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Action mechanisms of natural antimicrobials against food-borne pathogenes

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Abstract

Consumer demand for minimally processed, and ready-to-eat foods with a reduced content of synthetic preservatives has stimulated the research of alternative preservation strategies. Essential oils (EOs) or their components represent one of the most promising natural feasible alternatives to improve food safety, shelf-life and quality. Although their antimicrobial properties are well documented few and fragmented are the information about their mechanisms of action, cellular targets and on the stress response strategies of microorganisms after the exposure to such compounds. In this framework, the main aim of the PhD project was to investigate on the effects of one hour exposure to sublethal concentrations of selected natural antimicrobials, such as citral, carvacrol, (E)-2-hexenal and thyme EO, on the food-borne pathogens *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655. The action mechanisms of the natural antimicrobials and the cellular targets were studied through multiple approaches able to give information on cell morphological, physiological, tracriptome and proteome changes. In particular, the transcriptome of *L. monocytogenes* Scott A was studied by RT-qPCR on a pool of gene representative of different metabolisms: energetic, ferric uptake, stress response, gene transcription, cell division, virulence, motility, while the proteome effects were determined by bi-dimensional electrophoresis (2DE). By contrast, the transcriptome changes on *Escherichia coli* K12 MG1655 were evaluated using the microarray technology. In addition, for both the microbial strains the effects on the membrane fatty acid profiles were studied using GC/MS approach while single cell responses to the one hour exposure to natural antimicrobials of the whole populations were studied by flow cytometry. Finally the antimicrobial effect of (E)-2-hexenal, in combination with high pressure homogenization or traditional thermal treatments was verified in a real food system, i.e. apple juice, deliberately inoculated with spoilage and pathogenic microorganisms including pathogens *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655.

The results obtained allowed to define for each strain and each antimicrobial the cell targets and the response mechanism, respectively. The use of the different multi-parametric approaches provided useful information on citral, carvacrol, (E)-2-hexenal and thyme EO action mechanisms on microbial cell targets as well as to elucidate the behavior and the stress response strategies used by *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655 after the one hour exposure to such natural antimicrobials. The validation in apple juice allowed to understand the

real potential of one of the antimicrobials (chosen on the basis of its sensory compatibility with the food matrix) to improve food safety and shelf life. The data obtained can speed up the explotation at industrial level of natural antimicrobials as alternative food preservatives.

Essential oils

Essential oils (EOs), in agreement with the European Pharmacopoeia, are defined as: ‘Odorous products, usually of complex compositions, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating’ (EDQM, 2008). All plants are able to produce volatile compounds, but the production of essential oils belongs only to specific plant families. Produced as secondary metabolites, they are ubiquitously distributed in the whole plant and for this reason they can be extracted and isolated from different organs like roots, stems, seeds, flowers, buds, leaves, wood, twigs, fruits or bark (Franz and Novak, 2009). EOs are involved in different physico-chemical processes like cell to cell communication, pollination, defense against insects, herbivores and volatiles. Until now, about 3000 EOs are known, of which about 300 have a commercial interest, especially for pharmaceutical, agricultural, food, health, cosmetics and perfumes. They are generally recognized as safe (GRAS) by different international food authorities such as EFSA, FDA and FSCJ (Newberne et al., 2000;Patrignani et al., 2015).

1.1 Extraction of Essential Oils

EOs are usually extracted from plants through several different methods, including steam (Perineau et al., 1992;Reverchon and Senatore, 1992;Babu and Kaul, 2005;Masango, 2005), hydro-distillation (Perineau et al., 1992;Golmakani and Rezaei, 2008) or also, solvent extraction (Areias et al., 2000;Pizzale et al., 2002;Koşar et al., 2005) and in the last years, supercritical carbon dioxide (Hawthorne et al., 1993; Jimenez-Carmona et al., 1999; Senorans et al., 2000;Deng et al., 2005; Gironi and Maschietti, 2008). The method of extraction depends on the use of the oil. For pharmaceutical and food purposes, the extraction by steam distillation is preferred, whereas for other uses extraction with lipophilic solvents or supercritical carbon dioxide is favored. The extraction method is one of the main factor that determines the quality of EOs. Inappropriate extraction procedure can lead to the damage or alter the action of chemical signature of essential oil (Tongnuanchan and Benjakul, 2014).

1.2 Chemistry of Essential Oils

Essential oils (EOs) are complex natural mixtures which can contain about 20–60 components at different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) that mainly contribute to the essential oil biological properties (Bakkali et al., 2008). For example, the major components of *Origanum compactum* are thymol (27%) and carvacrol (30%), while linalool is the major component (68%) of the *Coriandrum sativum. Cinnamomum camphora* essential oil is mainly characterized by 1,8-cineole (50%), while- menthol (59%) and menthone (19%) are the main components of Mentha piperita. Essential oil components belong to three different low molecular weight groups (Betts, 2001; Bowles, 2003;Pichersky et al., 2006):

- terpenes;
- terpenoids;
- aromatic compounds.

1.2.1 Terpenes

Concerning EOs, terpenes represent the most important group of natural products. They are defined as chemicals composed by isoprene (2-methylbutadiene) units, formed by a unidirectional coupling process named head-to-tail coupling. The branched end of the chain is referred to as the head of the molecule while the other is the tail. Although isoprene back bone is easily recognized in the molecular terpenoid structure, it is not an intermediate in the biosynthesis. Terpenoid bio-synthesis starts with the mevalonic acid, which is made from three molecules of acetyl CoA. After the phosphorylation, followed by elimination of the tertiary alcohol and concomitant decarboxylation of the adjacent acid group, mevalonic acid is converted into the isopentenyl pyrophosphate (IPP). The head-to-tail coupling of two 5-carbon units of IPP gives a 10-carbon unit, geranyl pyrophosphate, and further additions of isopentenyl pyrophosphate lead to 15-, 20-, 25-, and so on carbon units backbone. Secondary enzymatic redox reaction of the skeleton attributes specific and heterogeneous functional properties to terpenes. On the base of the number of isoprenil units (and carbon atoms), terpenes are classified as:

- Hemiterpenoids (C5)

- Monoterpenes (10C);
- Sesquiterpenes (15C);
- Diterpenoids (20C);
- Triterpenoids (30C);
- Tetraterpenoids (40C);

Only hemiterpenoids, monoterpenes, and sesquiterpenes are sufficiently volatile to be components of EOs. Monoterpenes are the most representative molecules constituting 90% of the EOs and allow a great variety of structures, like: alcohols, aldehydes, ketones, esters, ethers, peroxides, phenols (Bakkali et al., 2008). Examples of terpenes include cymene (p-cymene), limonene, terpinene, citral (mix of two isomers of the same aldehyde geranal and neral) (Figure 1).

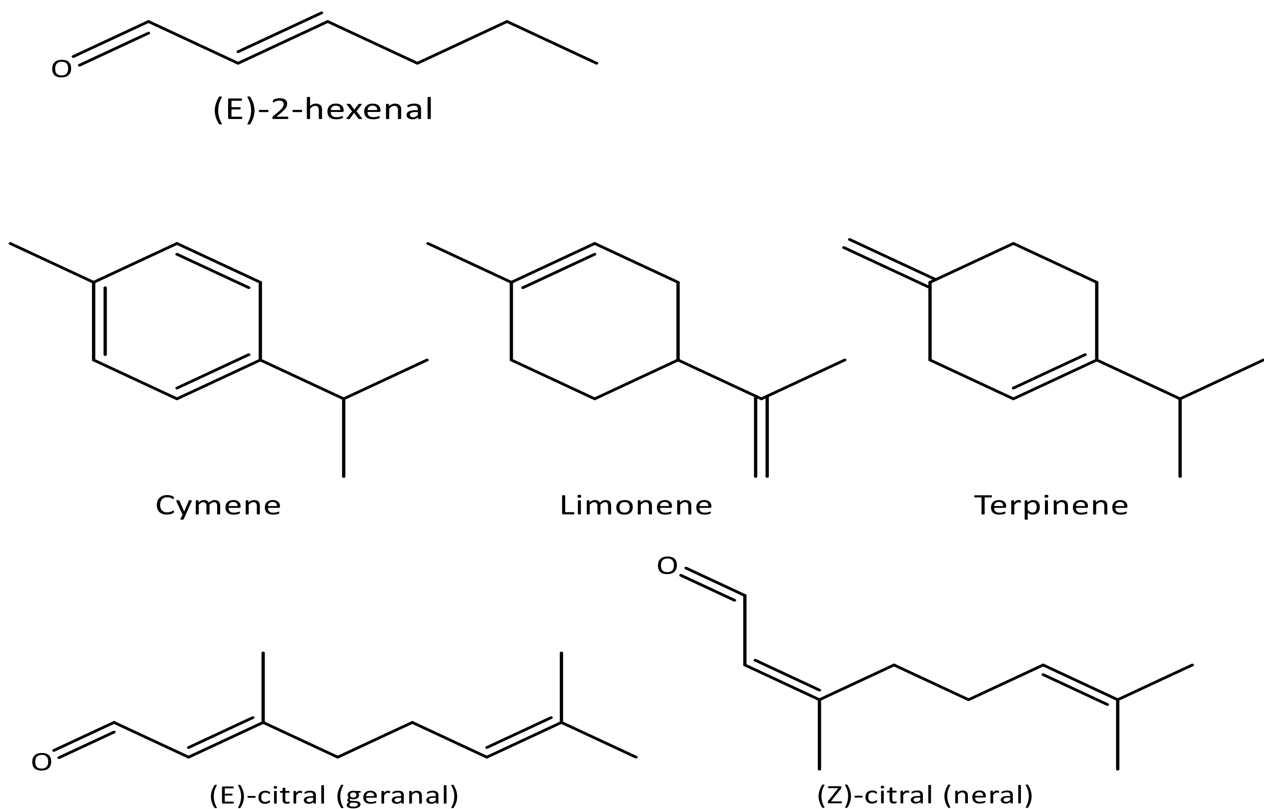


Figure 1: Chemical structure of some hemiterpenes (E)-2-hexenal and monoterpenes cymene, limonene, terpinene and citral (both isomeric forms).

When the terpene molecule is optically active, the racemic form is the most frequently encountered, for example (±)-citronellol is widespread, but the form (+) is characteristic of *Eucalyptus citriodora*, while the form (-) is common to the rose and geranium EOs (Bakkali et al.,

2008). In some cases, the biosynthesis process is stereochemical selective and plants accumulate only one specific enantiomer: (+)-α-pinene from *Pinus palustris*, (-)-β-pinene from *Pinus caribaea*, (-)-linalol from coriander, (+)-linalol from some camphor trees (Bakkali et al., 2008). The sesquiterpenes are formed from the assembly of three isoprene units (C15). The extension of the chain inc reases the number of cyclisation which allows a great variety of structures (Figure 2). This also results in a lower volatility and enhanced boiling point than monoterpenoids. Therefore, few of them (in percentage) contribute to the odor of EOs, but those that do often have low-odor thresholds and contribute significantly as endnotes. They are also important as fixatives for more volatile components (Sell, 2015).

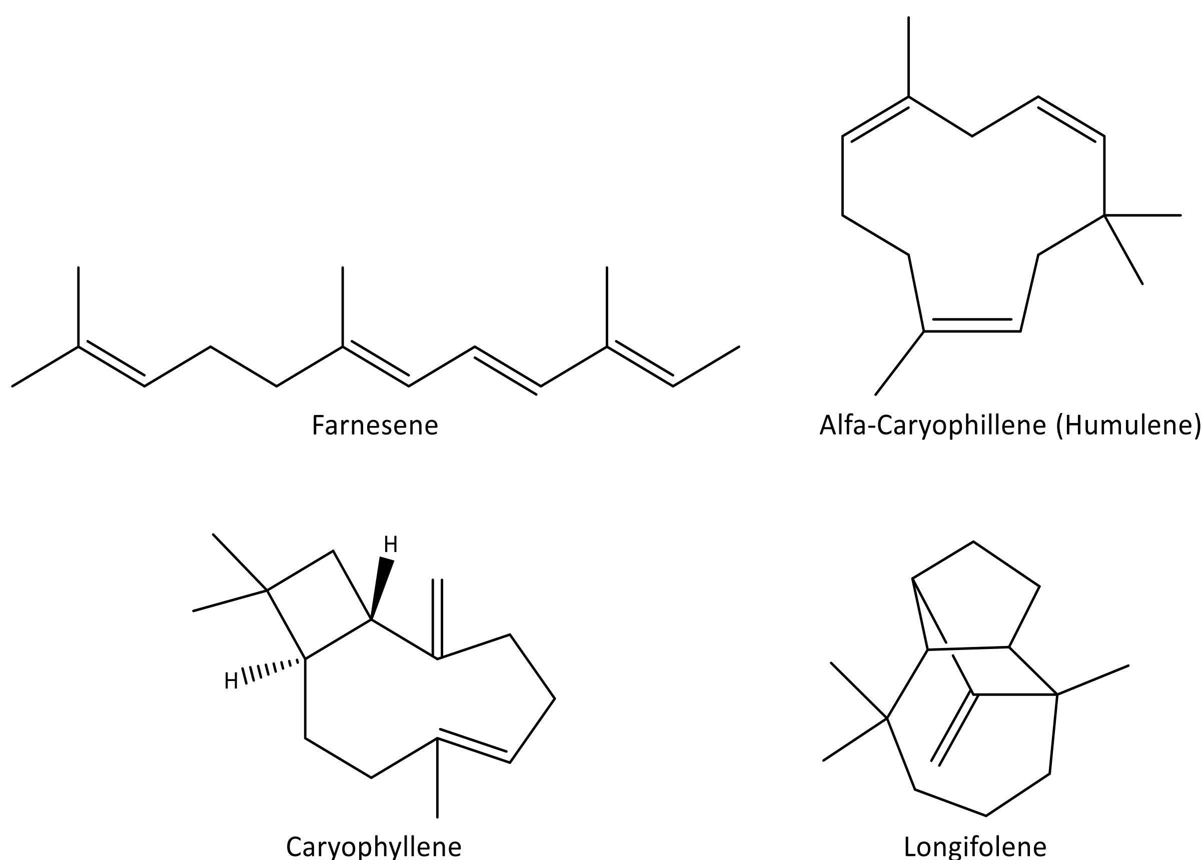


Figure 2: Examples of acyclic (farnesene), monocyclic (humulene), bicyclic (caryophyllene) and tricyclic (longifolene) sesquiterpenes.

1.2.2 Terpenoids

Terpenoids are terpenes that undergo to biochemical modifications via enzymes that add oxygen molecules and move or remove methyl groups (Caballero et al., 2003). Depending on their chemical modifications, they are subdivided into alcohols, esters, aldehydes, ketones, ethers,

phenols, and epoxides. Examples of terpenoids are thymol, carvacrol, linalool, citronellal, piperitone, menthol, and geraniol (Figure 3).

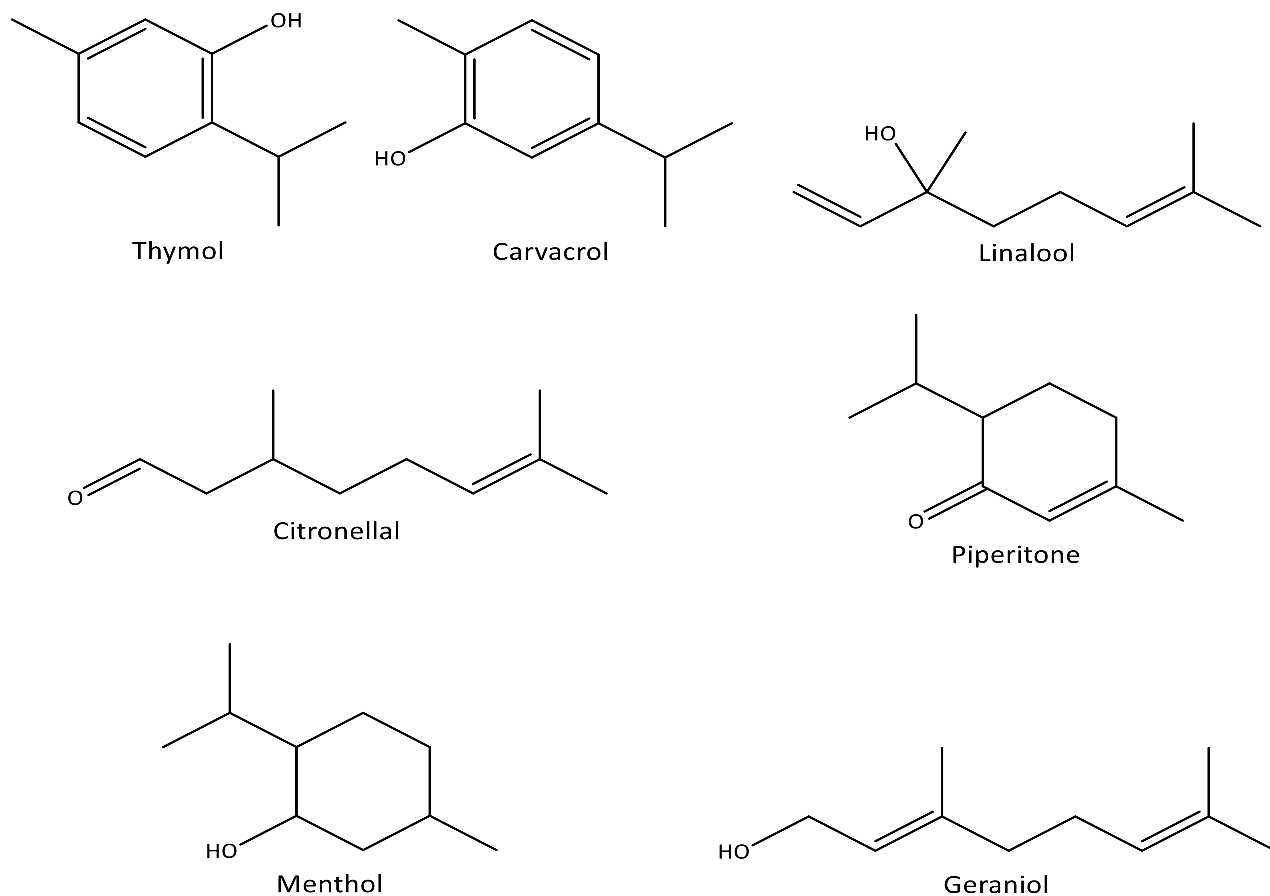


Figure 3: Chemical structure of some selected terpenoids.

1.2.3 Aromatic compounds

Derived from phenylpropane, the aromatic compounds occur less frequently than the terpenes. Their biosynthetic pathways are generally separated in plats but in some cases they coexist with one major pathway taking over. An example is represented by the cinnamon EOs with the aromatic compound cinnamaldehyde as major and the terpene eugenol as the minor constituent (Bakkali et al., 2008).

1.3 Essential oil antimicrobial properties

Essential oil (EO) antimicrobial properties against pathogenic microorganisms are related to their physiochemical features (Dorman and Deans, 2000;Delaquis et al., 2002a). Their antimicrobial properties have been studied by several authors (Juven et al., 1994; Basilico and Basilico, 1999;

Lambert et al., 2001; Patrignani et al., 2015;Siroli et al., 2015). Although aromatic compounds are structurally heterogeneous, they possess similar physical properties. The physic-chemical properties able to affect the antimicrobial activity of EOs are the solubility and volatility. The solubility of these molecules is strictly related to the length of the carbon atom chain. As the number of carbon atoms rises, the solubility in polar solvent decreases while the solubility in apolar solvent increases. For linear aldehydes, the water solubility decreases with the increasing of the length of the carbon chain. The same relation can be applied for 1 to 9 carbon atom alcohols, methyl-esters and ketones. Lipophilicity is important for the biological activity of volatile organic compounds. In fact, the solubility in fats enables to volatile molecules, to permeate in cytoplasmic membranes and the waxy cuticle. Volatility, described as the tendency of the molecules to pass from the liquid phase to the vapor one, is described by the partition coefficient expressed as the ratio at equilibrium and constant temperature between the amount of volatile compound dissolved in one milliliter of air and the amount dissolved in one milliliter of water. This relation is valid only for pure aqueous solutions below the saturation point. Volatility in water is higher for ketones, esters and aldehydes compared to alcohols. Addition of other solutes such as salts or sugars considerably changes the volatility. Under the same conditions of temperature and concentration, the vapor pressure of a solute depends on the water activity of the system and on the concentration and nature of the other solutes (Guerzoni et al., 1994). The compounds in the gaseous phase accumulate rapidly in the cytoplasmic membranes of cells acceptor than they do if solubilized in the carrier. Reached the cytoplasm there are not physiological differences between volatile and non-volatile compounds (Guerzoni et al., 1994).

EOs and their constituents have large differences in the antimicrobial activities. The most used tests to assess EOs ability to injury microbial cells are the disk diffusion (Farag et al., 1989;Cimanga et al., 2002; Packiyasothy and Kyle, 2002; Wilkinson et al., 2003;Burt, 2004;Bakkali et al., 2008;Faleiro, 2011), agar wells method (Dorman and Deans, 2000), agar dilution (Hammer et al., 1999) and the broth dilution one (Lambert et al., 2001;Delaquis et al., 2002b;Smith-Palmer et al., 2002;Ultee et al., 2002;Burt, 2004). In broth dilution studies, the end point is determined using photometric methods (optical density) and the total viable count (Burt, 2004). Information about the antimicrobial activity are also obtained using scanning electron microscopy (SEM), for the evaluation of cell wall and membrane morphological changes (Lambert et al., 2001;Burt and Reinders, 2003), and time-kill trials for the kinetical evaluation of antimicrobial properties (Tassou et al., 1995;Ultee et al., 2002). Although different techniques can be applied for the study of EO

antimicrobial properties, there is not a standardization (Burt, 2004). In fact, the antimicrobial activity is affected by some factors such as the method used to extract the EO from plant material, the volume of inoculum, growth phase, culture medium used, pH of the media and incubation time and temperature (Burt, 2004;Lanciotti et al., 2004). Moreover, a solvent has to be used to vehicular the EOs and dissolve them in a water solution and for this purpose several solvents have been used: ethanol, methanol, Tween-20, Tween-80, acetone in combination with Tween-80, polyethylene glycol, propylene glycol, n-hexane, dimethyl sulfoxide and agar (Burt, 2004). The solvent used as carrier for EOs strongly affects the antimicrobial activity (Burt, 2004). Despite many Authors have highlighted the antimicrobial properties of EOs and their components in the past (Holley and Patel, 2005), their mechanisms of action and their cellular targets are not fully understood (Nazzaro et al., 2013). In fact, the high heterogeneity of the chemicals compounds present in the EOs makes difficult to define a univocal mechanism of action and targets in the microbial cells. Consequently, the chemical structure of the individual compounds present in the EOs affects their precise mode of action and their antibacterial activity (Viuda-Martos et al., 2008;Picone et al., 2013). However, many studies indicate the cell membrane as the primary target of bioactive aromatic compounds (Burt, 2004) causing different effects on the cells physiology.

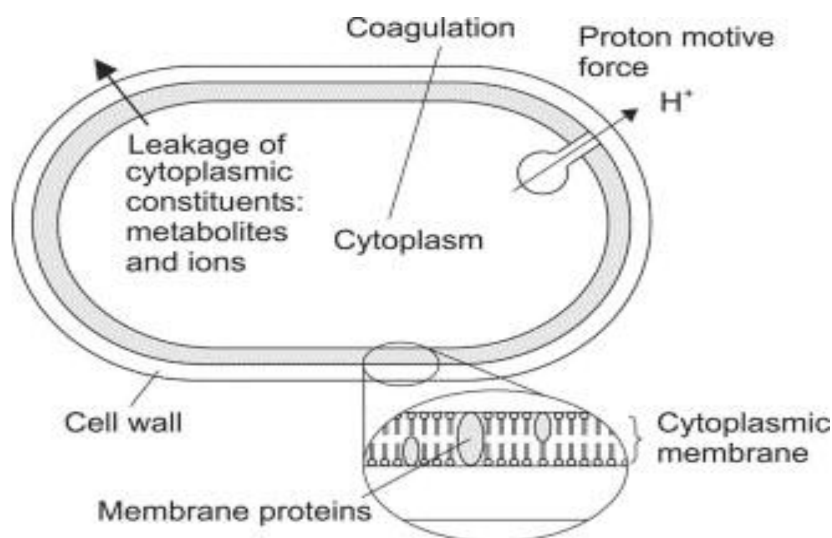


Figure 4: Locations and mechanisms in the bacterial cell thought to be sites of action for EO components(Burt, 2004): degradation of the cell wall (Helander et al., 1998); damage to cytoplasmic membrane (Sikkema et al., 1994;Ultee et al., 2000;Ultee et al., 2002); damage to membrane proteins (Juven et al., 1994;Ultee et al., 1999); leakage of cell contents (Cox et al., 1998;Gustafson et al., 1998;Helander et al., 1998;Lambert et al., 2001); coagulation of cytoplasm (Gustafson et al., 1998) and depletion of the proton motive force (Ultee et al., 1999;Ultee et al., 2000;Ultee et al., 2002).

