3.2	17.7	28.6	9.1	0.5	9.6	5.6	14.7	0.5	3.0	2.2	0.05	1569
carvacrol 20ppm	0.0	1.3	0.5	4.5	16.0	22.2	5.4	0.5	16.7	4.8	9.1	0.5	9.7	4.3	0.12	1585
carvacrol 35ppm	0.1	1.3	0.4	3.9	13.3	19.7	3.3	1.8	19.8	3.3	6.6	0.6	13.0	8.4	0.18	1603
carvacrol 50ppm	0.0	0.3	0.7	2.8	14.2	20.3	4.5	1.1	18.4	6.2	8.2	2.4	11.3	5.8	0.18	1605
citral 50ppm	0.0	1.6	0.4	2.6	17.1	25.5	7.0	0.5	11.1	6.0	15.4	0.7	4.3	4.2	0.07	1579
citral 85ppm	0.1	0.7	0.3	2.5	14.8	24.1	7.2	0.9	15.3	5.4	11.6	1.1	6.0	5.7	0.10	1591
citral 125ppm	0.1	1.0	0.4	3.6	16.4	23.7	4.8	0.6	15.0	4.8	10.2	3.7	6.5	5.4	0.16	1589
oregano 30ppm	0.1	0.5	0.6	3.2	8.3	11.4	3.0	4.1	23.8	3.5	5.9	1.7	13.8	14.0	0.24	1638
oregano 40ppm	1.0	1.1	2.0	2.9	6.6	9.5	2.5	2.5	22.4	2.4	7.9	1.8	16.3	17.9	0.25	1645
oregano 50ppm	0.0	1.1	0.2	2.9	9.0	13.7	6.4	2.4	18.9	5.2	10.0	0.5	10.7	15.8	0.15	1633
thymol 40ppm	0.1	1.3	0.1	3.4	17.4	33.6	8.1	0.1	7.8	5.8	15.4	0.2	2.8	1.2	0.04	1561
thymol 70ppm	0.0	0.4	0.1	2.5	16.8	29.7	8.8	0.3	9.2	6.3	15.4	0.6	3.2	2.5	0.06	1577
thymol 100ppm	0.1	0.4	0.2	2.6	15.1	30.1	4.3	0.2	9.7	8.8	17.6	0.8	3.9	2.1	0.06	1581
thyme 40ppm	0.6	0.2	0.7	2.6	5.3	18.9	5.0	0.6	16.2	4.7	16.4	0.7	6.1	17.8	0.11	1640
thyme 70ppm	0.0	0.2	0.1	0.6	10.9	26.7	6.5	0.1	10.3	7.9	21.9	0.1	4.1	8.3	0.05	1611
thyme 100ppm	0.5	0.3	0.7	0.6	7.8	23.1	6.1	0.2	11.1	7.1	26.0	0.5	4.8	7.9	0.08	1623

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters.

The results are means of three repetitions of three independent experiments. The coefficients of variability, expressed as the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

* Unsaturation level calculated as $[\text{percentage monoenes} + 2(\text{percentage dienes}) + 3(\text{percentage trienes})] / 100$

** Mean chain length calculated as $(\text{FAP} * \text{C})$ (where FAP is the percentage of fatty acid and C the number of carbon atoms).

1 **Table 3.** Membrane fatty acid composition of *E. coli* 555 in relation to the stress condition applied