As shown in Figure 4, the damages to the cell wall and in the membranes may lead to loss of macromolecules up to cell lysis. In particular, the loss of specific ions (Cox et al., 1998;Gustafson et al., 1998; Helander et al., 1998; Lambert et al., 2001), due to the action of the aromatic molecules on the cell membrane, has dramatic effects on the proton motive force, by decreasing the content of intracellular ATP (Ultee et al., 1999;Ultee et al., 2000;Ultee et al., 2002). Viuda-Martos et al., (2008) suggested that components of the EOs cross the cell membrane, interacting with the enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately, their death. In addition EOs can also coagulate the cytoplasm (Gustafson et al., 1998;Picone et al., 2013) and cause damage to lipids and proteins (Juven et al., 1994;Ultee et al., 1999). Cristani et al., 2007 reported that the antimicrobial activity of terpenes is related to their ability to affect not only permeability but also other functions of cell membranes. These compounds might cross the cell membranes, penetrating into the interior of the cell and interacting with critical intracellular sites.

The antimicrobial properties of EOs is mainly attributed to their lipophilic constituents. Different aromatic compounds of EOs such as, cyclic monoterpenes are characterized by a high hydrophobicity allowing them to have a good partition coefficient in the lipids promoting their diffusion among the cytoplasmic lipidic bilayer (Patrignani et al., 2015). As described from different authors cyclic monoterpenes increase in fluidity and permeability of the membrane, which leads to an inhibition of membrane enzymes (Nazzaro et al., 2013) and ultimately to the cell membrane disruption (Lanciotti et al., 2004; Holley and Patel, 2005; Viuda-Martos et al., 2008;Liolios et al., 2009;Nazzaro et al., 2013).

The antimicrobial properties of many EOs also appears to be connected with the presence of phenolic compounds. Various studies, concerning oregano species have shown that their oils possess strong antimicrobial activity; this activity could be attributed to their high percentage of phenolic compounds and, specifically, carvacrol, thymol, p-cymene and their precursor c-terpinene (Liolios et al., 2009).

1.4 Applicative potential of essential oils and their components

Despite the strong antimicrobial activity against food-borne pathogens and spoilage microorganisms shown by EOs (Tassou et al., 2000;Oussalah et al., 2007), their practical

application in food industry is currently limited due to their strong impact and changes they cause in food products (Gutierrez et al., 2009). Moreover, the limited use is due to i) the variability of the composition of EOs (due to the geographic origin, agricultural techniques, season, methods of extraction, etc.) able to influence their effective overall antimicrobial activity (Burt, 2004); ii) the interaction of bioactive molecules with the food matrix (in particular with proteins, lipids, starch, etc.) limiting the contact of these molecules with the microbial cells, thereby reducing the effects on cell viability (Gutierrez et al., 2009); iii) the lack of knowledge of the interaction between technological and composition parameters and their activity; iv) the lack of knowledge of the mechanisms by which these molecules exert their antimicrobial activity. In fact, their use in food industry can not disregard from the knowledge of their mechanisms of action. In this perspective, the main aim of my PhD thesis will we to elucidate some of these mechanisms for *L. monocytogenes* and *E.coli* when exposed to some natural antimicrobial compounds and EOs, in order to favour a possible application in foods.

Among EOs and natural antimicrobials, the most investigated are citral, hexanal, E-2-hexenal, oregano and thyme essential oils, due to their potential applications as natural antimicrobials in minimally processed fruits and vegetables and beverages.

1.4.1 Citral

A wide literature shows the great potentilas antimicrobials in model and food systems of EOs deriving from citrus fruit peels (Espina et al., 2011;Settanni et al., 2012). In particular, citral (3,7-dimethyl-2-7-octadienal), is an acyclic unsaturated monoterpene aldehyde found naturally in the volatile oils of citrus fruits, lemongrass, and other herbs and spices. It consists of a mixture of two isomers, geranial and neral, and is used for flavouring citrus-based beverages. Its antimicrobial properties and pleasant fruity scent could make citral a suitable antimicrobial ingredient for wider use in the food industry (Somolinos et al., 2008) (Figure 5).

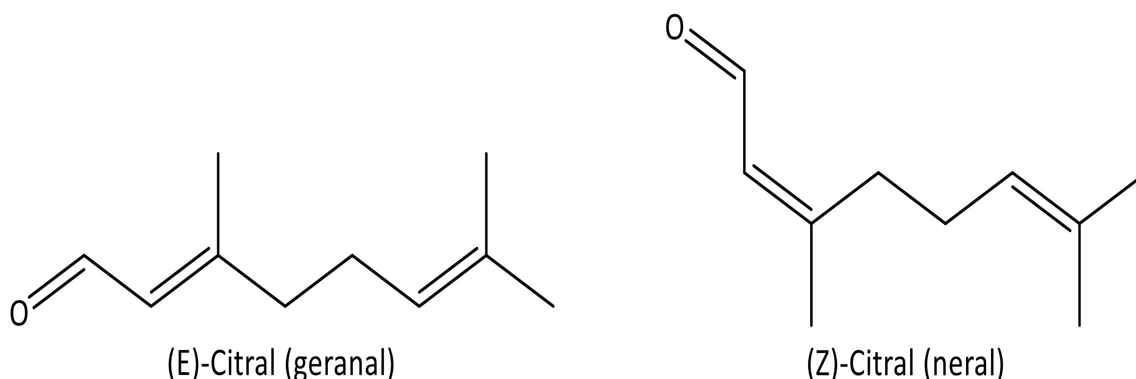


Figure 5: Chemical structure of some citral (both isomeric forms)

Citral its commonly founded in leaves and fruits of several plant species including myrtle trees, lemons, limes, lemongrass, oranges and bergamots. Because its use does not present risk to the health of the consumer (Burt, 2004), it is consider as a generally recognized as safe (GRAS) additive from different international food authority like Food and Drug Administration and European Food Safety Authority. For this reason is commonly used in food industries as flavor and taste enhancer of citrus-based beverages and products (Somogyi, 1996;Ress et al., 2003;Lalko and Api, 2008). The citral mechanism of growth inhibition, cell injury and inactivation is not fully understood. In general, due their hydrophobic nature, the plasma membrane is the primary site of toxic action of terpenes (Burt, 2004;Hyldgaard et al., 2012).Consisting to this hypothesis several author observed damage and permeabilization of the cell membrane after the exposure to citral on different microorganisms (Uribe et al., 1985;Williams and Barry, 1991;Cox et al., 1998;Prashar et al., 2003;Inoue et al., 2004;Somolinos et al., 2008;Park et al., 2009;Somolinos et al., 2010). As a result of the cell membrane disruption the leakage of specific ions has dramatic effects on proton motive force, the intracellular ATP content and the overall activity of microbial cells (Poolman et al., 1987;Sikkema et al., 1994;Helander et al., 1998;Lanciotti et al., 2004;Turina et al., 2006). Citral and citron EO, at concentration compatibles with sensorial features of fruits, were able to significantly prolong the shelf-life of the fruit based salads in syrup (Belletti et al., 2007), and the stability of fruit based soft drink (Belletti et al., 2007).

1.4.2 Carvacrol

Carvacrol, or cymophenol, is a monoterpenoid phenol, naturally occurring in the essential oil fraction of oregano and thyme (Figure 6).

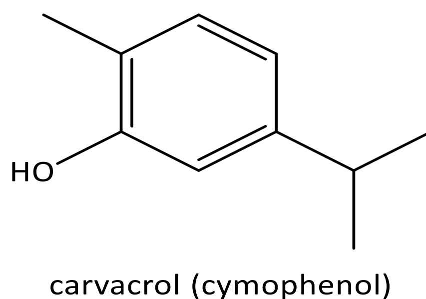


Figure 6: Chemical structure of carvacrol

The antimicrobial properties of carvacrol are related to its physio-chemical features. Carvacrol is a short hydroxylated aromatic compound able to interact with the cytoplasm membrane phospholipids and cell membrane embedded enzymes, increasing the cell membrane permeability and fluidity (Nikaido and Vaara, 1985;Nikaido, 1994). Different authors proposed that microbial species change their membrane fatty acid composition as a stress mechanism adaptation to carvacrol exposure (Ultee et al., 2000;Lambert et al., 2001; Di Pasqua et al., 2006, 2007). This hypothesis 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; Xu et al., 2008). Carvacrol hydroxyl functional group has been proposed as a transmembrane carrier of monovalent cations across the membrane, carrying H^+ into the cell cytoplasm and transporting K^+ back out (Ultee et al., 1999;Ben Arfa et al., 2006), resulting in a loss of the cell membrane potential. Cell membrane potential is fundamental for the cellular metabolic activity especially for the intracellular pH homeostasis and ATP synthesis. Carvacrol has been also proposed to interact with membrane, periplasmic and intracellular proteins (Juven et al., 1994). Only few information are available about this topic and one example of direct interaction between carvacrol and membrane components is reported by the Gill and Holley's study. In this study, carvacrol interaction with membrane components was evaluated by monitoring the cellular ATPase activity (Gill and Holley, 2006). The grow of *Escherichia coli* cells in presence of carvacrol increased the expression of the GroEL chaperon complex, indicating that protein folding was affected. Furthermore, it inhibited the synthesis of flagellin (Burt et al., 2007) causing a decrease in motility as carvacrol concentration increased. In this case, carvacrol disrupted the membrane potential and thereby the proton motive force needed to drive flagellar movement (Gabel and Berg, 2003; Burt et al., 2007; Xu et al., 2008).

1.4.3 (E)-2-hexenal

(E)-2-hexenal (trans-2-hexenal) is an α-β-unsaturated aldehyde (Figure 7), produced by throughout the lipoxygenase pathway, that have a protective action towards microbial proliferation in wounded areas (Casey et al., 1999).

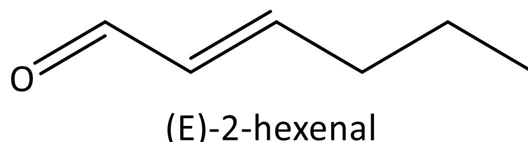


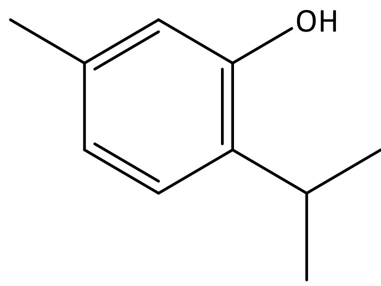
Figure 7: Chemical structure of (E)-2-hexenal

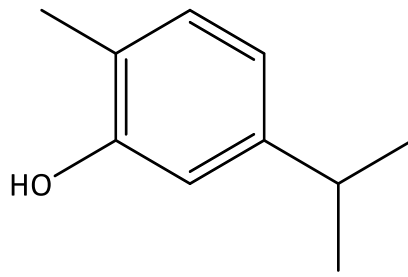
In addition, (E)-2-hexenal has been demonstrated to possess a significant antimicrobial activity against several microfungal, gram-positive and gram-negative bacterial strains (Gardini et al., 1997; Kubo et al., 2004; Lanciotti et al., 2004; Patrignani et al., 2008; Patrignani et al., 2015; Siroli et al., 2015). The antimicrobial properties of 2-(E)-hexenal are strictly related to its chemical structure. This aldehyde is reported to act as a surfactant. Due to the interaction with the microbial cell membrane bilayer, 2-(E)-hexenal permeates by passive diffusion across the plasma membrane. Once inside cells, the aldehyde group reacts with biologically important nucleophilic groups (Kubo and Fujita, 2001). This aldehyde moiety is known to react with sulphydryl groups mainly by 1,4-additions under physiological conditions (Kubo and Fujita, 2001). Sulphydryl groups in proteins and lower-molecular-weight compounds such as glutathione are known to play a key role in living cells. The mechanisms of antimicrobial action of other aldehydes, such as glutaraldehyde and ortho-phthalaldehyde, are likely to involve interaction with the cytoplasmic membrane and increase in its permeability (Ramos-Nino et al., 1998; Simons et al., 2000; Tsuchiya, 2001).

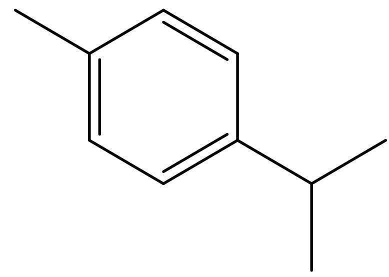
1.4.4 Thyme essential oil

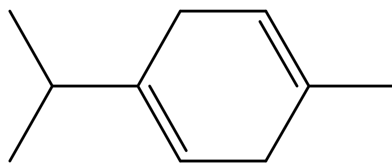
Thyme species (Lamiaceae) are well known aromatic herbs used not only as spice but also as treatment of different diseases (Jamali et al., 2012). *Thymus vulgaris* has been applied for different indications such as dry cough, bronchitis, and digestive problems (Grosso et al., 2010;Tsai et al., 2011). Thyme essential oil contains thymol, carvacrol, p-cymene, γ-terpinene, and linalool (Figure 8), where the thymol and carvacrol are present in relatively high percentage, up to 41.6% and

7.9%, respectively (Grosso et al., 2010;Aazza et al., 2011;Asbaghian et al., 2011). Thymol and carvacrol are considered to be major responsible of the antimicrobial features of thyme oil (Jamali et al., 2012). Thymol is structurally related to carvacrol (Figure 8), having the hydroxyl group at a different position on the phenolic ring. Although the primary mode of antibacterial action of thymol is not fully known, it involves outer and inner membrane disruption, and interaction with membrane proteins and intracellular targets (Sikkema et al., 1994; Bakkali et al., 2008; Xu et al., 2008; Hyldgaard et al., 2012). The thymol interaction with the cell membrane has been documented by the loss of membrane potential, cellular uptake of ethidium bromide, and leakage of ATP and potassium ions of microbial cell exposed to lethal and sublethal thymol concentrations (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, different authors have documented thymol's interaction with membrane proteins and intracellular targets. Interaction with membrane proteins was 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 up-regulation of genes involved in synthesis of outer membrane proteins and chaperonins. 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).









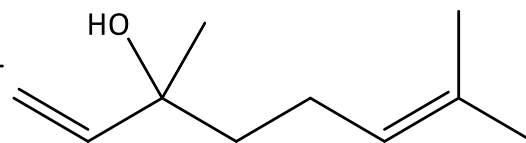


Figure 8: major constituents of Thyme spp. essential oil.

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Listeria monocytogenes

2.1 Taxonomy, morphology and main characteristics

Listeria monocytogenes (originally named *Bacterium monocytogenes*) is a Firmicutes, Gram-positive, non-spore forming, facultative anaerobic, catalase positive and oxidase negative rod shaped bacterium, first described in 1926 in United Kingdom, as a cause of infection with *monocytosis* in laboratory rodents (Murray et al., 1926). Due the low percentage of guanine/cytosine bases genome, *L. monocytogenes* is strictly related to *Bacillus, Staphylococcus, Streptococcus* and *Clostridium* species (Wilkinson and Jones, 1977;Roccourt et al., 1982;Fersu and Jones, 1988;Collins et al., 1991;Hartford and Sneath, 1993;Glaser et al., 2001) and for this reason it belongs to Bacilli class and Bacillales order. Although originally described as monotypic genus containing only *L. monocytogenes sensu lato,* now the *Listeria* genus comprises six species: *Listeria grayi (Larsen and Seeliger, 1966), Listeria innocua* (Seeliger, 1981*), Listeria ivanovii* (Seeliger et al., 1984), *Listeria monocytogenes* (Seeliger et al., 1984)*, Listeria seeligeri* (Rocourt and Grimont, 1983) and *Listeria welshimeri* (Rocourt and Grimont, 1983)*.* Only *L. monocytogenes sensu stricto* and *L. ivanovii* are pathogens: the first for humans and the second for ungulates (Santagada et al., 2004). On the base of serological reactions of somatic (O-factor) and flagellar (H-factor) antigens with specific antisera, *Listeria* species are classified into different serotypes. *L. monocytogenes* includes the serovars 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7 (Wagner and McLauchlin, 2008). Molecular techniques allowed to dive *L. monocytogenes* serotypes into three different lineages:

1. **Lineage I** contains serovars 1/2a, 1/2c, 3a, and 3c; and lineage III contains serovars 4a and 4c;
1. **Lineage II** contains serovars 1/2a, 1/2c, 3a, and 3c;
2. **Lineage III** contains serovars 4a and 4c;

Lineage III can be further divided into three subgroups on the base of the rhamnose assimilation and virulence:

1. **Subgroup IIIA** is made up of typical rhamnose-positive avirulent serovar 4a and

virulent serovar 4c strains;

2. **Subgroup IIIB** is made up of atypical rhamnose-negative virulent non-4a and non-4c strains, some of which may be related to serovar 7;
3. **Subgroup IIIC** is made up of atypical rhamnose-negative virulent serovar 4c strains.

Cells form regular, short rods, 0.4–0.5 by 1–2 µm with parallel sides and blunt ends and usually occur singly or in short chains or arranged in V and Y forms (Rocourt and Buchrieser, 2007); in older or rough cultures, filaments of 6 µm in length may develop. A tumbling motility characterizes *Listeria monocytogenes as the* result of the production of a peritrichous flagellum when cultured between 20 to 25°C but not at 37°C. The metabolism is aerobic and facultatively anaerobic. Growth occurs between pH 5.2 and 9 and between 0 and 45°C; optimal growth occurs between 30 and 37°C. Cytochromes are produced and homofermentative anaerobic catabolism of glucose results in production of L(+)– lactic acid, acetic acid, and other end products. Acid but no gas is produced from other sugars When cultured on artificial media, like Brain Heart Infusion agar, *Listeria monocytogenes* form nonpigmented colonies after 24–48 h with a diameter between 0.5–1.5, rounded, translucent, low convex with a smooth surface and entire margin, and with a crystalline central appearance. After 3-7 days colonies became larger (3–5 mm in diameter), and have a more opaque appearance; sometimes, rough colonial forms may develop with a sunken center (Wagner and McLauchlin, 2008).

2.2 Listeriosis

Until 1980s, human listeriosis remained a relatively obscure disease attracting limited attention, although large outbreaks of considerable morbidity and mortality but of unknown transmission occurred**.** Listeriosis occurs in various animals, including humans, and most often affects the uterus at pregnancy, the central nervous system, or the bloodstream. Human listeriosis are, in almost the cases, due to *L. monocytogenes* (McLauchlin, 1997) and the most susceptible populations are pregnant womens, immunocompromised subjects and elders. Although infection can be treated successfully with antibiotics, the human infection has a mortality of 20–40% (Farber and Peterkin, 1991). The principal contamination route is represented by the consumption of contaminated food. *L. monocytogenes* can be found in a wide variety of raw and processed foods like fish, vegetables, beef and pork meats, milk and dairy products. Also ready to eat (RTE) foods and minimally processed foods have been associated to cases of listeriosis. In fact, in general, they

are subjected to mild treatment and held at refrigeration or chilled temperature for long time. Due to its psycrotrophic nature, to the ability to survive to high NaCl concentration and low pH, L. *monocytogenes* represents a species able to adapt its-self to different conditions (Buchanan et al., 2017).

As showed from different authors *L. monocytogenes* strains are widely distributed in food processing environments (TOMPKIN, 2002;Carpentier and Cerf, 2011;FERREIRA et al., 2014). Different are the contamination sources: raw products, materials, equipment and the movement of people. *Listeria* is also able to persist due to ineffective cleaning and sanitation, poor design or condition of food equipment or environment or insufficient controls of movement of people or equipment (Carpentier and Cerf, 2011). Improved control measures starting in the 1990s have greatly reduced the incidence of *L. monocytogenes* in many food categories, particularly in meats and meat products, but listeriosis still represents a severe cause of food-borne illness. In the 2013 as reported from the European Food Safety Authority (EFSA), 1763 human cases of listeriosis were confirmed in 27 member states with an increase of the 8.6% compared to 2012 and a mortality rate of the 15.6 % (European Food Safety et al., 2015). In the same year, five UE member states also reported seven food-borne listeriosis outbreak. Implicated Food vehicles were: mixed salad (1 case), meat product (1), pig meat (1) and crustaceans, shellfish and mollusks (3) (European Food Safety et al., 2015). The new rules in Europe, defined by the EC directive 2073/2005, can allow the presence of *L. monocytogenes* (100 CFU in 5 sample units) in ready-to-eat foods placed on the market at the end of their shelf-life in relation to the category of use, and the physio-chemical features of food (European Commission, 2005). Generally for foods dedicated to babies, pregnant women, immune depressed people, or food characterized by high pH and water activity, L. monocytogenes must be absent in 25 g of products. On the other hand, for foods not able to support the growth of L. monocytogenes, this pathogenic species can be present at 100 CFU/g of products at the end of the shelf-life (European Commission, 2005). Foods not supporting the growth of L. monocytogenes can be those having pH <4.4, Aw <0.920 or combination of pH <5 and aw< 0.940, or product with a shelf-life shorter than 5 days. However, not all food regulators have taken this approach, and the United States has had zero tolerance of any *L. monocytogenes* in a processed food since the 1980s.In Italy, at the end of 1990 (DM 15.12.1990, listeriosis has been included in the list of nationally notifiable diseases. The minimal infective dose for listeriosis is hard to be defined and vary considerably between individuals. In general, the consumption of food with levels below 100 colony-forming units (CFUs) per gram is associated very low listeriosis

risk (Wagner and McLauchlin, 2008). The world health organization defined the DL_{50} for human Listeriosis in of 1.9 * 10^6 CFU. In the majority of the cases, in foods an average contamination level of 10^2–10^6 CFUs/mL/g is associated with an infection (Dawson et al., 2006).

2.3 Stress survival strategies

The ability of *L. monocytogenes* to survive to different adverse environmental conditions and stresses encountered both in its natural environment and subsequently within the host and it is at the base of its pathogenicity (Stack et al., 2008).
During the foods industrial processing, many factors may affect the *L. monocytogenes* growth including traditional (*e.g.* heat, low temperatures, high salt content, low or alkaline pH, chemical additives) and novel (*e.g.* HHP, HPH, ionizing radiation, PEF, MAP…) food preservation techniques, but also competition and metabolites produced by other microorganisms (microbial antagonism).
The ability of *L. monocytogenes* to withstand severe environmental stresses depends on its efficient stress response mechanisms. Although, environmental stresses are different, the response mechanisms passthrough the regulation of the alternatives RNA polymerase sigma factor. One of the most important sigma factor is σ^B (encoded by the *sigB* gene). Mutation induced in the *sigB* gene lead a lower acid and osmotic stress resistence. Another transcription regulator gene strictly related to the stress response is *prfA* . PrfA transcription activator protein is involved in the response to several environmental factor including temperature, pH and have a key role in the *Listeria monocytogenes* virulence mechanism (see chapter 2.4 Virulence).
Four are the mayor stresses encountered by *L. monocytogenes* during its life cycle:

- Heat stress;
- Cold stress;
- Acid stress;
- Osmotic stress

2.3.1 Heat stress response

Exposure to temperatures above the range for normal cell growth leads to progressive loss of bacterial viability. *L. monocytogenes* is exposed to lethal or sublethal heat stress in food

processing environment as the result of thermic treatments for preserving foods (i.e., blanching, pasteurization, and sterilization). Many factors can influence the thermo-tolerance of *L.* monocytogenes and are related to the age of the culture, growth conditions and the properties of food matrixes in term of salt content, water activity, acidity, presence of inhibitor (Doyle et al., 2001) *Listeria monocytogenes* has evolved specific mechanisms to resist and survive to the heat stresses generally defined as heat shock response. That kind of response is a highly conserved defense mechanism characterized by the transiently induced over-expression, biosynthesis and accumulation of heat shock proteins (Hsps) (Yura and Nakahigashi, 1999). Heat shock proteins are mainly divided into two different groups: adenosine triphosphate (ATP)-dependent proteases (ATPases) and molecular chaperones. The exposure to heat stresses may provoke the denaturation of the cellular proteins compromising their biological function. Misfolded proteins have a collapsed structure and may aggregate forming precipitates. The denaturation is associated with the exposure on the protein surface of hydrophobic residues of proteins become damaged and that are normally inside the protein structure. Chaperones bind hydrophobic residues and attempt to refold these proteins while proteases degrade those denatured proteins unable to adopt their native conformation (Georgopoulos and Welch, 1993;Gottesman, 1996). The mayor proteins involved in the heat stress responses of *L. monocytogenes* are the chaperonins GroES, GroEL, DnaK, DnaJ, and HtrA, Clp and complex. GroEL is one of the most conserved proteins in nature (Zeilstra-Ryalls et al., 1991) and with GroES act to maintain the intracellular protein stability under adverse environmental conditions (Hendrick and Hartl, 1993). The chaperone function of DnaK has been well characterized (Craig et al., 1993). Exposure to sublethal heat shock stresses induces the expression of both GroEL and DnaK. (Bunning et al., 1990;Gahan et al., 2001). The GroESL complex and DnaK proteins are induced also after the exposure to other environmental stresses such as low pH, high salt and ethanol indicating a protective role in the general stress response (Hill et al., 2002). Clp (caseinolytic protease) protein complexes play a critical role in energy-dependent proteolysis . The Clp complex is composed of a proteolytic subunit, ClpP, which associates with a Clp ATPase. Clp ATPases are ubiquitous among prokaryotes and eukaryotes and are members of the highly conserved Clp/Hsp100 family of proteases, whose function is to regulate ATP-dependent proteolysis and also play a role as molecular chaperones involved in protein folding and assembly. HtrA (hiGh-temperature requirement**)** is the best characterized protein of the High Temperature Requirement cluster encoding a serine protease (Foucaud-Scheunemann and Poquet, 2003). The protein acts both as a protease in the degradation of

unfolded proteins and as molecular chaperone in the refolding of proteins (Foucaud-Scheunemann and Poquet, 2003).

2.3.2 Cold stress response

Listeria are psychrophilic organisms highly adapted to low temperatures (2–4 °C) and the survival occurs also at temperatures below 0°C, representing an issue for the food industry. *Listeria monocytogenes* have the ability to adapt the cell membrane fluidity to the adverse environmental conditions modulating the membrane fatty acids composition to preserve the proper solute exchanges (Gandhi and Chikindas, 2007). The lipidic cell membrane composition of *L. monocytogenes* is characterized (over 90%) by odd-numbered branched-chain fatty acids (BCFAs) like heptadecanoic (C17:0) and pentadecanoic in its iso and anteiso forms (C15:0 iso, C15:0 ante) acids. The BCFAs are synthetized from alpha-keto acids precursors like isoleucine, leucine, valine by the Alpha- keto acid dehydrogenase (BKD) complex. The relative abundance of these branched fatty acids shift with the growth temperature. As showed by (Beales, 2004), when *L. monocytogenes* is cultured at 7 °C, the membrane concentrations of branched pentadecanoic acids (C15:0 ante) rise, while the concentration heptadecanoic acid decrease. The lower carbon-carbon interaction between neighboring C15:0 acid chains reduce the overall cytoplasmic membrane viscosity taking back the membrane fluidity to the optimum degree.

Moreover, a change from C15:0 iso to C15:0 ante was observed when *L. monocytogenes* was grown at 5°C (Annous *et al.,* 1997).

The *L. monocytogenes* cold stress response is also mediated by specific pathway shared with the heat shock response (GroESL and ClpPB) and in the osmoadaptation processes (see osmotic stress response).

2.3.3 Acid stress response

Acid exposure and stresses represent one of the most frequently adverse environmental condition encountered by microorganisms and they can be defined as the combined biological effects of low pH and weak (organic) acids present in the environment (Stack et al., 2008). Acidification is a common strategy used to preserve foods, and it is achieved by fermentative processes or by direct addition of organic preservatives acids like citric, ascorbic, and lactic acid. Weak organic acid in the protonated form are able to pass thought the cell wall and cytoplasmic membrane. Inside the cell due the higher cytoplasmic pH, they dissociate releasing a proton and leading to acidification of

the cytoplasm causing a cytotoxic effect (Bearson et al., 1997). Like the majority of food-borne pathogens, *L. monocytogenes* is a neutrophile with an optimum of growth between pH 6 – 7 (Yura and Nakahigashi, 1999). The ability of L. *monocytogenes* to keep regular cytoplasmic pH (pHi), despite variation of the external pH, is fundamental to its survival and a prerequisite for infection (Stack et al., 2008). To preserve pH homeostasis, *L. monocytogenes* has developed different acid tolerance response (ATR) systems. During the ATR response the organism produce acid shock proteins (ASPs) which are also involved in the virulence. Different authors (Foster and Hall, 1991;Kroll and Patchett, 1992;Foster et al., 1994;O'driscoll et al., 1997) showed a correlation between the acid tolerance response and the *Listeria monocytogenes* pathogenic ability. Another acid stress resistance strategy is represented by glutamate decarboxylase (GAD) and the arginine deiminase (ADI) systems. Glutamate decarboxylase enzyme irreversibly decarboxylates a extracellular glutamate sourced molecule producing γ-aminobutyrate (GABA). The reaction consumes one intracellular proton mitigating the proton excesses as the result of acid hostile environment. Glutamate: GABA antiporter subsequently exchange the GABA produced in this pathway with is a extracellular molecule of glutamate causing an alkalization of the environment due the lower acidity of GABA (Stack et al., 2008). Arginine deaminase complex is formed by three proteins: arginine deiminase (ADI), catabolic ornithine carbamoyltransferase (cOTCase), and carbamate kinase (CK). In 2006, (Ryan, 2006) characterized in *L. monocytogenes* the ADI systems and demonstrated its role for this system tor the growth and survival in acidic conditions. This detoxification pathway is less effective compared to the Glutamate - γ-aminobutyrate system. The ADI complex provokes the intracellular accumulation of NH_3 as the result of the arginine deamination to ornithine. Ammonia may react with free cytoplasmic protons forming NH_4^+ ions and increasing the pHi and maintaining pH homeostasis (Ryan, 2006). Also the energetic metabolism is involved in the acid stress resistance. The F0F1ATPase, known as ATPsyntase complex or ATPase pump, couples ATP synthesis/hydrolysis with a transmembrane proton translocation. This enzyme can maintain intracellular pH homeostasis. The complex normally synthetizes, in aerobic condition, ATP depleting the proton motive force (PMF). When cytoplasm becomes acid, ATPsyntase is able to alkalinize the intracellular pH inverting the process. By the ATP hydrolysis the complex pump outside the cell protons restoring the proton motive force. Although the *Listeria* F0F1ATPase has been demonstrated have a role in the acid-tolerance response the response to these stresses is not dependent on the activity of this complex.

2.3.4 Osmotic stress response

The maintenance of intracellular osmotic pressure is critical for the survival in osmotic stress conditions. Osmotic stress can be defined as the increase or decrease in the osmotic strength of the environment of an organism (Csonka, 1989;Csonka and Hanson, 1991) as a result of low water activity caused by dehydration or high amounts of osmotically active compounds. Internal osmotic pressure, normally is higher than that of the surrounding medium generating cell turgor which is fundamental for cell extension, growth, and division. The ability of bacteria to adapt to osmotic stress is called osmoadaptation. In *Listeria monocytogenes* this process involves both changes in the physiological cell properties (presence of osmotically active solutes in the cytoplasm) and in the gene expression profiles (Hill et al., 2002) allowing to survive in presence of high salt concentrations, making the control of this pathogen in foods difficult. One of the mechanism at the base of *Listeria* osmoadaptation is represented by the cytoplasmic accumulation of K^+ as potassium glutamate. This provokes the rise of the inner cell concentrations of small organic compounds like glycine, glycine betaine, proline, proline betaine, acetylcarnithine, carnitine, γ-butyrobetaine, and 3-dimethylsulphoniopropionate, that function as osmoprotectants, with glycine (Patchett et al., 1992). These molecules are characterized by a high solubility and are unable to cross the membrane without active transport systems. In *L. monocytogenes* the uptake of glycine betaine and carnitine is well characterized and mediated by the transporters: BetL, Gbu (for glycine betaine) and OpuC (carnitine transporter) (Ko and Smith, 1999;Fraser et al., 2000;Sleator et al., 2003).

As described by (Sleator et al., 2003), *L. monocytogens* mutants with a *betL* reduced activity, are not able to survive in elevated osmolarity environments. Another mechanism used by *Listeria* to combat osmotic stress, is represented by the expression of osmotic stress response proteins like RelA (Okada et al., 2002), Ctc (Gardan et al., 2003a;Gardan et al., 2003b), KdpE (Brøndsted et al., 2003), ProBA (Sleator et al., 2001),and BtlA (Begley et al., 2003).

relA, is the gene encoding a (p)ppGpp synthetase. (p)ppGpp is stress-response-related factor and it is considered an activator of the osmotic stress response (Okada et al., 2002). Ctc is a osmotic stress protein in *L. monocytogenes* belonging to the L25 family of ribosomal proteins and has been shown to facilitate growth in minimal media under conditions of high osmolarity. KdpE forms in combination with *kdpD*, *kdpE,* and *orfX,* a transcriptional response factor, active to prevent plasmolysis and restore turgor pressure. (Kallipolitis and Ingmer, 2001;Sleator et al., 2001;Brøndsted et al., 2003).

ProBA, is a protein involved in proline biosynthesis. The role of proline as an osmolyte protector has previously been described. As showed by (Begley et al., 2003), in presence of elevated osmolarity (7%), *L. monocytogenes* has been shown the activation of the BtlA protein, a bile tolerance locus which play a role in also in the osmotic stress response.

2.4 Virulence

The *Listeria monocytogenes* virulence is close related with the stress response. The stress adaptation to adverse environmental condition represents the evolutionary advantage that allows *Listeria* to infect and survive inside mammalian cells. Not surprisingly many protein involved in the stress response are also involved in the virulence like the transcriptional promoter pfrA. The *Listeria monocytogenes* pathogenic ability involve the activation of specific genes called pathogenic islands. This gene cluster is mainly regulated by prfA protein. Following ingestion of contaminated foods, *Listeria monocytogenes* strains enter through the cells in Peyer's patches through either macrophage or epithelial cells. The process is mediated by two proteins InlA, required to invade epithelial cells and InlB, a, is involved in hepatocyte invasion (Gregory et al., 1997) and the extracellular invasion protein p60. InlA and InlB are two acid-surface proteins characterized by a C-terminal ends rich leucine tandem. These sequences represent an internalization signal recognized the mammalian cell E-cadherin receptor. After the internalization process *Listeria* becomes trapped inside vacuoles. The release from vacuoles is mediated by the listeriolysin pore-forming protein encoded by *hly*A gene. Reached the cytoplasm *Listeria* cells immediately start the duplication process with a generation time, depending on the strain of about 20-40 minutes. The intracellular growth of *Listeria* is stimulated by the oligopeptide binding protein OppA (Borezee et al., 2000) and also by p60 invasion protein. After the primary infection *Listeria* is capable to infect surrounding cells without leaving the cells. The process involves the *act*A gene product and *Listeria* starts to produce form one cellular pole actin-like filaments. The formation of actin tails propels bacteria towards cell plasma membrane results in formation of a protrusion into the neighboring cells and the formation of single cell vacuoles after the invasion. Vacuoles are now disrupted again by the activity of listeriolysin in synergy with a phospholipase C class protein PclB. The entire process occurs in about 5 hours.

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Escherichia coli

3.1 Taxonomy, morphology and main characteristics

The genus *Escherichia* (named in honor after its discoverer Theodor Escherich) is a large Gram-negative, rod-shaped, motile, non-spore-forming bacilli (known as ing bacilli (tive, rod-shaped, motileProteobacteria phylum. Six species are comprise in the genus *Escherichia*, i.e., *Escherichia albertii*, *Escherichia coli*, *Escherichia fergusonii*, *Escherichia hermannii*, *Escherichia marmotae*, and *Escherichia vulneris*, (Kaper et al., 2004;Liu et al., 2014). Among reported species, *E. coli* represents the predominant facultative anaerobe in the gastrointestinal tract of warm-blooded animals, including humans, and it has been linked to nutrition due its role in the food breakdown and as a source of vitamin K. Although its benefit to the human health, some specific strains are pathogen and have developed the ability to colonize mucosal surface of intestine and of the urinary tract (Kaper et al., 2004). The pathogenic potential is related to specific virulence genes and their combination which determine the *E. coli* intestinal and extra-intestinal pathotypes. The intestinal *E. coli* strains are classified in: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), Shiga-toxin-producing enteroaggregative(STEAEC), enteroinvasive (EIEC), diffusely adhering (DAEC), cell detaching (CDEC), necrotoxic (NTEC), and adherent invasive (AIEC) (Makobe et al., 2012;Lozer et al., 2013). Three pathotypes are responsible of extraintestinal infections: septicemia-causing *E. coli* (SCEC), neonatal-meningitis-causing *E. coli* (NMEC), and uropathogenic *E. coli* (UPEC) (Kaper et al., 2004).

Serological analysis of surface antigens allowed to differentiate up to 200 serogroups, on the base of specific combinations of O and H antigens (e.g., O157:H7). Recently *Escherichia coli* strains were also classified on the base of genetic information into six different phylogenetic groups: A (saprophyte), B1, B2 (pathogen), C, D (pathogen), E and *Shighella* (Touchon et al., 2009). Although its close phylogenetic relationship, *Shigella* has continually been treated as a separate genus. *Escherichia coli* appears as a rod-shaped bacterium of about 0.6 µm in diameter and 2 µm in length. Cells are also characterized by *fimbriae* and *flagella.* Due a chemoheterotroph metabolism using a large variety of sugars or amino acids, *E. coli* grows rapidly in nutrient riched broths. Although only few stains have a single auxotrophic requirement such as thiamin, the growth of many strains may be inhibited by the presence of single amino acids such as serine, valine, or

cysteine (Liu, 2017). *E. coli* tolerates temperature between 8°C and 48°C and grows optimally at 39°C. The bacterium survives between pH 6.0 and 8.0 (even if there are some strains able to survive to pH5 in relation to the food composition) , but is unable to grow in media containing >0.65 M NaCl. In response to changes in the osmotic pressure of the medium, *E. coli* increases its concentration of ions, especially K+ and glutamate (Liu, 2017).

3.2 Stress survival strategies

E. coli withstand in adverse environmental condition is related to the stress response mechanisms. During its life cycles, *E.coli* is exposed to different stresses like weak acids, starvation, high osmolarity, and high or low temperature (Lange and Hengge-Aronis, 1994;Buchanan, 1997). As previously described for *Listeria monocytogenes*, *Escherichia coli* response to adverse conditions and environments largely relies on transcriptional reprogramming *via* activated by the alternative sigma factors. One of the most important alternative sigma factor is RpoS (also named σ^{38}) (Amato et al., 2013) and its activation is associated with the general stress response mechanisms involving the expression of more than 35 genes. Stress response mechanisms also involve sigma factor σ^{32} and σ^{24}. The mayor stresses encountered by *E. coli* during its life cycle are:

- Heat stress;
- Cold stress;
- Acid stress;
- Osmotic stress

3.2.1 Heat stress response

When *E. coli* is exposed to lethal or sublethal thermal stress conditions, the withstand is allowed by the transiently induced biosynthesis and accumulation of over 30 heat shock proteins (HSPs) (Chung et al., 2006;Nonaka et al., 2006). This stress response is mainly mediated by σ^{32}. In *E. coli*, during the heat stress response, transcription initiation is regulated largely by σ32 alternative factor and as previously described for *L. monocytogenes* the mayor protein involved in the heat stress responses are the chaperonins DnaK, DnaJ, GroEL, GroES, ClpB and the protease Lon, ClpP, FstH (see chapter 2.3.1). The exposure to heat stress induces the modulation of some regulator genes, such as *mlc*, *arcA*, *pflA* and *ldhA* and adhE (Ye et al., 2012). The activation of *mlc* is followed

by the down-regulation of glucose uptake, while the up-regulation of *arcA* is associated with a reduce activity of the TCA cycle and glyoxylate pathway. Thermal shock also induce the up-regulation of PflA , ldhA and the down regulation of AdhE. As a consequence, the formate and lactate yield increased, while the ethanol yield decreased (Hasan and Shimizu, 2008).

3.2.2 Cold stress response

The cold climate *Escherichia coli* adaptation involve modifications of the membrane fluidity (Russell et al., 1990) and the maintenance of the structural integrity of proteins and ribosomes complexes (Jaenicke, 1991;Berry and Foegeding, 1997). When the temperature decreases, *E. coli* changes the membrane lipid composition in order to maintain the proper fluidity that allows an optimal nutrient exchanges with the environment. At low temperature in *E. coli,* the concentration of unsaturated cis-vaccenic acid (C18:1) rises, while the concentration of the saturated palmitic acid (C16:0) decreases. The reduced interaction among the neighboring fatty acids chains due to the stereochemical properties of the unsaturated chains led to an increased membrane fluidity (Garwin and Cronan, 1980). The cold adaptation requires also the activation of specific gene products formally named cold shock proteins (CSPs). These proteins are involved in large variety of fundamental cell function transcription, translation, mRNA degradation, protein synthesis, and recombination in *E. coli* (Jones and Inouye, 1994;1996;Jiang et al., 1997;Graumann and Marahiel, 1998). One of the most conserved cold shock protein between non-pathogenic and pathogenic *E.coli* strains is the CspA. This protein is induced at low temperature and act as a s a e and act a CspA destabilize secondary mRNA structure preventing the RNase digestion (Jiang et al., 1997).

3.2.3 Acid stress response

Acid stress can be defined as the combined effect of environmental protons and weak organic acids (Zhao et al., 1993;Miller and Kaspar, 1994). *Escherichia coli* Enteroinvasive, enteropathogenic, and enterohaemorrhagic strains are more acid tolerant than nonpathogenic strains such as *E. coli* K12 (Gorden and Small, 1993). The development of acid tolerance is strictly related to cell physiological properties and can involve pH-dependent, pH-independent or a combination of both types of systems (Lin et al., 1995). In all *E. coli*, two pH-dependent mechanisms were described: one induced in the log phase and another activated in stationary growth phase (Small et al., 1994).

During the log phase, the exposure to low pH value provokes the cytoplasmic accumulation of many compounds like glucose, glutamate, aspartate, FeCl3, KCl, and L-proline, phosphate and cAMP (Goodson and Rowbury, 1989;Foster, 2000). The acid stress response during the stationary phase is mediated by glutamate decarboxylase and the arginine deiminase complexes. Three acid resistance (AR) systems have been identified in all *E. coli* (Lin et al., 1995;Bearson et al., 1997;Audia et al., 2001): AR1, AR2 and AR3. The acid resistance system 1 is an oxidoreductive complex, while AR2 (glutamate decarboxylase) and AR3 (arginine decarboxylase) are fermentative complex and their activation depends on medium used for the growth (Audia et al., 2001). The oxidative system is dependent upon σ^s and it not require amino acids for the proton depletion after the exposure to acid stress (Lin et al., 1995). As described for *Listeria monocytogenes* (see chapter 2.3.3) the amino acid decarboxylation systems involving the AR2 and AR3 complex appeared to act as inducible pH homeostasis systems, and play an important role in the maintenance of the cytoplasmic pH_i . The pH-independent response to acid environment pass thought changes in the cell membrane composition. As reported by (Brown et al., 1997;Jordan et al., 1999), the exposure to acid increases the bioaccumulation in the cytoplasmic cell membrane of phospholipids containing membrane-stabilizing cyclopropane fatty acids.

3.2.4 Osmotic stress response

Increased osmotic pressure has been used to control the growth of food spoilage and pathogenic bacteria by desiccation or addition of high amounts of osmotically active compounds, which result in a decreased water activity (Chung et al., 2006). The maintenance of intracellular osmotic pressure is critical for the survival in osmotic stress conditions. When the osmotic pressure in the surrounding environment increases, cells activate osmoregulation systems to prevent shrinkage and eventual plasmolysis.

In *E. coli* the osmoadaptation induces the expression of Postexponential protein (Pex) and heat shock proteins HPSs (Christman et al., 1985;Jenkins et al., 1988;Schultz et al., 1988;Jenkins et al., 1990). Pex proteins are intracellular sensors (Rowbury, 1997) and their expression, which occurs in a large variety of environmental stress conditions, results in an increased resistance to heat, oxidation, and osmotic pressure. As described for *L. monocytogenes ,* osmoregulation (see chapter 2.3.4 is achieved by the uptake form the environment or bio-synthesis of different osmoprotectants solutes like: trehalose, proline, glycine, betaine and carnitine (Pichereau et al.,

2000). When exposed to osmotic stre ss, *E. coli* accumulates betaine and proline under *via* two specific transport systems ProU and ProP (Booth et al., 1994;Csonka and Epstein, 1996).

3.3 Stress response and virulence

The pathogenic ability of different enteropathogenic *E. coli* strains is strictly related with their response mechanisms to the adverse growth condition (Benjamin et al., 1991;Fang et al., 1992;Garcia-del Portillo et al., 1993;Gahan and Hill, 1999). The virulence of *E. coli* O157:H7 depends on its ability to resist to the acid environment of the gastrointestinal tract (Benjamin (Benjamin and Datta, 1995;Tuttle et al., 1999;Law, 2000;Smith, 2003). As reported from many authors, sub-lethal stress conditions can influence the Stx toxin production. After the exposure to cold environments or heat and acid shocks, *E. coli* O157:H7 showed during the recovery an enhanced Stx toxin production.

Leenanon et al., (2003) highlighted, using RT-qPCR and ELISA assays, an increased expression of the *stx-II* gene during the exposure to acid stress without an increase of the Stx toxin. However, the growth in acid environments stimulated the expression of the virulence *eaeA* and *hlyA* genes (Chung et al., 2006). The first encode for the attaching and effacing protein and the second for the hemolysin. A similar effect on the Stx toxin regulation was observed by Yuk and Marshall, (2003) after the exposure of *E. coli* O157:H7 at high temperature. Heat adaptation induced a intracellular reduction of the Stx toxin concentration. However, the secretion of the protein was higher. *E. coli* proteins associated with thermotolerance HSPs contributed also to the bacterial macrophage survival (Delaney et al., 1993).

3.4 References

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General Objectives

Consumer demand for minimally processed and ready-to-eat foods, with a reduced content of synthetic preservatives, has stimulated the research toward alternative preservation strategies. Essential oils (EOs) or their components represent one of the most promising natural feasible alternatives to improve food safety, shelf-life and quality. Recognized as safe (GRAS) from international food authorities, they are traditionally used in food industry as flavor and taste enhancers (Newberne et al., 2000). Moreover, a wide literature documents their application as natural preservatives also in different food matrices such as meat (Fratianni et al., 2010; Barbosa et al., 2015; Radha krishnan et al., 2015), dairy products (Amatiste et al., 2014; Ehsani et al., 2016; Ben Jemaa et al., 2017), minimally processed fruits and vegetables (Patrignani et al., 2015; Siroli et al., 2015b, c) and beverages (Kiskó and Roller, 2005; Chueca et al., 2016). Among the natural antimicrobials, thyme EO, and some components of citrus and officinal EOs, such as citral, carvacrol, and (E)-2-hexenal, are very promising alternatives to traditional preservatives (Ivanovic et al., 2012). In fact, they are widely reported to be able to improve safety and shelf-life of several foods also when used at concentrations lower than their bactericidal ones and compatible with the product sensory properties (Lanciotti et al., 1999; Gardini et al., 2002; Lanciotti et al., 2003; Lanciotti et al., 2004; Belletti et al., 2008; Siroli et al., 2014; Zanini et al., 2014a; Zanini et al., 2014b; Patrignani et al., 2015; Silva-Angulo et al., 2015; Siroli et al., 2015). However, although their antimicrobial properties are well documented, their practical application is currently limited due to the strong impact and changes they cause in food products (Gutierrez, Barry-Ryan, & Bourke, 2008). Moreover, the limited use is due to i) the variability of the composition of EOs (due to the geographic origin, agricultural techniques, season, methods of extraction, etc.) able to influence their effective overall antimicrobial activity (Patrignani et al., 2015); ii) the interaction of bioactive molecules with the food matrix (in particular with proteins, lipids, starch, etc.) limiting the contact of these molecules with the microbial cells, thereby reducing the effects on cell viability (Gutierrez et al., 2008); iii) the lack of knowledge of the interaction between technological and composition parameters and their activity; iv) the lack of knowledge of the mechanisms by which these molecules exert their antimicrobial activity.

In fact, few and fragmented are the information about their mechanisms of action, their cellular targets and on the stress response strategies that microorganisms take in place after the exposure to such compounds (Burt, 2004; Hyldgaard et al., 2012; Patrignani et al., 2015). Although cell

membrane, energetic metabolism and cytoplasm coagulation are regarded as the main target of EOs and their components, few are the information of the gene and transcriptome modifications induced by a short term exposure to natural antimicrobials such as citral, carvacrol, (E)-2-hexenal and thyme essential oil in food-borne microorganisms. In addition, also the information on the contribute of each cell, within a population, to the microbial resistance are not fully understood. The heterogeneity in microbial population resistance to different stresses is reported to occur as a monomodal Gaussian with a narrow or broad distribution, or as a multimodal distribution comprising subpopulations of similar or vastly different numbers of individuals (Dhar and McKinney, 2007). For these reasons, an holist approach should be considered for the evaluation microbial cell responses and mechanisms of action of EOs (Caccioni et al., 1997). In fact, the detailed knowledge of the action mechanisms of citral, carvacrol, (E)-2-hexenal and thyme EO and the microbial stress response is mandatory for their implementation at industrial level as innovative preservation strategies even when they are used sublethal concentrations in combination with other food preserving non-thermal strategies. The implementation processes should be also related to the food matrices and production processes.
In this framework, the main aim of the PhD project was to investigate the effects of one hour exposure to sublethal concentrations of selected natural antimicrobials such as citral, carvacrol, (E)-2-hexenal and thyme EO on the food-borne pathogens *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655 using multiple approaches. In particular, the effects of the natural antimicrobials and the stress response were evaluated using molecular approaches in order to highlights the shifts on the transcriptome and proteome. For *Listeria monocytogenes* Scott A, the effects of the exposure to natural antimicrobials on the transcriptome was studied by RT-qPCR on a pool of gene representative of different metabolisms: energetic, ferric uptake, stress response, gene transcription, cell division, virulence, motility, while the proteome effects were determined by bi-dimensional electrophoresis (2DE). The transcriptome changes of *Escherichia coli* K12 MG1655 were evaluated using the microarray technology. In addition, for both the selected microbial strains, the effects of the natural antimicrobials on the membrane fatty acid profiles and the single cell responses to of the whole populations were studied using GC/MS approach and the flow cytometry, respectively.