	Total Fatty acids (%)											UL*	CL**
	C 12:0	C 14:0	C12 cyc	C 16:1cis9	C 16:0	C17 cyc	C 18:2(cis-cis)	C 18:1cis9	C 18:1trans9	C 18:0	C19 cyc		
control	8.01	8.01	7.44	0.51	37.23	12.16	1.02	4.34	1.92	5.91	9.99	0.10	157
ethanol 1%	0.22	5.22	5.77	0.98	45.86	17.01	1.26	4.99	4.22	2.84	10.30	0.13	163
oregano 50ppm	0.51	4.33	11.72	2.16	40.84	7.56	1.58	10.71	6.15	5.64	3.80	0.2	1606
oregano 80ppm	0.22	3.90	10.91	5.27	42.10	6.45	1.48	11.80	8.61	5.33	2.09	0.3	1615
oregano 120ppm	1.23	4.07	16.45	1.97	31.96	1.84	2.82	19.27	6.09	11.45	1.64	0.1	1606
trans-2-hexenal 100ppm	0.20	3.33	5.02	1.69	52.91	11.50	1.17	5.87	7.04	4.47	4.88	0.1	1635
trans-2-hexenal 170ppm	0.20	2.49	6.76	0.97	53.89	7.43	1.36	6.05	3.40	11.20	4.17	0.1	1630
trans-2-hexenal 250ppm	0.40	3.30	6.49	3.42	46.65	13.81	0.64	3.50	9.37	4.58	5.87	0.1	1633
thymol 40ppm	0.12	4.40	1.23	0.36	50.64	13.41	0.34	2.99	4.71	9.68	8.34	0.0	1611
thymol 70ppm	0.92	6.60	7.66	1.71	46.60	10.88	0.65	2.68	4.41	9.68	7.50	0.1	1636
thymol 100ppm	1.50	3.08	8.07	0.69	39.23	15.87	0.91	6.60	5.16	12.15	5.90	0.2	1647
thyme 70ppm	0.09	3.28	6.19	2.76	44.72	10.22	1.01	9.73	7.35	10.57	2.57	0.2	1627
thyme 120ppm	3.69	4.82	4.86	0.56	42.00	5.00	1.58	10.67	8.71	15.77	0.00	0.3	1645
thyme 170ppm	0.39	3.65	4.52	7.36	48.49	6.17	1.29	5.52	13.89	4.84	2.23	0.2	1636
carvacrol 40ppm	1.23	1.69	3.46	0.72	34.12	11.50	2.63	17.51	4.48	12.93	5.84	0.3	1666
carvacrol 70ppm	0.86	3.15	1.76	2.30	35.62	12.84	1.50	15.60	6.89	10.68	6.45	0.3	1687
carvacrol 100ppm	0.33	2.75	6.69	5.01	31.41	10.64	2.81	11.16	13.58	9.39	4.48	0.3	1664

2

3 The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters.

4 The results are means of three repetitions of three independent experiments. The coefficients of variability, expressed as
5 the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

6 *Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100

7 ** Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon
8 atoms).

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Table 4. Membrane fatty acid composition of *S. enteritidis* E5 in relation to the stress condition

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	Total Fatty acids (%)										UL*	CL**
	C 12:0	C 14:0	C12 cyc	C 16:1 cis 9	C 16:0	C17 cyc	C 18:1 cis 9	C 18:1trans9	C 18:0	C19 cyc		
control	0.6	4.3	5.9	0.8	51.8	13.9	5.3	2.1	6.4	6.8	0.11	1630
ethanol 1%	0.87	5.52	0.04	1.68	47.88	20.67	2.48	5.21	1.75	13.05	0.10	1664

carvacrol 40ppm	0.24	6.40	1.41	2.96	52.19	7.30	2.43	13.87	7.17	4.22	0.21	1649
carvacrol 70ppm	3.46	10.21	0.52	2.44	34.60	5.21	0.00	18.12	8.16	3.70	0.32	1639
carvacrol 100ppm	0.26	2.89	1.21	6.79	49.47	6.06	4.01	18.81	4.95	3.71	0.32	1662
thyme 50ppm	0.90	4.42	0.16	2.72	34.14	20.28	6.92	9.87	3.89	13.57	0.23	1691
thyme 90ppm	0.26	4.25	0.05	3.22	47.87	14.42	1.86	11.97	3.17	8.68	0.19	1665
thyme 135ppm	0.50	3.09	0.36	5.08	43.13	15.88	4.37	12.04	2.56	9.34	0.28	1677
thymol 40ppm	0.39	5.64	0.17	2.56	51.30	11.77	3.04	11.47	2.95	8.07	0.19	1641
thymol 70ppm	0.71	4.07	0.48	2.88	46.26	10.84	3.91	14.66	4.87	9.36	0.23	1673
thymol 100ppm	1.25	5.56	0.32	3.62	49.53	7.25	3.57	16.00	5.20	4.96	0.26	1656
oregano 60ppm	0.00	3.61	14.52	1.20	48.83	6.21	4.50	4.38	11.67	4.02	0.12	1595
oregano 100ppm	0.70	6.99	18.85	2.02	44.13	8.17	3.63	5.07	5.20	2.37	0.14	1550
oregano 150ppm	0.18	2.29	13.89	6.73	43.78	7.97	1.51	10.60	7.41	2.14	0.20	1560

18

19 The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters.

20 The results are means of three repetitions of three independent experiments. The coefficients of variability, expressed as
21 the percentages ratios between the standard deviations and the mean values, ranged between 2 and 5%.

22 *Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100

23 **Mean chain length calculated as (FAP*C) (where FAP is the percentage of fatty acid and C the number of carbon
24 atoms).

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27 **Highlights**

28 Natural antimicrobials induced marked changes in pathogen membrane fatty acid profiles

29 Natural antimicrobials induced marked changes in pathogen volatile molecule profiles

30 The data contributed to the comprehension of the pathogen stress response mechanisms

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ACCEPTED MANUSCRIPT