Finally the effect of (E)-2-hexenal, in combination with high pressure homogenization or traditional thermal treatments, was evaluated on the safety, shelf-life and quality of apple juices inoculated with different food-borne pathogens and spoilage agents.

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Gene expression responses of *Listeria monocytogenes* Scott A exposed to sub-lethal concentrations of natural antimicrobials

Introduction

Microbial cells have adopted proficient defence systems to survive a variety of physicochemical adverse conditions and to face to environmental stresses. The modulation mechanisms are different depending on the species, the strains and the physiological state of the cells (Patrignani et al., 2008;Picone et al., 2013;Patrignani et al., 2015) and regard a wide spectra of metabolic pathways such as a perturbance of the cell wall, membrane, pH and intra-cytoplasmic environment, DNA injuries as well as to ATP production, protein synthesis, and *quorum sensing* (Faleiro, 2011;Siroli et al., 2015a). The persistence of *L. monocytogenes* strains in food-related environments suggests niche adaptation of these strains and therefore constitutes a major risk to consumer health and results in economic losses for the food producers (Cabrita et al., 2015). The ability of *L. monocytogenes* to grow in a wide spectrum of environments depends on its metabolism and to its responses to environmental stress (Dutta et al., 2013). One of the main characteristics of *L. monocytogenes* is the heat resistance and also the resistance to refrigeration temperatures (between 0 and 4°C). In fact, *L. monocytogenes* is able to grow in refrigerated foods such as lettuce (Koseki and Isobe, 2005) and other ready to eat products (Dutta et al., 2013;Siroli et al., 2015c) and generally its growth is not affected by the modified atmospheres applied for fresh-cut vegetables and fruits (Thomas et al., 1999;Castillejo Rodríguez et al., 2000). Moreover, *L. monocytogenes* is able to activate protection strategies to different stressing conditions commonly applied in food processing and storage (Gomes Neto et al., 2015). One of the emerging strategies proposed to prevent the presence and the growth of *L. monocytogenes* in food products, is the use of natural antimicrobial compounds such as essential oils (EOs) or their components alone or in combination with other mild hurdles (Kamdem et al., 2011;Ngang et al., 2014). In addition, the consumer demand for foods with no or few chemical preservatives, has created a market demand for natural, non-thermal and feasible technologies for ensuring the microbial safety of foods (Sivakumar and Bautista-Baños, 2014). EOs and every pure compound extracted from EOs have a specific action and specific target in the microbial cells (Viuda-Martos et al., 2008;Picone et al.,

2013). Their activity depends from their stereo-chemical properties (Helander et al., 1998) and their interaction or synergies with the matrix components (Picone et al., 2013;Siroli et al., 2015a). The use of citral (a mixture of monoterpene aldehydes: genial and neral) and citron EO at different concentrations demonstrated their synergic activity against spoilage and pathogenic microorganisms in fruit salads avoiding the undesirable effects attributable to the cytotoxicity of pure citral (Belletti et al., 2008). Moreover, some EOs, such as rosemary, thyme and oregano and some components of essential oils, like carvacrol, thymol, citral, hexanal and (E)-2-hexenal, are promising natural alternatives to traditional preservatives, since their antimicrobial activity is well documented both in model and real foods (Ivanovic et al., 2012;Patrignani et al., 2015;Siroli et al., 2015b;Siroli et al., 2015c). The presence of thyme and rosemary EOs implied a reduction in the population of *L. monocytogenes* (about 2 log UFC/g) in *Sous Vide* cook-chill beef (Gouveia et al., 2016) the combination of moderate heat, carvacrol and thymol (Guevara et al., 2015) or curcuma (Ngang et al., 2014) explicated a synergistic effect leading to inactivation kinetics values three or four times lower than when using heat alone, also in pineapple juice (Ngang et al., 2014). Exposure to sub-lethal concentration of natural antimicrobials outlined effects on the cell wall components and energy metabolism. A common characteristic of these compounds is their hydrophobic nature. Microbial cells accumulate these natural antimicrobials into the cytoplasmic membrane, where they can elicit several toxic effects that may eventually lead to cell death (Burt, 2004). In particular, in *L. monocytogenes* the grown in presence of sub-lethal concentrations of oregano EO, thyme EO, thymol, carvacrol and citral increased the unsaturated level of fatty acids in the cytoplasmic cell membrane (Dowd et al., 2011). Moreover, the exposure with four different concentrations of carvacrol in *Escherichia coli* 555 afflicted the energy metabolism, proton motive force and glucose accumulation in cells (Picone et al., 2013). These mechanisms were also confirmed by the response of *Salmonella enterica* ser. Thompson MCV1 to sub-lethal concentration of thymol. After thymol exposure *Salmonella enterica* cells become more sensitive to oxidative and osmotic stress due the decompensation in ATP synthesis and outer membrane channel system TolC (Di Pasqua et al., 2006). In this framework, the aim of this study was to evaluate the gene expression mechanisms, by RT-qPCR, of *L. monocytogenes* Scott A exposed for 1 hour to different sub-lethal concentrations of (E)-2-hexenal, citral carvacrol and thyme EOs in order to evaluate the effects of the antimicrobials on some genes involved in cell division, cell wall synthesis, membrane function, transcriptional regulation, nucleotide and protein synthesis, metabolism, and cell motility. *L. monocytogenes* Scott A was selected because this strain possess

one or more virulence determinants that make able to cause systemic infection following inoculation via the gastro-intestinal (Czuprynski et al., 2002). The comprehension of the response mechanisms of food pathogens strains exposed to EO molecules is a fundamental step for the up-scale of the use of those compounds as natural preservatives directly in the food processing (Faleiro, 2011).

Material and methods

Natural antimicrobials

Citral, carvacrol and (E)-2-hexenal were obtained from Sigma-Aldrich (Milano, Italy), while thyme EO was purchased from Flora s.r.l. (Pisa, Italy). Natural antimicrobials stock solutions were diluted in absolute ethanol (Sigma-Aldrich, Milano, Italy) and stored at 4°C until use and for up to a month.

Bacterial strain

L. monocytogenes Scott A belongs to the Department of Agricultural and Food Sciences DISTAL), University of Bologna and stored at -80°C. Before the experiments culture was cultured in brain heart infusion (BHI) broth (Thermo-fisher, Milano, Italy) at 37°C for 24 h. Before the experiment, *L. monocytogenes* was preliminarily grown in BHI broth incubated at 37°C for 24 h.

Exposure to natural antimicrobials

For each exposure assay, 250 mL of fresh BHI broth was inoculated with 2.5 mL of *L. monocytogens* Scott A and incubated at 37°C (4 log CFU/mL). The growth was monitored by the optical density (OD) at λ=600 nm (Spectrophotometer Jenway, Staffordshire, United Kingdom). Reached the middle of the exponential growth phase (OD=0.4, λ=600 nm) cells were exposed to citral, carvacrol, (E)-2-hexenal, thyme EO and 1% absolute ethanol as control. Two hundred µL of natural antimicrobial stock solutions were added to 20 mL of liquid cultures in order to obtain the concentrations reported in Table. Exposure regarded the 1/5, 1/3 and 1/2 of the minimal inhibition concentration (MIC) calculated for *L. monocytogenes* Scott A (Table) (Siroli et al., 2015a).

Cultures were incubated for 1 h at 37°C. All the exposure trials were performed in triplicate. After the treatments 3 aliquots of 1 mL of liquid culture were harvested. Each sample was added of an equal volume of RNA protect Bacteria Reagent (Qiagen Inc., Ontario, Canada) incubated at room temperature for 5 minutes and centrifuged for 7 minutes at 12000 rpm. Supernatants were discarded and cell pellets were stored at -80°C.

Table 1: Natural antimicrobials treatment concentration respectively as 1/5, 1/3 and 1/2 the minimally inhibitory concentration

MIC value	**citral (mg/L)**	**carvacrol (mg/L)**	**(E)-2-hexenal (mg/L)**	**thyme EO (mg/L)**
1/5	50	20	150	40
1/3	85	35	250	70
1/2	125	50	400	100

Total RNA isolation and purification

RNA was extracted using the SV Total RNA Isolation System (Promega, Wisconsin, USA). The yield and the purity of each extraction was determined by measuring the ABS at 260 nm and the 260/280 nm ratio using a BioDrop µLITE (BioDrop, Milan Italy). The yields were about 15 ng/µL for all the samples and only samples with a ratio 260/280 nm above 1.9 were used for the reverse transcription reaction.

cDNA first strand synthesis

The reverse transcription into cDNA was performed according to Serrazanetti et al., 2015. Before real time assays, samples were properly diluted in DNAse/RNAse free water (Promega, Wisconsin, USA) to reach a final concentration of 5 ng/µL.

Reverse Transcription quantitative PCR (RT-qPCR)

The best reaction conditions for each primer stets, were investigated by end point PCRs using genomic DNA as a template. Different $MgCl_2$ final concentrations (2.00, 3.00, 4.00 mM) and annealing temperatures (AT) were tested. Amplification quality was verified by gel electrophoresis

using 1.5% agarose gels. Results are reported in the Table. RT-qPCRs were performed using a Rotor gene 6000 thermal cycler (Corbett Life Science, Mortlake, Australia). The list of genes and their function is reported in Table. The RT-PCR reaction mixture (25 µL) included 5 ng of cDNA, 12.5 µL of SYBR Premix Ex Taq II (TaKaRa Bio Inc., Japan), 0.5 µM of each primer and 6.5 µL DNAse/RNAse free water (Promega, Wisconsin, USA). Each reaction was performed in triplicate. For each gene, a threshold line and quantitative cycle (Cq) were determined using the Rotor-Gene series software (Qiagen Inc., Ontario, Canada). Genomic DNA standard curves (5 points of dilutions) for each of the target genes were included in each assay to account for differences in the amplification efficiencies (E) and to serve as a positive control points according to the model defined by . The E value for each primer pair is reported in Table.

Relative gene expression analysis

The relative gene expressions (RGEs) were determined according to the MIQE guidelines (Bustin et al., 2009) using the mathematical model proposed and reviewed by Pfaffl, 2012. Reference genes (RGs) were chosen from a pool of candidate genes: *ccpA* (catabolite control protein A), *rpoB* (DNA-directed RNA polymerase subunit beta), *tufA* (transcription elongation factor Tu) and 16S-rRNA (Lane, 1991) by the evaluation of different statistical parameters, using the BestKeeper© tool program (Pfaffl et al., 2004;Tasara and Stephan, 2007).

Statistical analysis

Three independent replicates were performed for all experiments. Means were compared using one way-ANOVA in order to assess the significance of the results obtained and the levels of gene over- and under- expression using the R software (R Core Development Team, 2017). Differences with $p<0.05$ were considered as statistically significant. Concerning the evaluation of relative gene expression levels: the overexpression was decided on the basis of the significance ($p<0.05$) of the differences with the untreated cells as major than 1.10 RGE, as well as the under expression was considered lower than 0.90.

Table 2: . RT-qPCR oligonucleotide primers used in this study.

	Gene	Forward primer (5'>3')	Reverse primer (5'>3')	Protein/function	Reference	Reaction efficiency E	$MgCl_2$ mM	Annealing temperature °C
Cell division/DNA repression and modulation	ftsE	AGGCTAAAGAGCCCACACAA	TCTTCGACGGGAGAAAATTG	Cell division ATP-binding protein FtsE	(Bowman et al., 2008)	2.13	4	56.3
	ftsZ	CAATGAAAGAAGCGGTGGAT	ATTCCCATAAGGGCAGAACC	Cell division protein FtsZ	(Bowman et al., 2008)	2.03	4	56.3
	hup	GCAGCGAAAGCAGTAGAAGC	AAGCGCTTTACCAGGTTTGA	DNA-binding protein HU	(Bowman et al., 2008)	1.88	3	57.3
Energy Metabolism	opuCA	ACATCGATAAAGGAGAATTTGTTTGTT	CGTTTTCCCACAACCACTTGGACCG	Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA	(Sue et al., 2004)	2.05	4	57.2
	pdhD	AACAGGATCTCGTCCAATCG	CTGGACCACCCTCAAGGATA	Dihydrolipoyl dehydrogenase (Dihydrolipoamide dehydrogenase)	(Bowman et al., 2008)	1.90	2	58.3
	pgm	TTGGCATGATGTGGACTTGT	TCAAGCGCCAAGATTTATGA	Phosphoglycerate mutase	(Bowman et al., 2008)	2.14	2	54.2
RNA and protein syntesis	fusA	GTGAAACCCATGAAGGTGCT	TGCATCTAGAACCGCAACAG	Translation elongation factor G	(Bowman et al., 2008)	2.13	2	57.3
	rpoC	CCGTATGCAAGGGGTAGAAA	TAGCTTCACGGTTGGCTTCT	DNA-directed RNA polymerase subunit beta'	(Bowman et al., 2008)	2.20	2	57.3

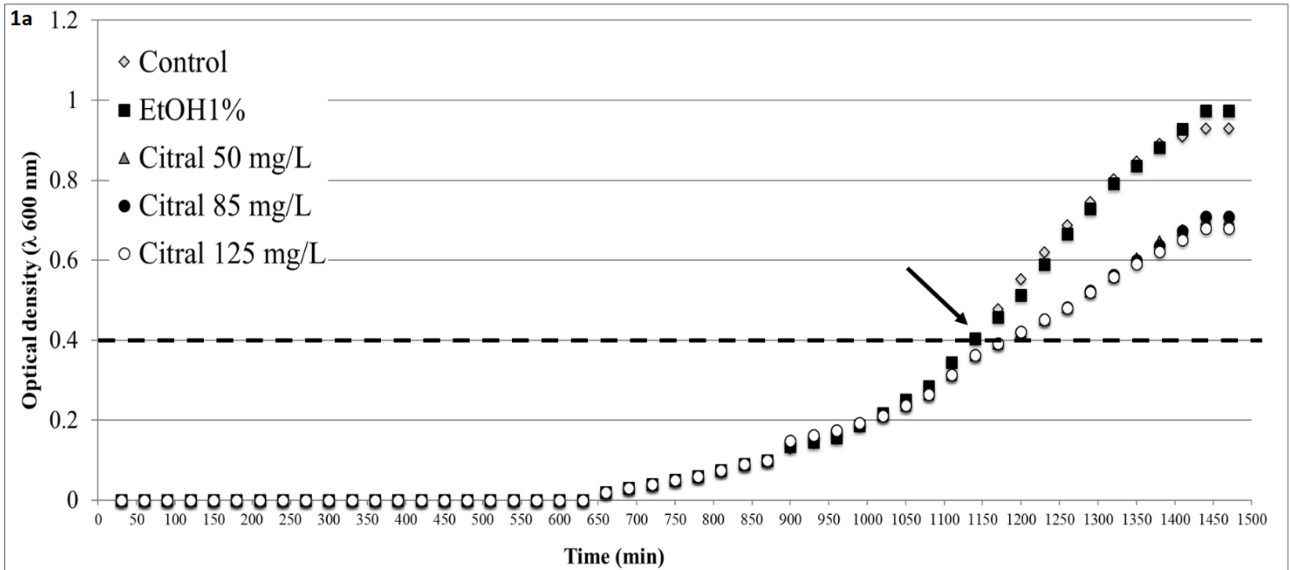
	Gene	Forward primer (5’>3’)	Reverse primer (5’>3’)	Protein/function	Reference	Reaction efficiency E	$MgCl_2$ mM	Annealing temperature °C
Stress Response	cspL	CGCAGAAAAAGGATTTGGTT	TGAACGTTAGCTGCTTGAGG	Cold shock-like protein CspLA	(Bowman et al., 2008)	2.20	2	55.2
	fri	TTACTAGCAATCGGCGGAAG	CATTGTCGCCTTCTTTGTCA	Non-heme iron-binding ferritin	(Bowman et al., 2008)	2.15	2	56.3
	gadB	AATACCTTGCCCATGCAGTC	AGTGGATATGCGGGAACTTG	Glutamate decarboxylase	(Bowman et al., 2008)	2.12	2	57.3
	lmo0669	TCAAGCTATCAAGGCGCTAATAAA	CCGACCAATTCCGGAGTCT	Lmo0669 protein putative oxidoreductase	(Sue et al., 2004)	2.11	3	58.2
	rpoE	GGGAGCGTCTTGTTCAATTT	CCAAGCTCTTCCACGATTTC	DNA-directed RNA polymerase subunit delta	(Bowman et al., 2008)	2.19	3	58
Virulence and Motility	bsh	GGCCTTAGTATGGCAGGACTCA	CTCATTGTCCTTACCTTCTGCAAA	Bile salt hydrolase	(Sue et al., 2004)	2.15	2	60.7
	flaA	CGTGAACAATCAATCCATCG	ACATTTGCGGTGTTTGGTTT	Flagellin	(Bowman et al., 2008)	2.15	2	54.2

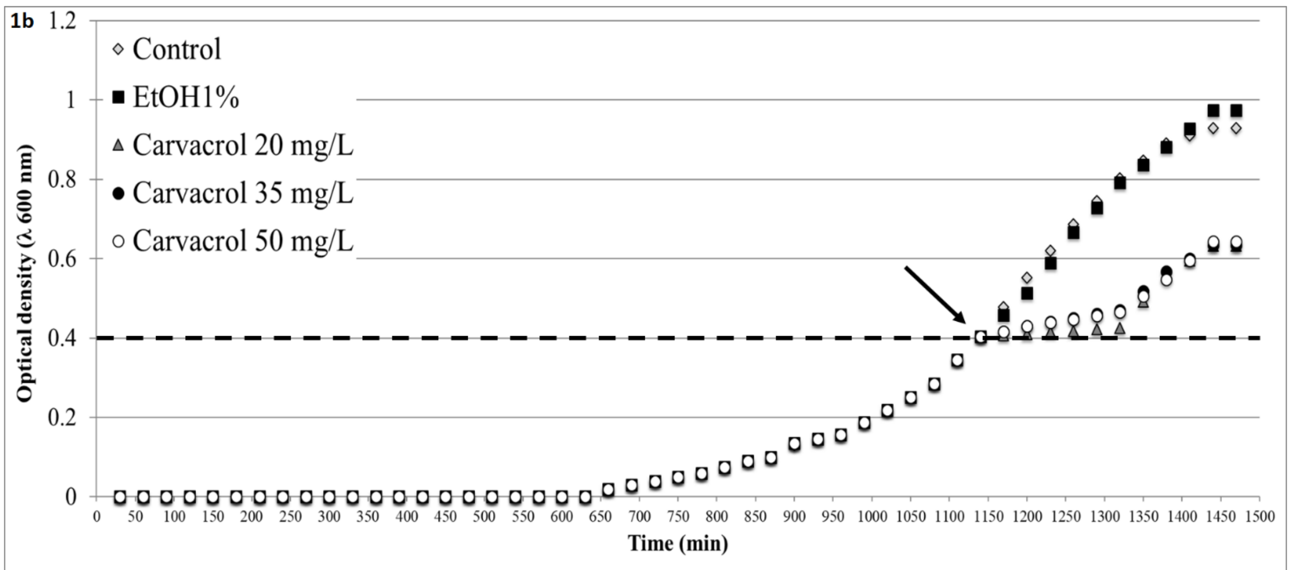
	inlA	GGTCTCACAAACAGATCTAGACCAAGT	TCAAGTATTCCACTCCATCGATAGATT	Internalin-A	(Sue et al., 2004)	2.11	3	64.6
Reference gene tested	16S rRNA	CCTACGGGAGGCAGCAG	GTATTACCGCGGCTGCTG	-	(Lane, 1991)	2.01		
	ccpA	GGAGCCGTTGATATGGAAAA	ATTTCATTTCGCGATTGACC	Catabolite control protein A	(Bowman et al., 2008)	2.18	3	54.2
	rpoB	TGTAAAATATGGACGGCATCGT	GCTGTTTGAATCTCAATTAAGTTTGG	DNA-directed RNA polymerase subunit beta	(Sue et al., 2004)	2.17	2	57.5
	tufA	TGGCGATGACATTCCTGTAA	CTGGCATCATGAATGGTTTG	Elongation factor Tu	(Bowman et al., 2008)	2.92	2	55.3

Results

Growth kinetics of *L. monocytogenes* Scott A in relation to different sub-lethal concentrations of natural antimicrobials

L. monocytogenes Scott A was subjected to different sub-lethal concentrations of carvacrol, citral, (E)-2-hexenal and thyme EO in order to comprehend the response in terms of growth kinetics also in relation to their concentrations. In particular, the concentrations used corresponded to 1/2, 1/3 and 1/5 of the MIC, previously assessed by (Siroli, Patrignani, Gardini, et al., 2015). In this study, the effects of the antimicrobials were determined on the basis of growth dynamics of *L. monocytogenes* exposed to the selected substance when OD = 0.4 (λ=600 nm) was reached in BHI medium. In figures 1a, 1b, 1c and 1d the growth curves, obtained by spectrophotometer absorbance, were reported on the basis of the molecule added as antimicrobials. In general, the exposition of *L. monocytogenes* Scott A cells to the different antimicrobials resulted both in a reduction of the curve slope and of the maximum OD levels reached in the presence of different antimicrobials tested (figures 1a, 1b, 1c and 1d). Regarding the exposition of the cells to 1% of ethanol, no differences were outlined compared to the control. The growth inhibition was dependent on the natural antimicrobial employed and on its concentration, with the exception of citral and carvacrol exposition. In fact, in the presence of these latter molecules, independently to their concentrations, the growth of *L. monocytogenes* was always retarded. By contrast, the presence of (E)-2-hexenal and thyme EO implied a growth response related to their concentrations. In particular, thyme EO added at 70 and 100 mg/L (1/3 and 1/2 of the MIC respectively) entailed a clear inhibition of the growth of *L. monocytogenes*. Also, the results obtained by plate counting confirmed this behaviour (data not shown).


1a
Control
EtOH1%
Citral 50 mg/L
Citral 85 mg/L
Citral 125 mg/L
Optical density (λ 600 nm)
Time (min)


1b
Control
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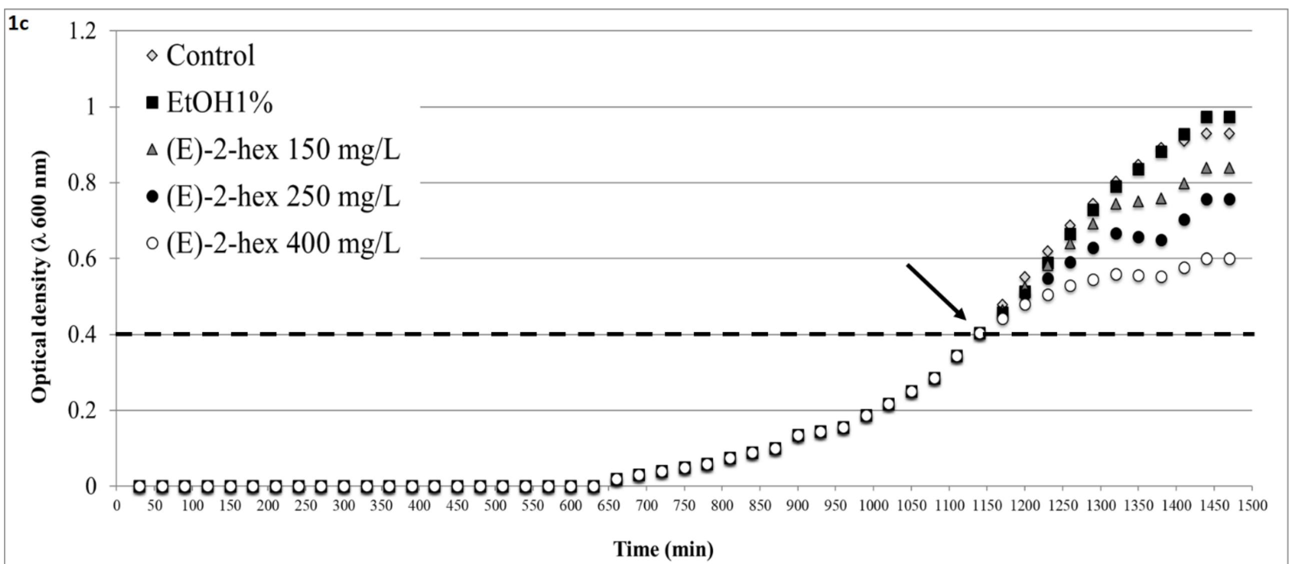
Carvacrol 20 mg/L
Carvacrol 35 mg/L
Carvacrol 50 mg/L
Optical density (λ 600 nm)
Time (min)


1c
Control
EtOH1%
(E)-2-hex 150 mg/L
(E)-2-hex 250 mg/L
(E)-2-hex 400 mg/L
Optical density (λ 600 nm)
Time (min)

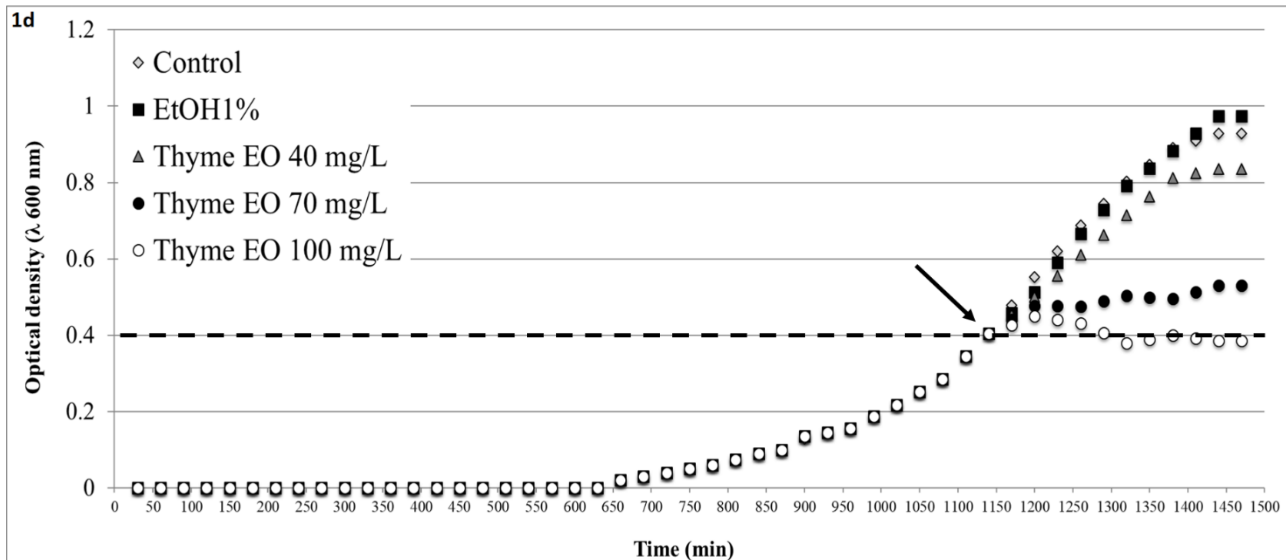


*Figure 1: growth curves of L. monocytogenes Scott A in optimal condition (BHI, 37 °C) and after the exposition to ethanol (1%) or the natural antimicrobials (1/2, 1/3 and 1/5 of the MIC): **1a**) citral; **1b**) carvacrol; **1c**) (E)-2-hexenal; **1d**) thyme EO. The exposition, for all the samples, started at the mid of the exponential phase (OD=0.4, λ = 600 nm), at the point indicated by the arrow.*

Selection of the most suitable reference genes: reference gene comparison by BestKeeper© Software

The expression stability of four potential reference genes (*ccpA*, *rpoB*, *ftsZ* and *16S rRNA*), in *L. monocytogenes* both in optimal growth condition and when exposed to 13 different stress conditions, was assessed according by BestKeeper© tool programme (Pfaffl et al., 2004) (data not shown). *ccpA,* codifying for catabolite control protein A, was selected as reference gene on the basis of its correlation and stability in the tested exposure conditions (data not shown). On the basis of the same parameters, also *rpoB,* codifying for the DNA-directed RNA polymerase subunit beta, was selected. In particular, in the sub-lethal stress condition imposed by natural antimicrobial exposition, the stability of the reference genes is ranked in the following order: *ccpA>rpoB>ftsZ>16S rRNA*.

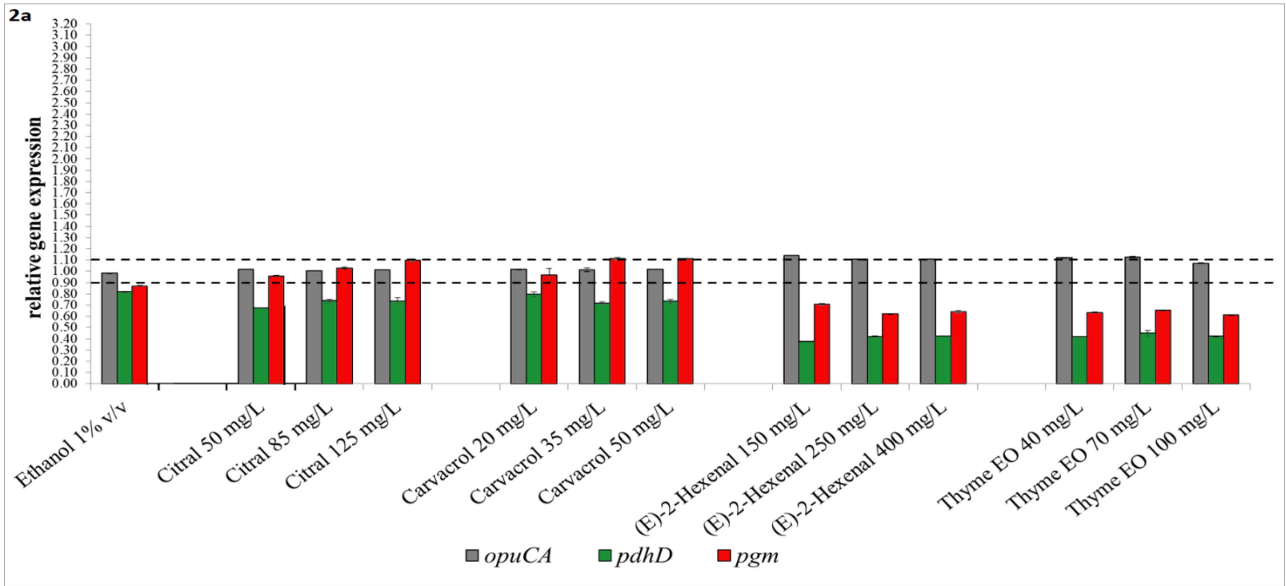
Relative gene expression of *L. monocytogenes* strain Scott A exposed to sub-lethal concentrations of natural antimicrobials on the basis of different metabolism pathways

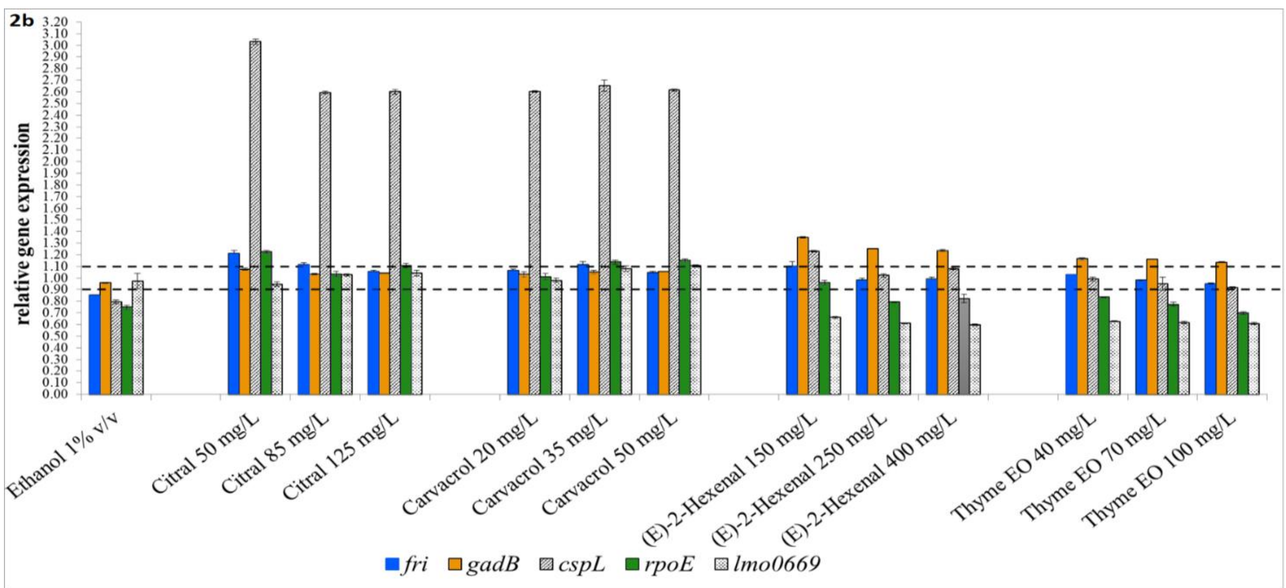
To assess the direct effect of sub-lethal concentrations of natural antimicrobials, citral, carvacrol, (E)-2-hexenal and thyme EO the relative level expression of the genes selected was analysed by RT-qPCR. In particular, the target genes selected (Table 2) are involved in pathways related to

different biological functions such as energetic metabolism, stress response, cell division, virulence and motility. Some of those belong to the alternative sigma factor involved both in the stress response (*opuCA* and *lmo0669*) and in the virulence mechanisms such as *inlA* (Sue, Fink, Wiedmann, Boor, & Kathryn Boor, 2004). The genes were sub-grouped on the basis of the metabolic pathways considered in order to pinpoint the role of the antimicrobial compounds utilised in relation also to their concentrations. It was observed that *L. monocytogenes* Scott A exhibited differential gene expression in relation to the molecule used as antimicrobial, while, in some cases, these differences are related to their concentration. Citral and carvacrol showed the same mechanism of action as well as (E)-2-hexenal and thyme EO giving raise the same gene response patterns. With respect to the exposition of *L. monocytogenes* to ethanol 1%, the presence of the natural antimicrobials tested induced changes mainly correlated ($p<0.05$) with the energy metabolism, the stress response, the cell division and DNA synthesis and repair.

In the figures 2a, 2b, 2c and 2d the results regarding the relative gene expression of *L. monocytogenes* exposed to ethanol at 1% and to different sub-lethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO are reported. It is important to outline that the same results were obtained also using *rpoB* as RG (supplementary data, table S4). The citral addition, when the cells of *L. monocytogenes* reached an OD = 0.4 (λ=600 nm), implied the modification of the expression of the genes involved in energy metabolisms (figure 2a), stress response (figure 2b), cell division/DNA repression and modulation (figure 2c) and virulence and motility (figure 2d). In fact, the presence of citral, independently on its concentration, caused the under expression of: *pdhD* (figure 2a), a dihydrolipoyl dehydrogenase (also known as dihydrolipoamide dehydrogenase) involved in the Krebs cycle; *hup* (figure 2c), a gene involved in the prevention of the DNA denaturation under extreme environmental conditions; and *flaA*, flagellar motility genes (figure 2d). In particular, the *hup* gene was repressed in all the conditions tested, with the exception of ethanol 1%. In the presence of (E)-2-hexenal and thyme EO, *hup* was strongly under expressed confirming that those conditions implied a reduction of the treated cells to repair the eventual DNA damage created by exposure to the molecules added as antimicrobials. The expression of *cspL* gene (figure 2b) increased, in particular when citral was added at 50 mg/L, corresponding to the 1/5 of the MIC. This treatment also induced the overexpression of *rpoE* (figure 2b), while the other concentrations didn't imply significant modifications of the expression of this gene belonging to the extra-cytoplasmatic sigma factor. An opposite behaviour was outlined by *lmo0669* (putative oxidoreductase) that was under expressed (figure 2b). In this case, it is possible

to underline how the concentration of 50 mg/L for citral can be considered a threshold concentration able to activate specific response in *L. monocytogenes* Scott A. Carvacrol exposition implied the same trend of gene expression described for citral, but in this case a threshold concentration for specific response levels, was not evidenced. Energy metabolism, general stress response and cell division were mainly affected by (E)-2-hexenal and thyme EO (figures 2a, 2b and 2c respectively). It is interesting to outline how (E)-2-hexenal and thyme EO implied a strong under expression of *pdhD* and *pgm* (figure 2a), involved in glycolysis, of *rpoE* and *lmo0669* (figure 2b), involved in the general stress response, and of *ftsE*, *hup* and *fusA* involved in the cell division and in DNA modulation and repression under environmental stress conditions (figure 2c). On the contrary, *rpoC* (DNA-directed RNA polymerase subunit beta) was overexpressed in the presence of (E)-2-hexenal, mainly at 150 and 400 mg/L, and thyme EO, at 40 and 70 mg/L (figure 2c). Also, *gadB* was minimally over expressed in the presence of (E)-2-hexenal (figure 2b). *bsh* gene, encoding the principal bile-resistance mechanism of *L. monocytogenes*, was significantly overexpressed (at different levels) in all the condition tested, in particular, when citral was added at 50 mg/L (figure 2d). As in part described before, the expression of *pdhD* was affected when the cells of *L. monocytogenes* were exposed to sub-lethal concentrations of ethanol, citral, carvacrol, (E)-2-hexenal and thyme EO (in all the conditions tested the relative gene expression of *pdhD* was always under 0.9), and contemporary also the expression of *pgm* was repressed in the presence of (E)-2-hexenal and thyme EO (figure 2a). Concerning the genes *opuCA*, probably responsible for energy coupling to the transport system and also involved in the osmoprotection and cryoprotection of the cells (figure 2a), *fri*, involved in the DNA protection of *L. monocytogenes* with respect to oxidative stress (figure 2b), *ftsZ*, coding for an essential cell division protein that forms a contractile ring structure (Z ring) at the future cell division site (figure 2c), and *inlA*, that mediates the entry of *L. monocytogenes* into cells, no modification have been detected in their expression in all the conditions tested.


2a
relative gene expression
Ethanol 1% v/v
Citral 50 mg/L
Citral 85 mg/L
Citral 125 mg/L
Carvacrol 20 mg/L
Carvacrol 35 mg/L
Carvacrol 50 mg/L
(E)-2-Hexenal 150 mg/L
(E)-2-Hexenal 250 mg/L
(E)-2-Hexenal 400 mg/L
Thyme EO 40 mg/L
Thyme EO 70 mg/L
Thyme EO 100 mg/L
opuCA
pdhD
pgm


2b
relative gene expression
Ethanol 1% v/v
Citral 50 mg/L
Citral 85 mg/L
Citral 125 mg/L
Carvacrol 20 mg/L
Carvacrol 35 mg/L
Carvacrol 50 mg/L
(E)-2-Hexenal 150 mg/L
(E)-2-Hexenal 250 mg/L
(E)-2-Hexenal 400 mg/L
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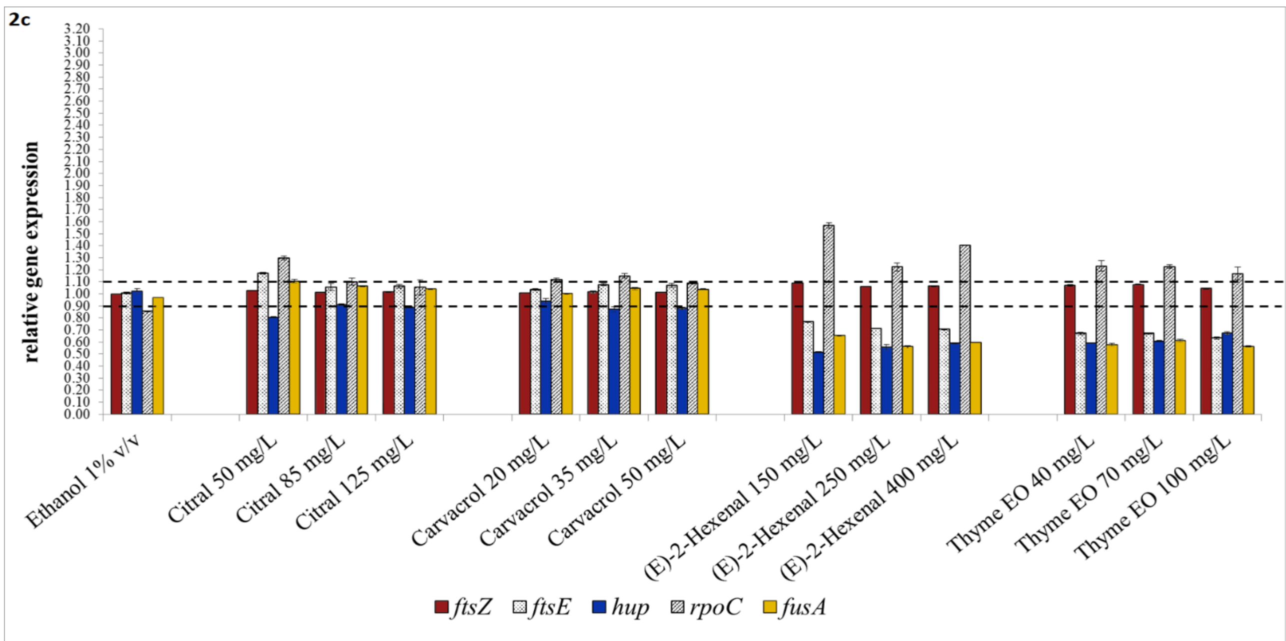
2c
relative gene expression
Ethanol 1% v/v
Citral 50 mg/L
Citral 85 mg/L
Citral 125 mg/L
Carvacrol 20 mg/L
Carvacrol 35 mg/L
Carvacrol 50 mg/L
(E)-2-Hexenal 150 mg/L
(E)-2-Hexenal 250 mg/L
(E)-2-Hexenal 400 mg/L
Thyme EO 40 mg/L
Thyme EO 70 mg/L
Thyme EO 100 mg/L
ftsZ
ftsE
hup
rpoC
fusA

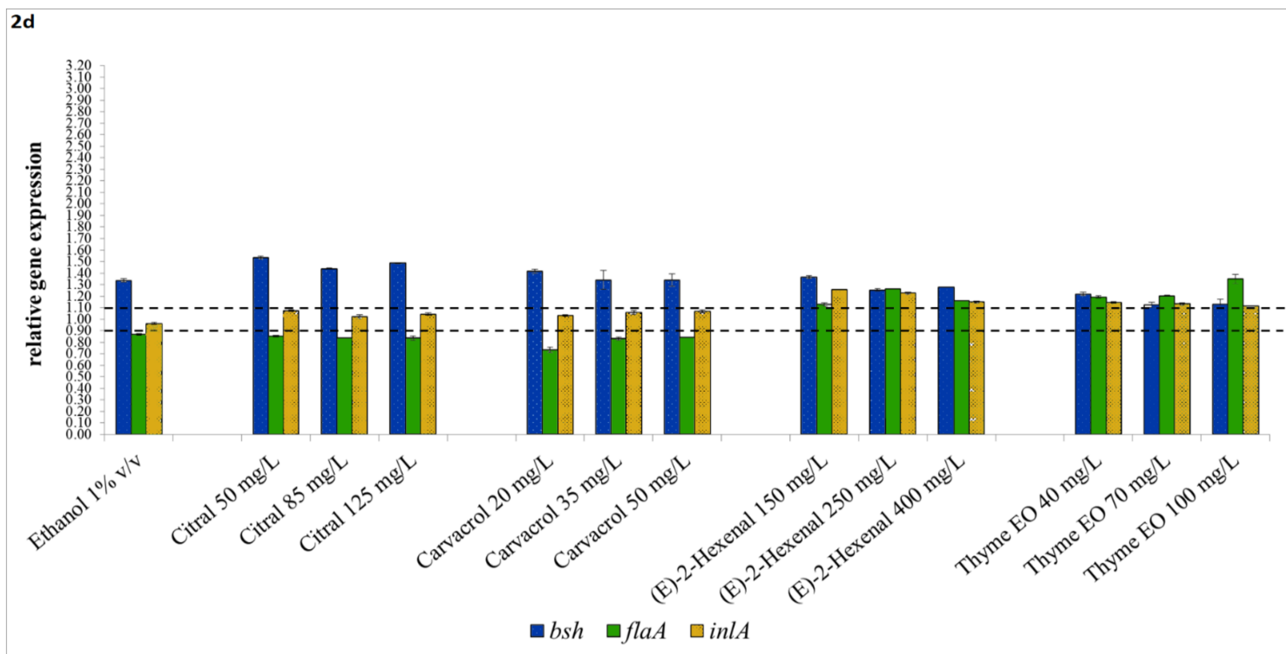

*Figure 2: Relative gene ex pression (rge) of the genes involved in the energy metabolism (**2a**), stress response (**2b**), in cell division, DNA repression and modulation (**2c**) and virulence and motility (**2d**) calculated with the untreated sample as a control and samples exposed to ethanol (1%), citral, carvacrol, (E)-2-hexenal and thyme EO at 1/2, 1/3 and 1/5 of the MIC. The gene products are as follows: opuCA, Glycine betaine/carnitine/choline transport ATP-binding protein OpuCA; pdhD, Dihydrolipoyl dehydrogenase (Dihydrolipoamide dehydrogenase); pgm, Phosphoglycerate mutase; fri, Non-heme iron-binding ferritin; gadB, Glutamate decarboxylase; cspL, Cold shock-like protein CspLA; rpoE DNA-directed RNA polymerase subunit delta; lmo0669, Lmo0669 protein putative oxidoreductase; ftsE, Cell division ATP-binding protein FtsE; ftsZ, Cell division protein FtsZ; hup, DNA-binding protein HU; rpoC, DNA-directed RNA polymerase subunit beta'; fusA, translation elongation factor G; bsh, Bile salt hydrolase; flaA, Flagellin; inlA, Internalin-A. Error bars indicate standard deviations of the means for the three experiments.*

Discussion

In this study, the generic response of *L. monocytogenes* Scott A to different natural antimicrobials (citral, carvacrol, (E)-2-hexenal and thyme EO), used at different sub-lethal concentrations (1/2, 1/3 and 1/5 of the MIC), was explored to determine how this microorganism reacts and can develop resistance mechanisms and to evaluate their potential use as natural preservatives in food industry. Unexpectedly, on the basis of the first tests developed in order to select the most suitable reference gene for the conditions analysed, *ccpA* evidenced the best features (data not shown). These data are not in agreement with previous studies in which this gene can modify its expression in dependence on the environment, particularly at low temperatures (Wouters et al.,

2000;Duché et al., 2002;Cacace et al., 2010). In fact, usually *16S rRNA* is commonly used as the best reference gene when *L. monocytogenes* is exposed to different stress conditions (Tasara & Stephan, 2007). On the other hand, different reference genes are reported for *L. monocytogenes* depending on the stress applied and on the tested environmental conditions (McGann et al., 2007;Werbrouck et al., 2007). The data obtained showed shared response mechanisms of *L. monocytogenes* Scott A to sub-lethal concentrations of citral and carvacrol and of thyme EO and (E)-2-hexenal outlining how the unsaturated aldehydes citral and (E)-2-hexenal, characterised by a common chemical structure, gave raise to completely different gene expression patterns. This common behaviour demonstrated as the specific responses in the microbial cells depends not only to the chemical structure but also to their MIC, to their activity and to the absolute volume of the antimicrobials added to the cells. In particular, the bactericidal or bacteriostatic effects of the tested compounds were evidenced by the response in the cell load after the exposition in the first 60 minutes and over the further incubation (figures 1a, 1b, 1c and 1d). The results reported in figure 2a, evidenced how *pdhD* was under expressed, while *pgm* was under expressed only in the presence of (E)-2-hexenal and thyme EO. These data evidenced the unbalance in the glycolysis pathway of *L. monocytogenes* in the presence of sub-lethal concentration of (E)-2-hexenal and thyme EO. These modifications outlined as, in the sub-lethal conditions tested, glycolysis has an important role in keeping up a minimal level of catabolic mechanism. The under-expression of *pgm,* after 30 min and the over expression of *pdhD* after 60 min of exposition to sub-lethal stresses in *L. monocytogenes* was previously described in the presence of increasing concentrations of NaCl (Duché et al., 2002). The phosphoglycerate mutase (*pgm*) is involved in the step 3 of the sub-pathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphatethis, and the second one (*pdhD*) is a pyruvate dehydrogenase that converts pyruvate to acetyl-coenzyme A, ethanol, lactate, or other small molecules. Their behaviour, in the presence of the antimicrobials assessed and, in particular, their repression in the presence of (E)-2-hexenal and thyme EO, suggests an hypothetical shift in the metabolism of *L. monocytogenes* Scott A from the oxidation to fermentation. The same metabolic switch was evidenced in *L. monocytogenes* in response to extracellular pH changes (Nilsson et al., 2013), in which a clear energy generation shift towards fermentation in the presence of alkaline environment was demonstrated in particular by the over expression of the same genes (*pgm* and *pdhD*). The proteins codified by *pdhD* and *pgm* were overexpressed also at 4°C (Cacace et al., 2010). Otherwise, it is known that bacterial metabolism is a complex network of interacting pathways, and negative effects on one pathway often lead to

compensatory adjustments in other pathways (a form of homeostasis), according to different mechanisms depending on the kind of stress and time of exposure (Goh et al., 2002). Consequently, different stresses can generate the same general metabolic switch (from respiration to fermentation) thought different coordinate changes in gene expression and rate of transcription demonstrating how *L. monocytogenes* is able to differently modulate its energy generation source in response to different growth conditions (Lungu et al., 2009). The exposure to the natural antimicrobials considered can also influence *L. monocytogenes* pathogenicity, as demonstrated by bile salt hydrolase (*bsh*) over-expression in most of the tested conditions. In fact, in the presence of ethanol, citral, carvacrol and minimally (E)-2-hexenal the expression of *bsh*, involved in the virulence and motility responses, increased. In particular, *L. monocytogenes* responded with the over expression of *cspL* and *bsh* to citral and carvacrol exposition. The gene *cspL* is involved in the cold shock stress response and can be activated also in the presence of other types of stresses such as HHP (Bowman et al., 2008). Bile salt hydrolase (*bsh*) is usually overexpressed, by *L. monocytogenes*, when subjected to bile salt stress in order to resist to the gastrointestinal tract adverse conditions and their bactericidal effect (Begley et al., 2010;Dowd et al., 2011). Bile tolerance is generally drive by many genes (*btlB*, *sigB*, *pva*, *prfA* and *btlA*) but the deletion of *bsh* implied a 2-fold reduction in the MIC of bile (Dowd et al., 2011). Another interesting result regarded the evidence of a threshold concentration for the activation of a repair response in the presence of citral 50 mg L^{-1}. In particular, a higher expression of *cspL* and *rpoC* was evidenced. Moreover, the conditions in which the cells were exposed to (E)-2-hexenal and thyme EO interfered negatively with the response to *L. monocytogenes* in terms to DNA protection, stabilization and prevention of its denaturation, with the translocation step during the translation elongation and with the cells division by the strong under expression of *hup*, *fusA* and *ftsE*, respectively (figure 2c). In optimal conditions the histone like proteins, in this case codified by *hup*, can be involved in the control of gene expression (Mekalanos, 1992). In the presence of the sub-lethal stresses induced by (E)-2-hexenal and thyme EO, *hup* was under expressed, showing a clear effect in the control of the cells biochemical mechanisms and in cells division (also *fusA* and *ftsE* were under-expressed). On the contrary, the expression of the proteins codified by *fusA* and *ftsE* were increased in the presence of cold adaptation of *L. monocytogenes* (Cacace et al., 2010). Glutamate decarboxylase *(gadB)* was lightly overexpressed only in the presence of (E)-2-hexenal. This gene is co-transcribed in tandem with an upstream gene, *gadC*, which encodes a potential glutamate/γ-aminobutyrate antiporter. Expression of this transcript (*gadB* and *gadC*), in *L.*

monocytogenes, is up-regulated in response to mild acid stress (Dowd et al., 2011). In fact, it is involved in the cell pH homeostasis, converting glutamate to γ-aminobutyrate (GABA). Consequently is also involved in the glutamate-dependent acid resistance in gastric fluid (Feehily et al., 2013). This assumption can suggest a minimal activation of the acidic stress response in the presence of (E)-2-hexenal. Moreover, the GABA obtained by the decarboxylation of glutamate, can be metabolised and converted to succinate. Hypothetically this pathway can compensate the incomplete tricarboxylic acid (TCA) cycle of *L. monocytogenes* (Feehily et al., 2013).

Conclusions

The results showed that citral and carvacrol when used at sub-lethal concentrations induce in *L. monocytogenes* Scott A an overexpression *cspL* and *bsh* genes. Citral at 50 mg/L represent a threshold concentration, able to maximize the expression of *cspL* and *bsh*. (E)-2-hexenal and thyme EO created a clear unbalance in the energy metabolism determining a shift from respiration to fermentation, under-expressing *pgm* and *pdhD* involved in glycolysis. Moreover, (E)-2-hexenal and thyme EO inhibited the expression of the genes involved in the stress response, in proteins synthesis and in DNA protection and repair after environmental shock. These data keep the attention on the need to choose the concentrations over the MIC to avoid the activation of virulence factors, such as bile salt hydrolase (*bsh*). However, since the natural antimicrobials used as a marked effect on the sensory properties of food, due to their low sensory threshold, their application at industrial level, as antimicrobials, requires the identification of combined strategies able to deactivate or under express also the virulence genes. This knowledge is fundamental to comprehend how those antimicrobials can be used in a conscious way applied in the food system in order to avoid *L. monocytogenes* resistance mechanisms.

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Response mechanisms of *Escherichia coli* to citral, carvacrol, (E)-2-hexenal and thyme essential oil.

Introduction

Essential oils (EOs) are aromatic and volatile compounds extracted from whole plants as well as from plant material such as flowers, roots, leaves, seeds, peel, fruits and wood (Hyldgaard et al., 2012). These molecules are produced by plants as secondary metabolites for defense purposes and some of the EOs are well known for their antimicrobial properties (Tajkarimi et al., 2010). The historical use of EOs was in medicine, perfumery, cosmetics, and they are also added to foods as part of spices or herbs. Generally, EOs contain 20–60 constituents at different concentrations. EOs are characterized by two or three major components at fairly high concentrations (20–70%) compared to other compounds present in trace amounts (Burt, 2004;Bakkali et al., 2008). In *vitro* studies showed that thyme EOs possess antimicrobial activity against a broad spectrum of Gram-negative or Gram-positive bacteria as well as yeasts and moulds (Burt, 2004;Solomakos et al., 2008). Carvacrol is, in addition to thymol, one of the main components of thyme and oregano EOs; it is a phenolic monoterpenoid with a strong antimicrobial activity against a wide range of pathogenic microorganisms (Bagamboula et al., 2004;Oussalah et al., 2007) and fungi (Kordali et al., 2008). Aldehydes such as (E)-2-hexenal and citral, which are components of the aroma of many fruits and vegetables, are characterized by a strong antimicrobial activity both in model and food systems (Lanciotti et al., 2004). In particular, antimicrobial action against bacteria, yeasts and moulds in different conditions has already been demonstrated for citral (3,7-dimethyl-2-7-octadienal), which naturally occurs in citrus EOs. It is an acyclic α,β-unsaturated monoterpene aldehyde that exists as the two isomers geranial and neral (Belletti et al., 2008;Leite et al., 2014).
In addition, some essential oils appear to exhibit particular medicinal properties that have been claimed to cure some organ dysfunctions or systemic disorders. EOs and some of their components are generally recognized as safe (GRAS) by the FDA and EFSA (Moreira et al., 2005;FDA, 2014).
EOs are used in the food industry as flavoring agents since many years. Because of the antimicrobial properties of some of the EOs (Cosentino et al., 2003), their application as food preservatives is very promising particularly in minimally processed fruits and vegetables (Siroli et

al., 2014;Siroli et al., 2015b), meat products (Jayasena and Jo, 2013), beverages (Patrignani et al., 2013) and dairy products (Lucera et al., 2012). However, their use as preservatives in traditional foods requires a deeper knowledge about the microorganisms they can target, their interaction with food matrix components and their modes of action. In fact, the mechanisms of action of most EOs are not or not fully understood (Hyldgaard et al., 2012;Picone et al., 2013)

Given their structural differences and the presence of different functional groups, the mechanism of the antibacterial activity of the various EO components will most likely not be the same and there may be several specific targets in the cell (Burt, 2004). Generally, it is accepted that EOs and their active molecules can lead to degradation of the cell wall, damage of the cytoplasmic membrane and membrane proteins, leakage of cellular contents, coagulation of cytoplasm, depletion of the proton motive force, or more general perturbation of energy metabolism (Burt, 2004;Picone et al., 2013). Evaluating the effect of carvacrol on the *Escherichia coli* 555 metabolome using 1H-NMR spectroscopy, Picone et al. (2013) showed a shift from respiration toward fermentation as the concentration of carvacrol increased due the decrease of fumarate, succinate and citrate present in the respiratory pathway of *E. coli*.

In order to promoting the use EOs and their components in the food industry it is necessary to better understand which stress responses are induced by the addition of these natural antimicrobials to pathogenic and spoilage microorganisms. Microorganisms come across several different stress conditions in, particularly, minimally processed foods and it is well known that they respond to these stresses by regulating gene expression and protein profiles. Stress responses can allow surviving more stringent conditions, augment resistance to consequent processing conditions, and/or increase virulence in pathogens (Chung et al., 2006). Thus, understanding the effects of stress on the physical tolerance of pathogens is important in order to evaluate and minimize the risk of food-borne illness (Chung et al., 2006).

Throughout the 1990s and nowadays, food-associated pathogens are a leading cause of food-borne diseases, which command a host of research and surveillance attention from governments and critical alertness from the food industry (Newell et al., 2010). In particular, enterohemorrhagic and enterotoxigenic strains of *E. coli* are widely recognized as very important causes of food-borne illness over the last two decades. Up to a decade ago, the sources of *E. coli* outbreaks were most often contaminated beef meat, but nowadays almost any source that could have been in contact with animal faeces is a potential risk, including vegetables, sprouts, fruits, meat products (such as dry fermented sausages), juices, unpasteurized apple cider and milk (both pasteurized and

unpasteurized) as well as faecally-contaminated drinking water and beverages (Newell et al., 2010). Researchers are currently trying to find natural methods to reduce pathogens and at the same time to ensure the safety and quality of the products (Severino et al., 2015).

The main aim of this work was to study the stress response to natural antimicrobials of the *E. coli* model strain K12 MG1655. The effects on whole-genome gene expression (the transcriptome) of sub-lethal concentrations of thyme EO and some of the major components of EOs such as carvacrol, citral and (E)-2-hexenal were studied in depth using DNA microarray technology.

Material and Methods

Natural antimicrobials

Citral, (E)-2-hexenal, and carvacrol were purchased from Sigma-Aldrich (Milano, Italy). Thyme EO was obtained from Flora s.r.l. (Pisa, Italy). The natural antimicrobials were stored at 4 °C. The chemical structure of citral, (E)-2-hexenal, and carvacrol is reported in Figure 1. Thyme EO used in this work was previously characterized through GC/MS-SPME analyses (Siroli et al., 2015b), and the composition and the relative percentages of each compound are reported in Table 1.

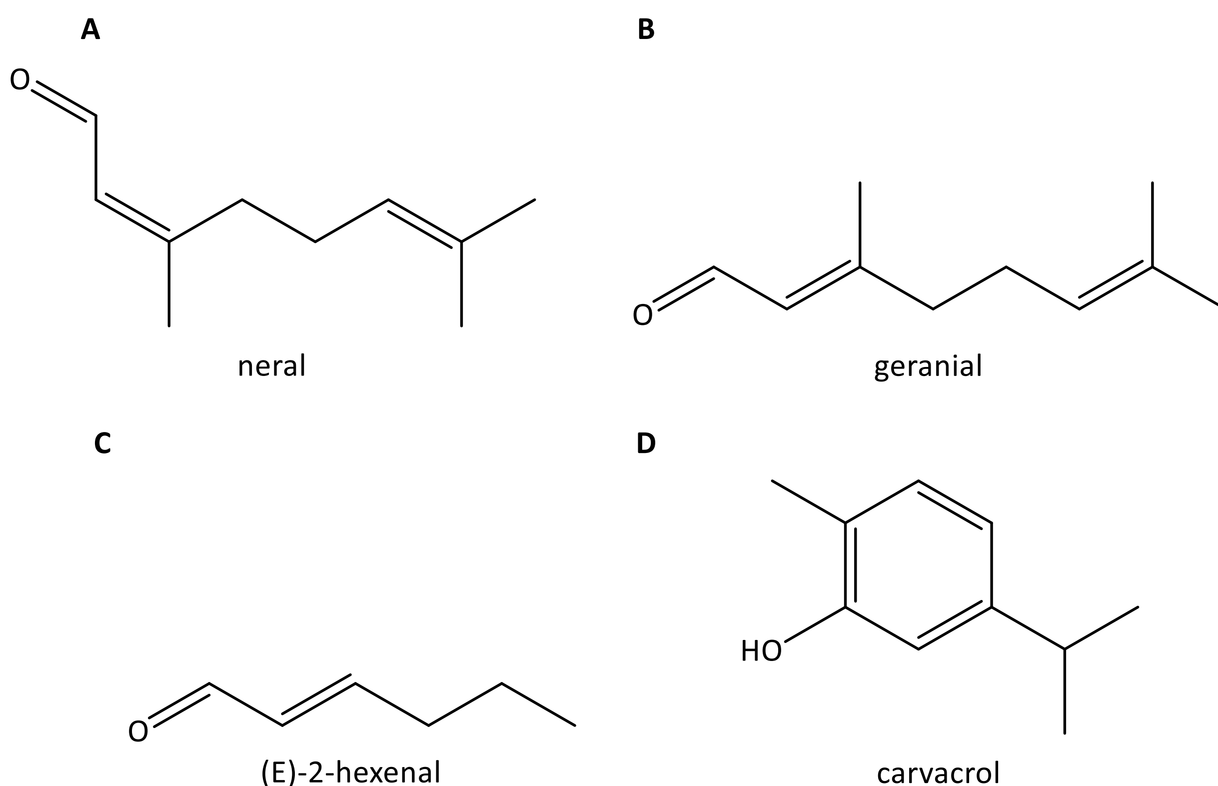

*Figure 1: Structural formulae of the component of EOs used in this work. Structural formulae of the two isomers of citral ((geranial (**A**) and neral (**B**)), (E)-2-hexenal (**C**), carvacrol (**D**).*

Table 1: *GC/MS-SPME characterization of thyme EO, in the table are reported the main molecules detected and the relative peak area/100000 and relative %*

Thyme EO		
Molecule	**peak area/100000**	**area %**
α-pinene	641.6	4.8
camphene	257.5	1.9
β-pinene	84.3	0.6
3-carene	29.3	0.2
β-myrcene	357.6	2.7
α-phellandrene	47.7	0.4
α-terpinene	512.4	3.9
Limonene	133.9	1.0
β-thujene	143.4	1.1
γ-terpinene	2035.6	15.3
p-cymene	3745.8	28.2
terpinolene	44.7	0.3
cis-β-terpineol	32.8	0.2
Linalol	270.6	2.0
bornyl acetate	8.0	0.1
thymol methyl ether	61.5	0.5
caryophyllene	908.8	6.8
Borneol	60.1	0.5
δ-cadinene	23.7	0.2
p-thymol	21.4	0.2
Thymol	1545.7	11.6
Carvacrol	2207.1	16.6
Total	**13290.7**	**99.1**

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

For the determination of the MIC and MBC values of (E)-2-hexenal, citral, carvacrol and thyme EO on the target microorganisms *Escherichia coli* K12 MG1655, 150 µL of Brain Heart Infusion (BHI, Oxoid Ltd. Basingstoke, United Kingdom), inoculated with the target microorganism at three different levels (2, 4 or 6 log CFU/mL) were added to 200 µL microtiter plate wells (Corning Incorporated, NY, USA). Fifty µL of the tested natural antimicrobials, properly diluted in BHI broth, and conveyed through 96% ethanol (VWR international, PROLABO, France) were added to each well to obtain the required concentration in the final volume (200 µL), and with a constant amount of ethanol (1% v/v in wells). A wide range of concentrations from 0 to 2000 mg/L with intervals of

100 mg/L was used preliminarily. Based on the first results, more confined ranges of concentrations with intervals of 25 mg/L were tested. Minimal inhibitory concentration (MIC) values, the lowest concentration of the compound preventing visible growth of the inoculated cells, were determined after 18 and 24h. Minimal bactericidal concentration (MBC) values, the lowest concentration of the compound that caused death of the inoculated cells, were determined after 24 h of incubation at 37° C with shaking. The MBCs were determined by spotting 10 µL of each well after 24 h onto BHI agar plates.

Treatment of bacterial cultures with natural antimicrobial compounds and cDNA microarray analyses

The concentrations employed were 200, 500, 60 and 125 mg/L for of (E)-2-hexenal, citral, carvacrol and thyme EO, respectively. Each compound was used at approximately half of the determined MIC values after 18h at an inoculum level of 2 log CFU/mL.

Overnight grown cultures were diluted to about 6 log CFU/mL in 1.0 L flasks containing 800 ml of BHI broth and incubated at 37 °C. The growth rate was monitored by measuring the optical density at 600 nm (OD600) every 30 min using a spectrophotometer UV-1204 (Shimadzu, Kyoto, Japan), until an OD600 of 0.4 was reached. Then, the cultures were aliquoted into 50 ml tubes and supplemented with the selected concentration of each compound dissolved in 1% v/v of ethanol to allow the solubility in water solution. The experiments were repeated three times on different days, and for each experiment, three tubes for each condition were used. Bacterial cultures to which 1% v/v of ethanol was added served as controls. Treatments were performed for 1 h at 37 °C. From each condition, the cells from two samples of 50 ml were harvested by centrifugation (6,000xg for 5 min in an eppendorf centrifuge (Eppendorf, Hamburg, Germany) at room temperature. The pellets were immediately frozen in liquid nitrogen prior to storage at -80 °C until the RNA extraction. The effects of the addition of the natural antimicrobials on the growth rate of the target microorganisms were also monitored after the treatment, by measuring the OD600 every 30 min of one 50 mL culture for each condition.

The RNA was extracted from the microbial pellet obtained from 50 mL following the methodology described by Kuipers et al., (2002). Single-strand reverse transcription (amplification) and labeling of 25–50 µg of isolated total RNA with Cy3-dCTP or Cy5-dCTP was done with the Invitrogen FluoroScript cDNA labeling system. Ultimately, the *E. coli* K12 cDNAs were hybridized to commercial *E. coli* gene expression 8×15K microarray slides (Agilent Technologies, Palo Alto, CA,

USA). After washing, the slides were scanned by using an Agilent G2565CA microarray scanner (Agilent Technologies). Each treatment condition was compared to the control. A biological replicate of each comparison as well as a dye swap were performed. DNA microarray slide pictures were analyzed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). The Limma R package (Sayings, 1999) was used to analyse the DNA microarray data using the 1% v/v ethanol control as the common reference. Fold changes were considered to be significantly changed when the Benjamini-Hochberg adjusted *p*-value is ≤ 0.01. To investigate the distribution of differentially expressed genes in relation to the stress applied, a Venn diagram was constructed by using the online tool venny (Oliveros, 2015).

Statistic tools

An in-depth analysis of the transcriptome data was performed with a variety of bioinformatics tools from the MolGen GENOME2D website (http://genome2d.molgenrug.nl). In order to compare the different treatments, the statistically relevant fold-change (FC) values were used.

Results and Discussion

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluation.

The MICs and MBCs of citral, (E)-2-hexenal, thyme EO or carvacrol against three density levels of the target microorganisms *E. coli* K12 are reported in Table 2. Differences in the MICs and MBCs were observed in relation to the substances and, in some cases, the inoculum level used. Otherwise, the effects of inoculation level on MIC and MBC values of EOs and their components were described previously (Lambert et al., 2001;Burt, 2004). Citral showed a low antimicrobial effectiveness against the target strain that was independent of the inoculation level. In fact, the MIC values were always higher than 2000 mg/L. Carvacrol showed the highest efficacy. The MIC and MBC values of carvacrol for *E. coli* K12 were not affected by the inoculation level showing MIC at 18 and 24h and MBC of 125 mg/L in all cases. This result is in agreement with other studies (Bagamboula et al., 2004;Klein et al., 2013). By contrast, the influence of the initial inoculum on MICs and MBCs was evident for (E)-2-hexenal and thyme EO. These molecules showed a good efficacy against *E. coli*, with MIC and MBC values ranging between 350-600 mg/L and 250-500

mg/L, respectively. The bacteriostatic effect of (E)-2-hexenal and thyme EO was evident at each inoculum level (2, 4, 6 log CFU/mL). In fact, the MIC values after 24h were always higher than those after 18h. The MIC at 18h and the MBC for (E)-2-hexenal decreased from 500 mg/L to 350 mg/L and from 600 to 425 respectively, with inoculation levels lowered from 10^6 to 10^2 CFU/mL. For thyme EO, the MIC at18h and the MBC decreased from 375 mg/L to 250 mg/L and from 500 to 300, respectively, with inoculation levels lowered from 10^6 to 10^2 CFU/mL.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of citral, (E)-2-hexenal, carvacrol and thyme EO against E. coli K12.

cell concentration	**6 log CFU/mL**			**4 log CFU/mL**			**2 log CFU/mL**		
MIC/MBC	**MIC 18h (mg/L)**	**MIC 24h (mg/L)**	**MBC 24h (mg/L)**	**MIC 18h (mg/L)**	**MIC 24h (mg/L)**	**MBC 24h (mg/L)**	**MIC 18h (mg/L)**	**MIC 24h (mg/L)**	**MBC 24h (mg/L)**
Carvacrol	125	125	125	125	125	125	125	125	125
(E)-2-hexenal	500	575	600	375	425	450	350	400	425
Citral	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000
Thyme EO	375	475	500	300	375	425	250	275	300

Transcriptional response of *E. coli* K12 treated with sub-lethal concentrations of natural antimicrobials

Employing a whole-genome DNA microarray approach, the transcriptional response of *E. coli* to sub-lethal concentrations of the natural antimicrobials, studied in this work, was assessed. The concentrations used for (E)-2-hexenal, citral, carvacrol and thyme EO were 200, 500, 60 and 125 mg/L, respectively. These concentrations corresponded to half of the respective MIC values, with the exception of that of citral. Cells in the mid-exponential phase of growth in BHI were exposed for 1 h to the antimicrobial substances. Since the antimicrobials were conveyed in 1% v/v ethanol, the common reference was a bacterial culture exposed for 1 h to 1% v/v ethanol. In order to verify the effects of the treatments on cell vitality, growth of the target microorganisms after the treatments was also monitored (Figure 2). The addition to *E. coli* of 1% v/v ethanol or 200 mg/L of (E)-2-hexenal did not affect the growth rate of the organism. On the contrary, carvacrol and, to a greater extent, thyme EO and citral strongly affected the maximum growth rate as well as the OD600 reached in stationary phase.

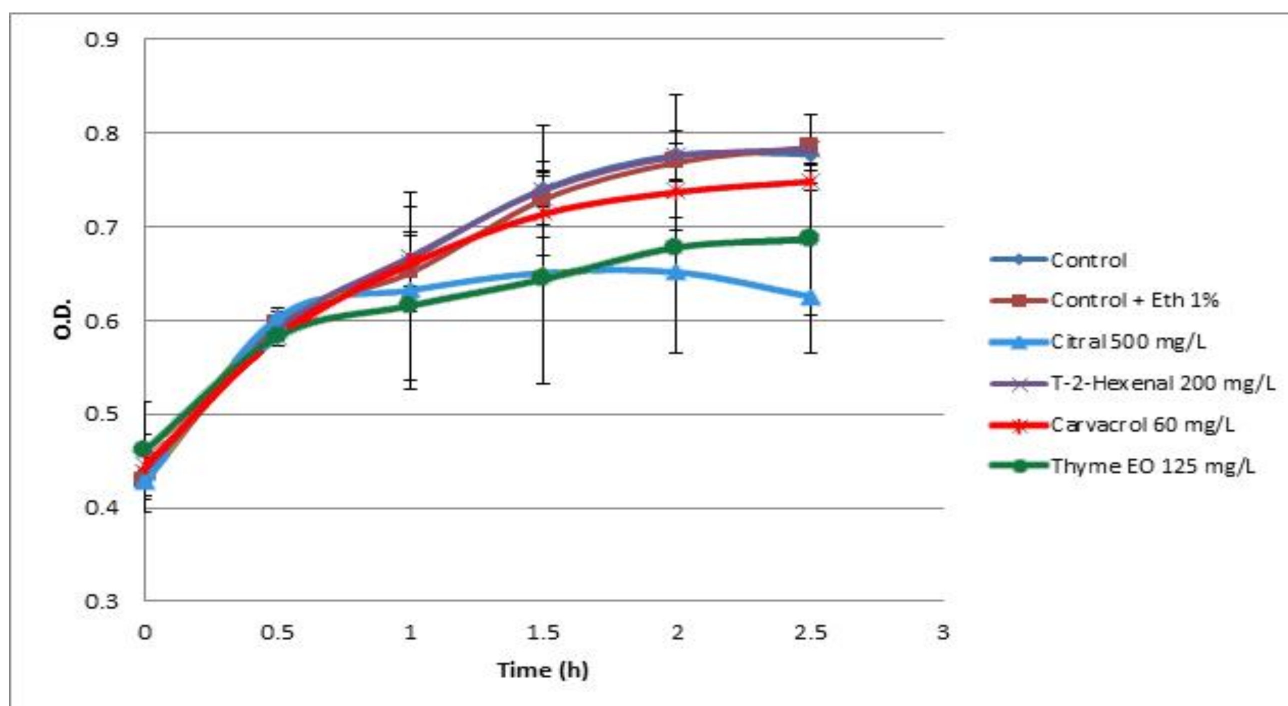

Figure 2: Effect of natural antimicrobials addition on the growth of E. coli. E. coli K12 growth in BHI broth at 37 °C is presented from the point at which the treatment with the indicated sub-lethal concentrations of natural antimicrobials was started. Control + ETh1%: E. coli to which 1% v/v ethanol was added. The arrows indicate the point at which cultures were collected for DNA microarray analyses. Growth was recorded as the change in OD600.

DNA microarray analysis was done on RNA isolated from parallel cultures after 1 h of exposure to the various compounds. The results revealed clear differences in the numbers of genes being significantly up- or down-regulated in the target microorganism *E. coli* K12 (Table 3). The highest number of genes of which the expression was significantly affected (550) was caused by the addition of thyme EO while for the carvacrol, citral and (E)-2-hexenal the number of genes significantly up- or down-regulated was quite similar and ranged from 352 to 411. In all cases, most of the affected genes belonged to the functional categories of energy metabolism, purine/pyrimidine metabolism, fatty acid and phospholipid metabolism, and protein synthesis.

Table 3: Number of significantly ($p<0.01$) up- or down regulated genes in *E. coli* K12 1655

	Escherichia coli K12			
	Citral	**(E)-2-hexenal**	**Carvacrol**	**Thyme EO**
UP	360	240	261	477
UNCHANGED	3798	3831	3857	3659
DOWN	51	138	91	73

A Venn diagram was constructed to investigate the distribution of differentially expressed genes in relation to the stress applied (Figure 3) (Oliveros, 2015). This analysis showed that exposure of *E. coli* to the selected compounds led to a gene expression response that is partially similar for all antimicrobials used. In fact, 70 genes were significantly differentially expressed in all four conditions. Moreover, approximately 31% of the differentially expressed genes were common among at least three conditions. The response of *E. coli* to sub-lethal concentrations of thyme EO and citral was very similar. In fact, the percentage of differentially expressed genes common to both conditions was around 45.

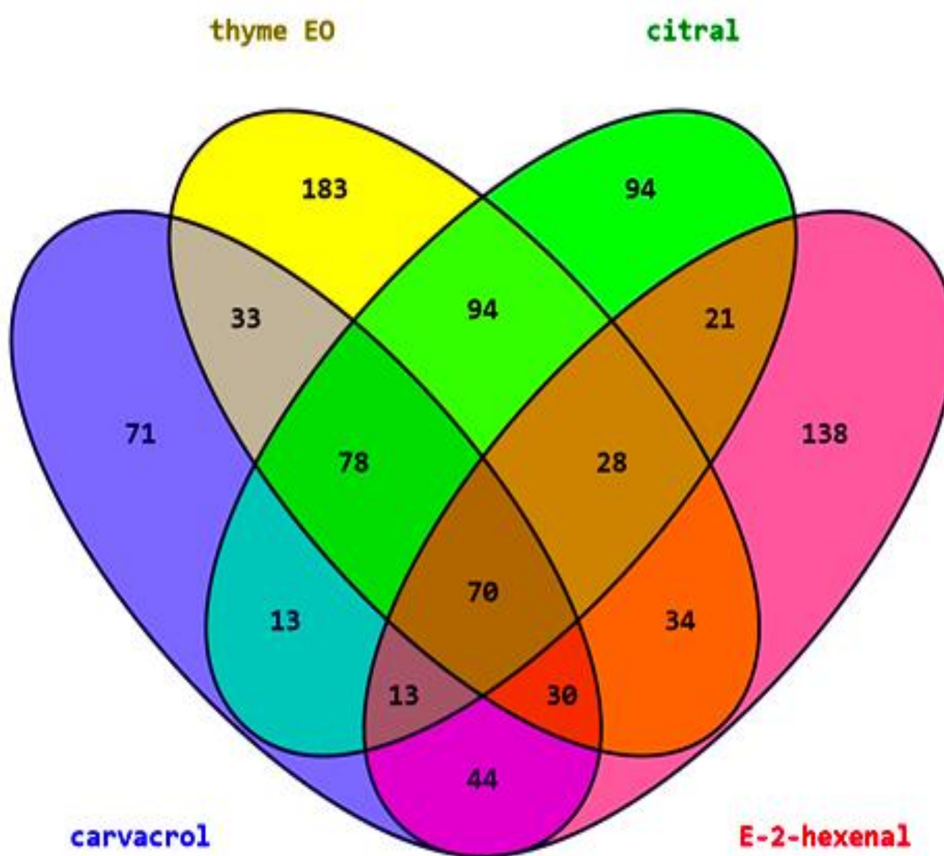

Figure 3: Distribution of differentially expressed genes in antimicrobial-treated E. coli K12 MG1655. The Venn diagram (Kestler et al., 2005) reports the numbers of unique and common differentially expressed genes.

The most significantly (*p*-value lower than 0.01) up- or down-regulated genes are reported in Table 4 and were further scrutinized. It is well known that one of the main targets of EOs is the cytoplasmic membrane (Burt, 2004;Nazzaro et al., 2013).

Table 4: Selected genes up- or down regulated in E. coli K12 after treatment with citral (500 mg/L), (E)-2-hexenal (200 mg/L), carvacrol (60 mg/L) and thyme EO (125 mg/L) (p<0.01)

		Fold Change			description
Gene	**Carvacrol**	**Thyme EO**	**Citral**	**(E)-2-hexenal**	
Ribosome					
b3321 (rpsJ)	2.52	2.90	2.67	1.63	30S ribosomal subunit protein S10 [b3321]
b3311 (rpsQ)	3.02	2.84	2.64	1.56	30S ribosomal subunit protein S17 [b3311]
b3230 (rpsI)	2.26	2.49	2.65	1.44	30S ribosomal subunit protein S9 [b3230]
b1717 (rpmI)	2.61	2.55	2.68	*	50S ribosomal subunit protein A [b1717]
b3231 (rplM)	3.05	7.35	2.78	*	50S ribosomal subunit protein L13 [b3231]
b3186 (rplU)	2.66	3.50	3.43	1.69	50S ribosomal subunit protein L21 [b3186]
b3185 (rpmA)	2.27	2.75	2.24	*	50S ribosomal subunit protein L27 [b3185]
b3312 (rpmC)	2.13	3.13	2.45	1.42	50S ribosomal subunit protein L29 [b3312]
b3320 (rplC)	2.19	2.54	2.31	1.45	50S ribosomal subunit protein L3 [b3320]
b1089 (rpmF)	2.71	2.33	2.63	*	50S ribosomal subunit protein L32 [b1089]
b3319 (rplD)	2.39	2.84	1.92	*	50S ribosomal subunit protein L4, regulates expression of S10 operon [b3319]
Glycerophospholipid metabolism					
b3426 (glpD)	-1.18	*	*	-2.89	sn-glycerol-3-phosphate dehydrogenase [b3426]
b2243 (glpC)	-1.60	-1.66	*	-3.34	sn-glycerol-3-phosphate dehydrogenase [b2243]
b2242 (glpB)	-1.28	*	*	-1.41	sn-glycerol-3-phosphate dehydrogenase [b2242]
Fatty acid biosynthesis					
b0180 (fabZ)	1.57	1.61	*	*	(3R)-hydroxymyristol acyl carrier protein dehydratase [b0180]
b1091 (fabH)	1.18	1.21	*	*	3-oxoacyl- [b1091]
b1092 (fabD)	1.24	1.39	1.33	*	malonyl-CoA- [b1092]
b1093 (fabG)	1.18	1.19	*	*	3-oxoacyl- [b1093]
b1095 (fabF)	1.14	1.17	1.15	*	3-oxoacyl- [b1095]
b1288 (fabI)	1.49	1.38	1.41	1.39	enoyl- [b1288]
b2316 (accD)	1.21	1.32	1.31	*	acetylCoA carboxylase, carboxytransferase component, beta subunit [b2316]
b3255 (accB)	*	1.47	*	*	acetylCoA carboxylase, BCCP subunit; carrier of biotin [b3255]
b3256 (accC)	1.32	1.47	1.32	1.28	acetyl CoA carboxylase, biotin carboxylase subunit [b3256]
Energetic metabolism					
b1651 (gloA)	*	1.72	*	2.87	lactoylglutathione lyase [b1651]
b2579 (yfiD)	1.57	1.56	1.44	2.58	putative formate acetyltransferase [b2579]
b0356 (frmA)	*	*	2.01	3.57	alcohol dehydrogenase class III; formaldehyde dehydrogenase, glutathione-dependent [b0356]
b1800 (yeaU)	*	*	*	3.08	putative tartrate dehydrogenase [b1800]
Purine, pyrimidine metabolism and transcription					
b3011 (yqhD)	*	*	5.13	8.36	putative oxidoreductase [b3011]
b4238 (nrdD)	*	*	*	2.82	anaerobic ribonucleoside-triphosphate reductase [b4238]
Hypothetical proteins					
b1112 (bhsA)	*	*	*	5.51	orf, hypothetical protein [b1112]
b1654 (grxD)	*	*	2.13	2.53	orf, hypothetical protein [b1654]
b3238 (yhcN)	1.73	*	2.41	7.04	orf, hypothetical protein [b3238]

b3914	*	4.29	3.82	*	orf, hypothetical protein [b3914]
Heat shock, acid shock, protease anddetoxification and protection					
b1597 (asr)	2.20	5.02	2.10	*	acid shock protein [b1597]
b0606 (ahpF)	1.69	2.80	2.03	1.49	alkyl hydroperoxide reductase, F52a subunit; detoxification of hydroperoxides [b0606]
b3686 (ibpB)	*	7.15	10.7 2	*	heat shock protein [b3686]
b3687 (ibpA)	*	2.35	3.43	*	heat shock protein [b3687]
b1305 (pspB)	*	2.92	2.19	*	phage shock protein [b1305]
b1307 (pspD)	*	2.57	1.63	*	phage shock protein [b1307]
b1304 (pspA)	*	4.10	2.39	*	phage shock protein, inner membrane protein [b1304]
b1531 (marA)	*	2.07	2.76	3.80	multiple antibiotic resistance; transcriptional activator of defense systems [b1531]
Outer and inner cell membrane					
b3035 (tolC)	*	*	5.96	*	outer membrane channel; specific tolerance to colicin E1; segregation of daughter chromosomes [b3035]
b0814 (ompX)	2.58	1.60	2.02	2.36	outer membrane protein X [b0814]
Replication and repair					
b3179 (rrmJ)	*	*	2.57	*	cell division protein [b3179]
Other functions					
b0849 (grxA)	*	2.85	*	*	glutaredoxin1 redox coenzyme for glutathione-dependent ribonucleotide reductase [b0849]
b1743 (spy)	*	*	2.60	*	periplasmic protein related to spheroblast formation [b1743]
b1454 (yncG)	*	*	*	-4.19	putative transferase [b1454]

***** *expression values were measured and there was no significant change*

As previously described by Siroli et al., (2015a), carvacrol, thyme EO and citral led to an increase in the level of unsaturation as well as of *trans*-isomers in *E. coli* membrane fatty acids. Unsaturated fatty acids (UFA) have been reported to play a crucial role in the response of bacteria to different stresses, including low or high temperatures, oxidative, acid, ethanol or salt stress, or the stress evoked by high pressure (Tabanelli et al., 2014). Moreover, the effect of EOs and their components on the modulation of the synthesis of cyclic fatty acids in Gram-negative bacteria is well reported (Zhang and Rock, 2008;Siroli et al., 2015a). The increase in the length of fatty acids is another important membrane modification that might increase survival in adverse environments e.g., with a low pH or containing antimicrobial compounds (Royce et al., 2015). In the present work, an up regulation of the genes involved directly in the biosynthesis of UFAs and the other fatty acids involved in the Gram-negative bacteria stress response was observed but with a fold-change ranging between 1.14 and 1.61, which is most probably due to the relatively short time of exposure (1 h) to the investigated compounds. In fact, up-regulation of the genes *fabZ, fabH, fabD, fabG, fabF, fabI, accB, accC, accD* was evidenced for all the tested antimicrobials. The *E. coli* accBCD genes specify the acetyl-carboxylase complex that activates acetyl coenzyme A (acetyl-

CoA) into malonyl-CoA, which represent the first step of fatty acid biosynthesis (My et al., 2015). The *fab* genes encode enzymes responsible for a series of condensation, reduction, and dehydration reactions, followed by elongation (My et al., 2013). These changes were accompanied by a down-regulation, in the presence of carvacrol and (E)-2-hexenal, of *glpC* and *glpD*, which are involved in glycerophospholipid metabolism. GlpC is the membrane-associated subunit of the heterotrimeric glycerol-3-phosphate dehydrogenase complex. Under anaerobic conditions this respiratory enzyme converts glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) using fumarate as the terminal electron acceptor (Varga and Weiner). GlpD is an aerobic glycerol 3-phosphate dehydrogenase catalyzing the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate (Austin and Larson, 1991). A response of *E. coli* K12 to the stresses applied here involves the over-expression of all the so-called phage shock genes (*pspA*, *pspB* and *pspD*) in particular in the presence of thyme EO and citral. The phage shock protein (Psp) stress response system in Gram-negative bacteria is responsible for repairing damage to the inner membrane of the cell (Kobayashi et al., 2007) and maintenance of the proton-motive force across the inner membrane (Darwin, 2007;Jovanovic et al., 2010). It is well reported that the Psp stress response is related to a block in protein export and fatty acid/phospholipid biosynthesis, exposure to organic solvents, extreme heat or osmotic shock, and exposure to high pH (Darwin, 2007;Jovanovic et al., 2010). PspA acts as an effector of Psp and is thought to prevent proton loss under conditions in which the *psp* operon is induced, but the precise mechanism is unknown (Kobayashi et al., 2007;Jovanovic et al., 2010;Huvet et al., 2011). Moreover, overexpression of *pspB* and *pspC* could prevent cytoplasmic membrane permeability caused by the passage of molecules through the mislocalized multimeric secretin channel (Horstman and Darwin, 2012). Secretins of Gram-negative bacteria are membrane proteins that form multiple systems associated to the secretion of specific proteins to the extracellular space and in assembly of fiber structures on the cell surface (Korotkov et al., 2011). The heat shock genes *ibpA* and *ibpB*, which codify for chaperones that protect cells from denaturation of protein induced by heat and oxidative stresses, (Goeser et al., 2015) are overexpressed in case of addition to *E. coli* of thyme EO and citral. According to Dodd et al., (1997) any stress condition (also chemicals) results in oxidative stress as a result of an imbalance between anabolism and catabolism. Of note, controversy exists as to oxidative stress being a mechanism of action of EO components. Khan et al., (2011) showed that treating *Candida albicans* with sub-lethal concentrations of three phenylpropanoid components of EOs (eugenol, methyl eugenol and estragole) caused oxidative stress, as demonstrated by the formation of

membrane lesions resulting from free radical cascade-mediated lipid peroxidation. In fact, they observed a significant increase (ranging between 1.69 to 3.27-fold) in catalase activity in cells treated with these antimicrobials. This rise in catalase activity is indicative for increased peroxide formation by phenylpropanoids. Chueca et al., (2014)on the other hand, suggest that the oxidative stress observed in *E. coli* after treatment with (+)-limonene, a component of citrus EOs, only takes place under specific conditions of drug concentration and a certain physiological state of the cells. In fact, they suggest that the mechanism of inactivation by (+)- limonene is mediated by ROS (superoxide and hydrogen peroxide) in exponentially growing cells, but not in cells in the stationary phase of growth. Considering the overexpression of *ibpA* and *ibpB* observed in this study we propose that oxidative stress plays a key role in the action mechanisms of thyme EO and citral against *E. coli*. (Kitagawa et al., 2000)have demonstrated that bacteria overproducing IbpA and IbpB proteins developed resistance to superoxide stress; moreover IbpA/B repressed the inactivation of selected enzymes by hydrogen peroxide and potassium superoxide *in vitro* (Kitagawa et al., 2002).

The natural antimicrobials also significantly affected genes involved in energy metabolism. In particular, an up-regulation was seen of the *frmA* gene in the presence of citral and (E)-2-hexenal and of *yfiD* for all three antimicrobials. As *frmA* encodes a glutathione-dependent formaldehyde dehydrogenase and the enzyme is part of aldehyde detoxification pathways (Gonzalez et al., 2006;Mills et al., 2009), its up-regulation is a probable attempt of the cell to inactivate the added aldehydes citral and (E)-2-hexenal. Previous reports have shown that several microorganisms can detoxify citral and (E)-2-hexenal by transforming them into alcohols (Siroli et al., 2015a).

Keating et al., (2014) studied the effects in *E. coli* of aromatic compounds from ammonia pre-treated lignocellulose and showed that expression of *frmA* increased in the presence of high levels of aromatic aldehydes and acetaldehyde while *frmA* transcript levels decreased again as the aldehydes were inactivated. It was hypothesized that the FrmAB system, for which formaldehyde is reported to be the only substrate, may in fact also act on acetaldehyde and other aldehydes.

The gene *yfiD* specifies a glycyl radical protein that can form a hetero-oligomeric complex with a C-terminally truncated form of pyruvate formate-lyase that mimics the oxygen-fragmented enzyme. After activation, this complex has pyruvate-formate lyase activity (Wagner et al., 2001). It has been reported that YfiD is a member of the FNR (fumarate and nitrate reduction regulator) regulon in *E. coli*. FNR is an oxygen sensor functioning mainly to activate the expression of genes required during anaerobic growth (Wyborn et al., 2002). Picone et al. (2013) observed a shift from

respiration to fermentation upon exposure of *E. coli* to carvacrol. Inhibition of respiration together with K^+ leakage, upon exposure of *E. coli* to sub-lethal concentrations of tea tree EO, has already been described (Cox et al., 1998).

As reported earlier, treatment with (E)-2-hexenal, thyme EO and carvacrol affects *E. coli* glycerol metabolism. In the present study, we observe a repression of the genes *glpC* and *glpD* upon treatment of the cells with (E)-2-hexenal, thyme EO and carvacrol. The *glpC* and *glpD* genes are related to glycerol-3-phosphate (G3P) metabolism via G3P dehydrogenase. The *glpD* gene encodes aerobic glycerol 3-phosphate dehydrogenase, a respiratory enzyme, and its down-regulation suggests that a shift occurs from respiration to fermentation when the cells are faced with (E)-2-hexenal, thyme EO and carvacrol.

The *ompX* gene, encoding a small outer-membrane (OM) protein forming an eight-stranded antiparallel β-barrel (Dupont et al., 2007), was up-regulated upon treatment of the cells with all tested compounds (Table 4). OmpX plays a key role in the down-regulation of porins in the OM of *E. coli* in response to environmental stresses that induce its overproduction (Dupont et al., 2007). Helander et al., (1998) have shown the effect of EOs on the OM permeability in Gram-negative bacteria: the monoterpene components of EOs such as carvacrol and thymol caused disintegration of the OM and release of OM-associated material.

An increase in expression of ribosomal subunit genes (*rps*, *rpm* and *rpl*) was evident after all three chemical stresses applied here. Several authors have reported up- or down-regulation of these genes under various stress conditions. A decrease in the expression of ribosomal subunit genes has been observed after an exposure of *E.coli* and *Salmonella enterica* to 30 min to triclosan, a member of the bisphenol biocide family that exhibits a broad spectrum of activity against many Gram-negative and Gram-positive bacteria. Down-regulation of ribosomal protein genes (*rpl* and *rps*) also occurred in *Campylobacter jejuni* upon a 15-min exposure to osmotic stress, coinciding with a temporary growth arrest, while the same genes returned to steady-state or greater expression levels with the resumption of growth (Cameron et al., 2012).

The results of the present work clearly show that the addition of sub-lethal concentrations of the natural antimicrobials employed here affects global gene expression in *E. coli*. The affected genes are mainly those involved in fatty acid biosynthesis, energy metabolism and protection against oxidative stress. These data add to previous literature studies showing that the cytoplasmic membrane of *E. coli* is, the major cellular target of EOs and their components. In addition, the shift

from respiration to fermentative growth, with a consequent drastic reduction of energy availability for the cell, is clearly demonstrated by the down-regulation of *glpD*.

Conclusions

The data contribute to the understanding in detail the mechanisms of actions of the natural antimicrobials tested. In fact, the use of new antimicrobials in food processing against pathogenic species is subordinate to the comprehension of their activities. However, further studies including real-time PCR analyses on the genes resulted significantly up/down regulated must be performed to validate the microarray results obtained in this work.

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Proteomic adaptation of *Listeria monocytogenes* Scott A to natural antimicrobial compounds

Introduction

Listeria monocytogenes, the etiologic agent of listeriosis, is one of the major serious food-borne illnesses worldwide (Swaminathan and Gerner-Smidt, 2007;Huang et al., 2014). Listeriosis is rare but causes lethal consequences greater than the cases of *Clostridium botulinum*. Listeriosis produces death for the 20-30% of patients showing the highest percentage in elderly, pregnant women, children or immune-compromised subjects (Forsyth et al., 1998;Mead et al., 1999;Orsi et al., 2011). *L. monocytogenes* is saprophytic species showing very high survival aptitudes in food ecosystems including raw, cooked and processed fruits and vegetables (Hadjilouka et al., 2015), meat (Liu et al., 2014;Gouveia et al., 2016), milk (Sadeghi et al., 2016) and fish (Rocourt et al., 2003). *L. monocytogenes* is able to growth in different niches with a strong ability to resist against environmental and technological stresses such as high/low temperatures and modified atmospheres (Gandhi and Chikindas, 2007;O'Byrne and Karatzas, 2008;Dutta et al., 2013;NicAogáin and O'Byrne, 2016). Within food chain, *L. monocytogenes* is able to adapt and survive to different stress conditions applied in food processing and storage (Gomes Neto et al., 2015). Consequently, *L. monocytogenes* constitutes a major risk to consumers and it causes also strong economic losses (Cabrita et al., 2015). European Commission, (2005) required that the levels of *L. monocytogenes* must not exceed 100 CFU/g, for the products not particularly favorable for the growth of this pathogenic species and not directed to infants. In minimally processed fruits and vegetables, the use of chemicals (e.g., ozone, H_2O_2, organic acids, calcium-based solutions and peroxyacetic acids) as disinfectants is not sufficient to statistical decrease the survival *L. monocytogenes* strains (Soliva-Fortuny and Martín-Belloso, 2003;Siroli et al., 2015). Based on consumer concern of chemical synthetic additives (Sivakumar and Bautista-Baños, 2014), one of the emerging strategies purposed to decrease the survival *L. monocytogenes* in food products is the use of natural antimicrobial compounds alone or in combination with other mild technologies (Kamdem et al., 2011;Ngang et al., 2014). Plants and plant products such as essential oils (EOs)

produced by lipoxygenase pathway, play a key role in plant defense against microbial proliferation. Today many EOs are generally recognized as safe (GRAS) and used to improve the sensory quality and shelf-life of fruits, vegetables, meat and dairy foods (Belletti et al., 2004;Burt, 2004;Belletti et al., 2010). The antimicrobial properties of EOs were mainly related to C10- and C15-terpenes with aromatic rings and phenolic-hydroxylic group forming hydrogen bonds with active sites of target enzymes (Picone et al., 2013). In addition, other EOs compounds such as alcohols, aldehydes and esters have antimicrobial effects. EOs differently affected bacterial and fungal viability depending to their composition and structural configuration as well as to the possible synergistic interactions among the components (Picone et al., 2013;Patrignani et al., 2015). Interestingly, some EOs have a wide spectrum of actions against pathogens including *L. monocytogenes* (Oliveira et al., 2013;Patrignani et al., 2015). Some EOs from rosemary, thyme and oregano such as citral (a mixture of monoterpene aldehydes composed by geranial and neral), carvacrol, thymol, hexanal and trans-2-hexenal seems to be good candidate as natural antimicrobials since they have inhibitory effects against bacteria and fungi in foods (Ivanovic et al., 2012;Patrignani et al., 2015). Compared to other common antimicrobial compounds and human pathogens, there are few information about the effect of natural antimicrobials to decrease the formation of biofilm, cell survival and environmental adaptation of *L. monocytogens* (Ahmad and Beg, 2001;Mahesh and Satish, 2008;Helke et al., 2017;Tracanna et al., 2017;Van Vuuren and Holl, 2017). Despite recent progress in discovering details of *L. monocytogens* genome, the mechanisms of cellular adaptation against natural antimicrobial compounds remain largely unclear (NicAogáin and O'Byrne, 2016;Van Vuuren and Holl, 2017). Studies on stress adaptations of *L. monocytogenes* to natural antimicrobial compounds are crucial to highlight the relationship between stress and virulence and to optimize the protocols for food production (Chaturongakul et al., 2008;Bowman et al., 2010;He et al., 2015).

Proteomic approaches liking genome and transcriptome to potential biological functions could highlight the molecular mechanisms of stress adaptations of *L. monocytogenes* to natural antimicrobial compounds (Guevara et al., 2015). Accordingly, this study aimed at investigating the proteomic adaptation of *L. monocytogenes* Scott A cells during exposure to natural antimicrobials (ethanol, citral, carvacrol, (E)-2-hexenal and thyme EO), used at sublethal concentrations to avoid their negative impact of food sensory properties. In fact, *L. monocytogenes* middle exponential phase cells were phase cells were exposed for an hour to 1/5, 1/3 and 1/2of the MIC values of the

antimicrobials considered and the proteomes analyzed by bi-dimensional acrylamide gel electrophoresis (2-DE) followed for the spot identification by MALDI TOF MS/MS approach.

Materials and Methods

Bacterial strains and culture conditions

L. monocytogenes Scott A was stored at −80 °C for long-term preservation. To acclimatize the strain to the experimental conditions, one mL of the culture strain was inoculated to nine mL of Brain Heart Infusion broth (BHI) (Thermo-fisher, Milano, Italy) and incubated for 24 h at 37 °C. After the growth, cells were propagated at 37 °C for 24 h in BHI broth using 1% of inoculum.

Antimicrobial treatment conditions

Cells of *L. monocytogenes* Scott A, grown in BHI broth for 24 h at 37 °C, were inoculated (1% v/v) in 1000 mL of fresh BHI broth at a final density of 4 log CFU/mL. Cells were cultivated at the optimum growth temperature (30 °C) until they reached the mid-exponential phase of growth (OD600 of ca. 0.4). Cells were harvested by centrifugation at 9,000 × g for 10 min at 30°C and resuspended in 1000 mL of fresh BHI broth alone (untreated cells, control) or added of antimicrobial compounds (treated cells). Antimicrobial treatments were performed using ethanol (final concentrations in BHI broth of 1% v/v) alone or added of citral (final concentrations in BHI broth: 85 and 125 mg/L), carvacrol (20, 35 or 50 mg/L), (E)-2-hexenal (150, 250 or 400 mg/L) and thyme essential oil (40, 70 or 100 mg/L). Citral (a mix of the two isomer of the same monoterpene aldehyde: geranial and neral), carvacrol, and (E)-2-hexenal were purchased from Sigma-Aldrich (Milano, Italy) while thyme essential oil (EO) was obtained from Flora s.r.l. (Pisa, Italy). After incubation at 37 °C for 1 h, control and treated cells were harvested by centrifugation at 6000 rpm at 4 °C for 10 min and used for total viable cell count or stored at -80 °C for protein extractions.

Measurement of antimicrobial tolerance

Harvested cells were showed in fresh BHI broth, harvested by centrifugation at 9000 rpm at 4 °C for 5 min and immediately resuspended in sterile physiological solution for plate count. Cell numbers were determined by plating on BHI agar medium. Numbers of C.F.U. were determined after 24 h incubation at 37 °C. The number of surviving micro-organisms was calculated as a

percentage of the cell number at time zero. The tolerance factor (TF) corresponded to the ratio of the survival of treated cells to that of control cells.

Protein extraction and 2-DE analysis

Exponential-phase cells harvested from BHI broth, were washed in Tris-HCl 50 mM pH 7.5 and proteins extracts were produced as described by De Angelis et al., (2001). The concentration of protein of the cell extracts was determined by the method of Bradford (1976). The same amount (60 µg for analytical runs or 200 µg for preparative runs for protein identification) of total protein was used for each electrophoretic run. Two-DE was carried out essentially as described by (Hochstrasser et al., 1988;Boguth et al., 2000), using a Pharmacia 2-D-Electro Focusing (EF) system (GE Healthcare). Gels were stained using Brilliant Blue G-Colloidal Concentrate (Sigma) or an MS-compatible silver method. The protein maps were scanned with LabScan software on ImageScanner (GE Healthcare) and analyzed with the ImageMaster 2D Platinum v.6.0 computer software (GE Healthcare). Three gels from three independent experiments were analyzed and spot intensities were normalized as reported by Bini et al., (1997). In particular, the spot quantification for each gel was calculated as relative volume (% VOL), which corresponded to the volume of each spot divided by the total volume over the whole image (De Angelis et al., 2001).

Protein identification

In-gel tryptic digestion was performed. Gel pieces were washed two times with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold with 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 µg/µL. Gel pieces were rehydrated by adding 10 µL of trypsin solution, and after 10 min enough 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37°C. A 1 µL aliquot of each peptide mixture was applied to a ground steel MALDI target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/mL solution of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% , trifluoroacetic acid (v:v). Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a range of 800-4000 *m/z*. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides

(des-Arg[1]-Bradykinin, 904.681; Angiotensin I, 1296.685; Glu[1]-Fibrinopeptide B, 1750.677; ACTH (1-17 clip), 2093.086; ACTH (18-39 clip), 2465.198; ACTH (7-38 clip), 3657.929.). Monoisotopic masses were obtained using a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

For each spot the ten strongest precursors, with a S/N greater than 30, were selected for MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 6. Bruker flexAnalysis software (version 3.3) was used to perform spectral processing and peak list generation.

Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.5.1), through the Bruker ProteinScape interface (version 2.1). Search criteria specified: Enzyme, Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M) and Deamidated (NQ); Peptide tolerance, 100 ppm; MS/MS tolerance, 0.5 Da; Instrument, MALDI-TOF-TOF. Results were filtered to accept only peptides with an expect score of 0.05 or lower.

Statistical analyses and bioinformatics

All experiments were carried out in triplicate and data were subjected to a one-way ANOVA (SAS, 1985), and pair-comparison of treatment mean values was achieved by Tukey's procedure at $p < 0.05$ using the statistical software Statistica for Windows (Statistica 6.0 per Windows 1998). Principal component analysis by statistical software Statistica for Windows and PermutmatrixEN software were applied to analyze the proteome profiles (De Angelis et al., 2008;De Angelis et al., 2015). To study changes in metabolic enzymes related to adaptation at different antimicrobial products, all identified proteins were mapped to Kyoto Encyclopedia of Genes and Genomes pathways using both the enter gene ID and/or EC number functions (www.genome.jp/kegg/pathway.html) (Bove et al., 2012). The comprehensive symbolic systems biology Pathway Tools (PT) software version 19.0 and the relative encompassed MetaCyc multiorganism database, were used to reconstruct metabolic pathways. The sample differences were normalized at reaction, pathway and mega-pathway hierarchical levels. custom scripts and

manually checking steps allowed us to improve the functional characterization considering EC numbers, KEGG codes, and multiple sub-units taking part of the same whole enzyme. In order to trace enzymes in our datasets, we used the REST-style KEGG API to link KEGG and MetaCyc pathways to EC numbers.

Results

Antimicrobial stress resistance

After the mild-exponential phase of growth was reached, cells were treated with sub-lethal doses of the antimicrobial compounds (ethanol, citral, carvacrol, (E)-2-hexenal and thyme essential oil). In details, ethanol was used at 1% v/v alone and also used for convey the other antimicrobial compounds. Citral was used at concentration levels corresponding to 1/3 and 1/2 of the MIC value. Carvacrol, (E)-2-hexenal and thyme essential oil were used at levels corresponding to the 1/5, 1/3, 1/2 of the MIC. According to MIC value, cell survival of *L. monocytogenes* Scott A was not affected by the exposure to the natural antimicrobials (Figure 1). The only exception was observed for cells treated with ethanol (1% v/v) and thyme EO (100 mg/L) showing a decrease of cell loads from 8.53 ± 0.36 to 7.20 ± 0.22 log CFU/mL, with a tolerance factor of 0.85, P=0.04. 4

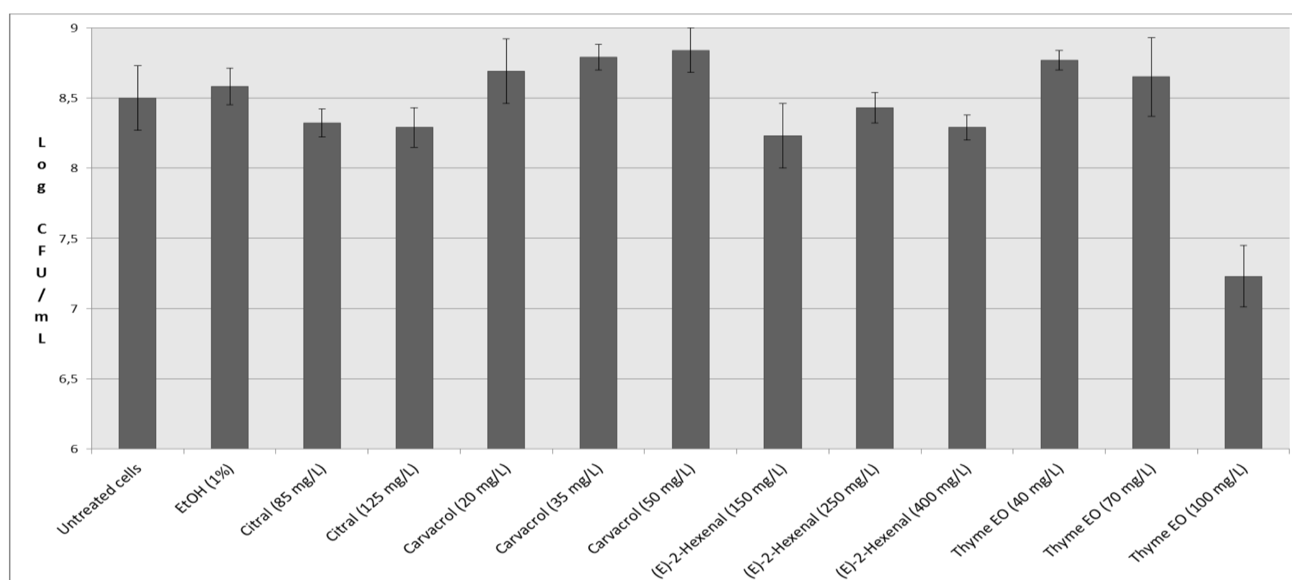


Figure 1: Cell density of Listeria monocytogenes Scott A untreated and treated for 1 h at 37°C with antimicrobial compounds.

Proteomic profile

Compared to control cells grown under optimal conditions, *L. monocytogens* Scott A treated for 1 h with antimicrobial compounds showed the increase or decrease (≥ or ≤ of 2 fold, $P<0.05$) of the levels of the synthesis of 223 protein spots (Figure 1-2 and Table 1).

In details, ethanol stressed cells increased the level of 87 protein spots compared to the untreated samples (Figure 1-3 and Table 1). On the contrary, the relative amount of other 17 protein spots was the highest in control cells. The presence of other antimicrobial compounds together with ethanol further modify the proteomic profile of *L. monocytogens* Scott A (Figure 1-2 and Figure 4 - 7Figure). The highest number of induced protein spots were found for cells treated with thyme essential oil at 70 (130 spots) and 100 (120 spots) mg/L and, especially, for carvacrol at 35 mg/L (161 spots). Within citral, carvacrol, (E)-2-hexenal and thyme essential oil exposures, the higher protein induction was found in cells exposed to doses corresponding to 1/3 of the MIC. On the contrary, the number of under-synthetized protein spots were the highest using concentrations of antimicrobial products corresponding to 1/5 (E-2-hexenal and thyme essential oil) and/or 1/2 (citral and carvacrol) of the MIC values. Proteins showing different relative amounts were analyzed by Principal Component Analysis (PCA). As shown in the 3-D plot (Figure 8), the proteome profiles differed among the treatments.

All proteins (223 spots), whose relative abundance was up- or down-regulated, were analyzed by MALDI-MS and MS/MS using a Bruker ultraflex III TOF/TOF. Except for hypothetical or unknown proteins, the identified proteins were arranged by functional categories according to the KEGG database. They are mainly involved in the following functional categories: i) cell morphology and motility; ii) ribosomal and regulation system proteins; iii) carbohydrate transport and metabolism and energy production; iv) nucleotide and nitrogen metabolism; v) cofactor and vitamin metabolism; and vi) stress response. Except for hypothetical or unknown proteins, the similarity profiles of identified proteins between samples was analyzed by PermutMatrixEN software (Figure 9). Samples were grouped in three different clusters. Cluster 1 showed two sub-clusters, which grouped control samples and cells treated with ethanol (1A), and (E)-2-hexenal at different concentrations (150, 250 and 400 mg/L) (1B). Clusters 2 and 3 grouped samples belonging to cells treated with thyme essential oil at different concentrations (70 and 100 mg/L) and citral (at 85 or 125 mg/L), respectively. The identified proteins were described in detail in the following paragraphs.

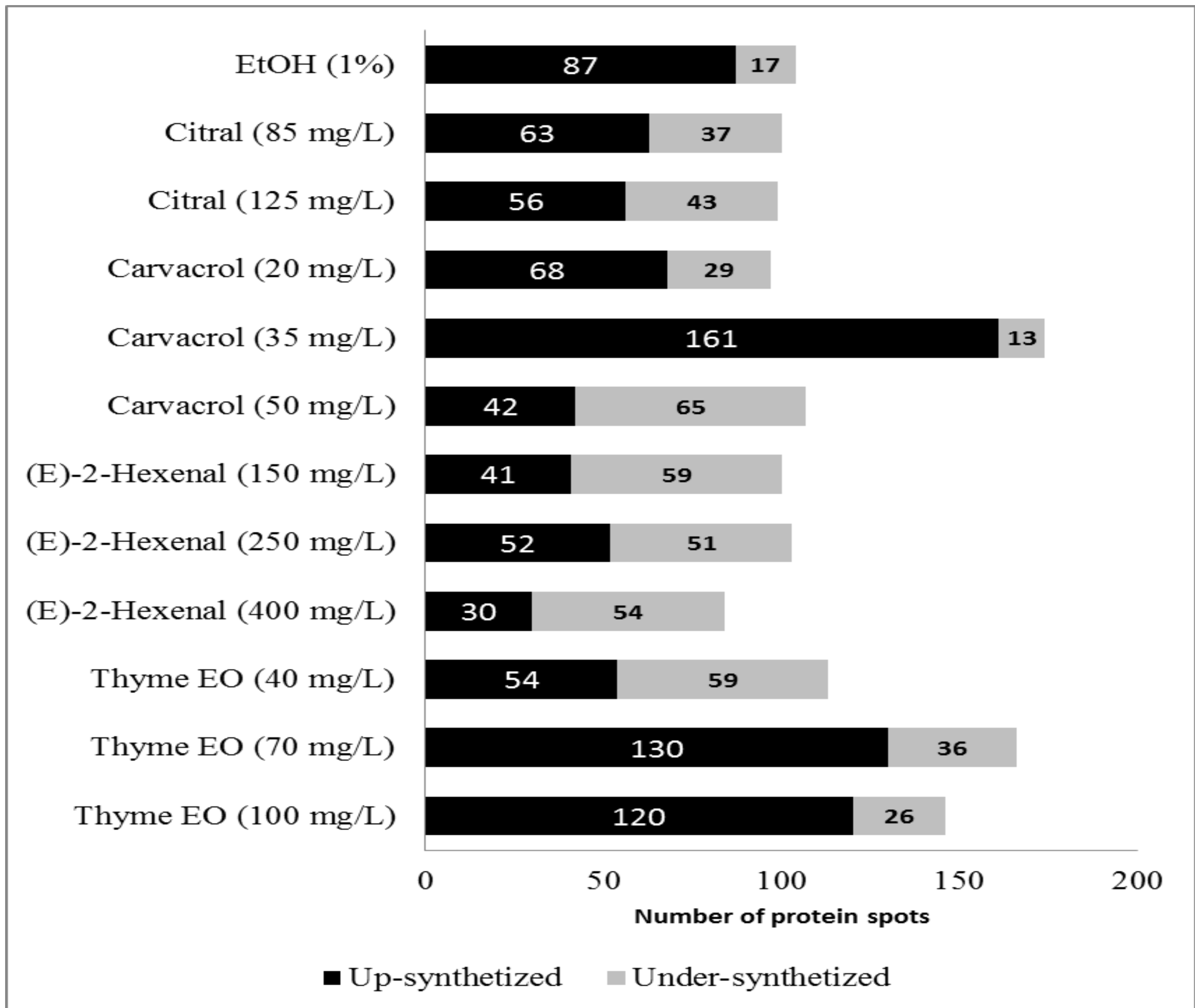


Figure 1: Number of protein spots showing increased and decreased (≥ or ≤ of 2 fold, P<0.05) levels of synthesis when Listeria monocytogenes Scott A cells were treated for 1 h at 37°C with antimicrobial compounds.

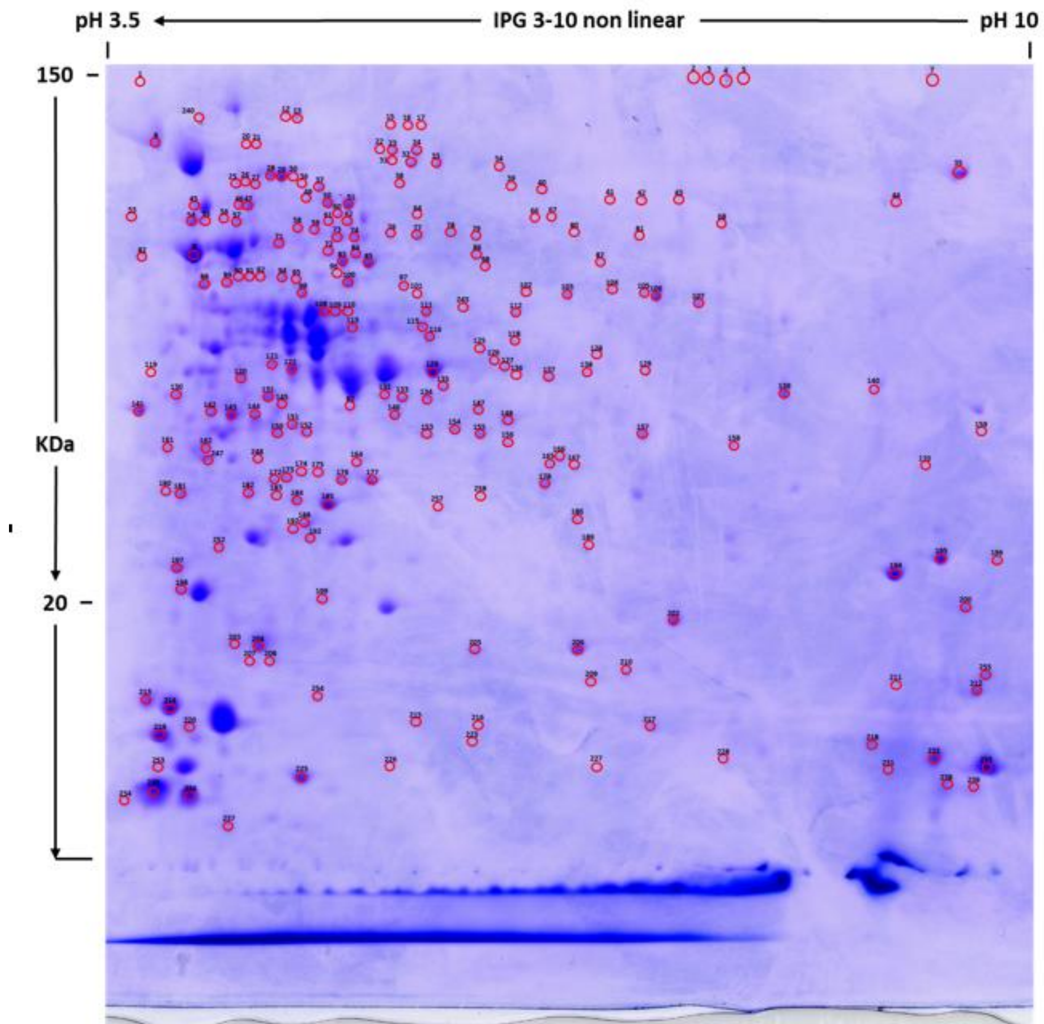


Figure 2: Two-dimensional electrophoresis analysis of intracellular proteins synthesized by Listeria monocytogenes Scott A cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached (untreated cells). The numbered circles refer to proteins with decreased or increased amount during treatment with antimicrobial compounds. Spot designation corresponds to that of the proteins in Table 2.

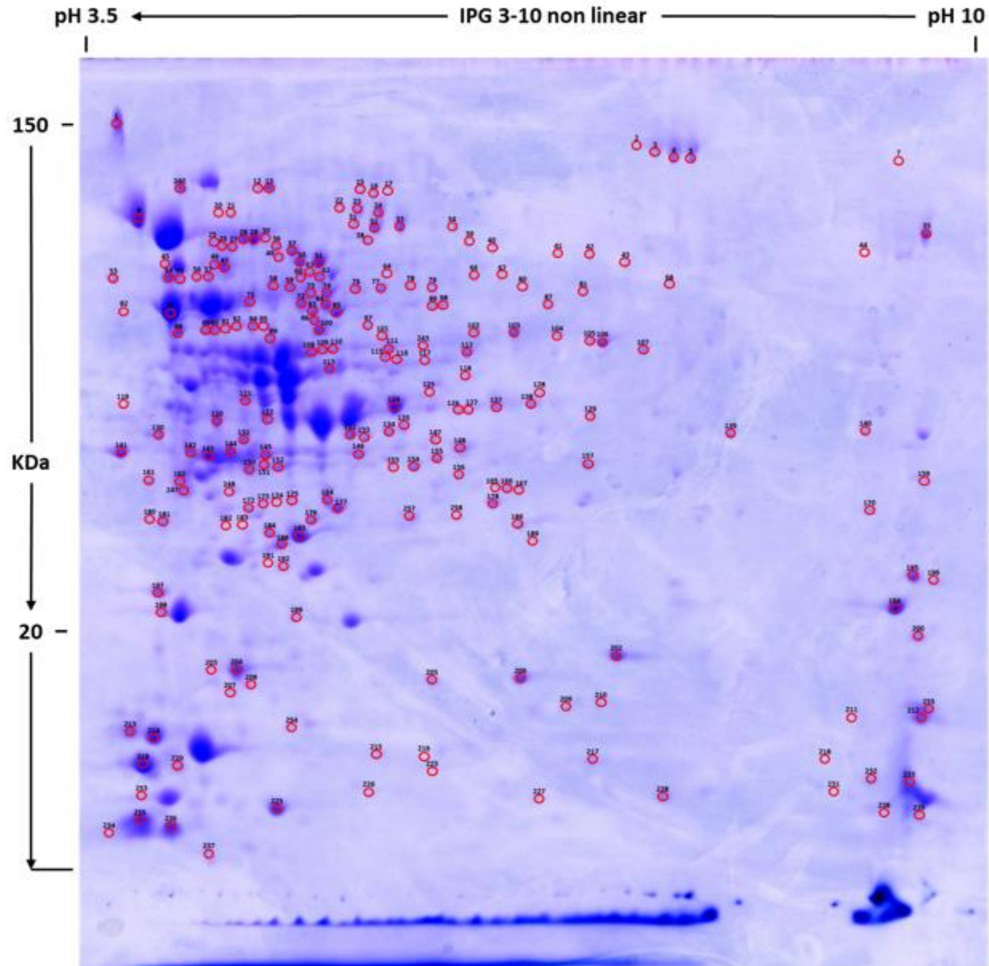
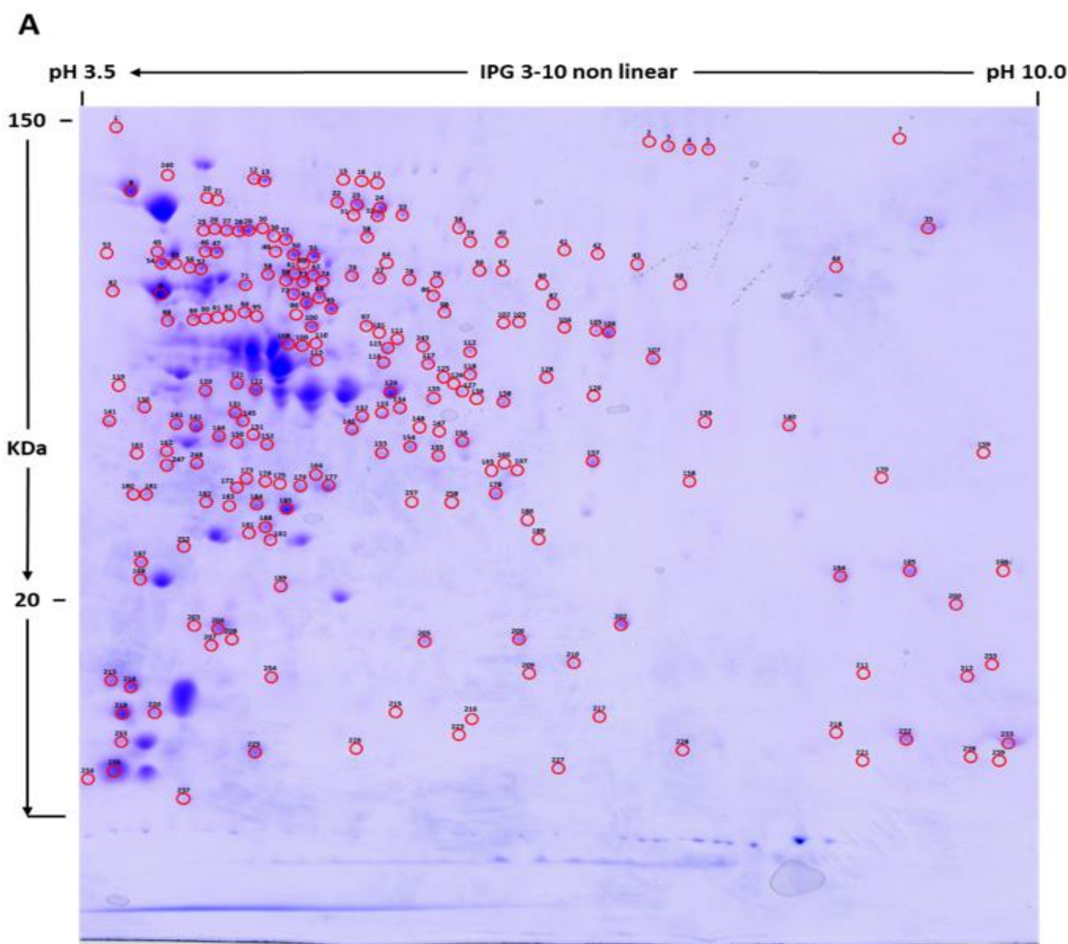


Figure 3: Two-dimensional electrophoresis analysis of intracellular proteins synthesized by Listeria monocytogenes Scott A cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached and treated for one hour to ethanol (1% v/v). The numbered circles refer to proteins with decreased or increased amount compared to un-treated cells and cells treated with other antimicrobial compounds. Spot designation corresponds to that of the proteins in Table 2.



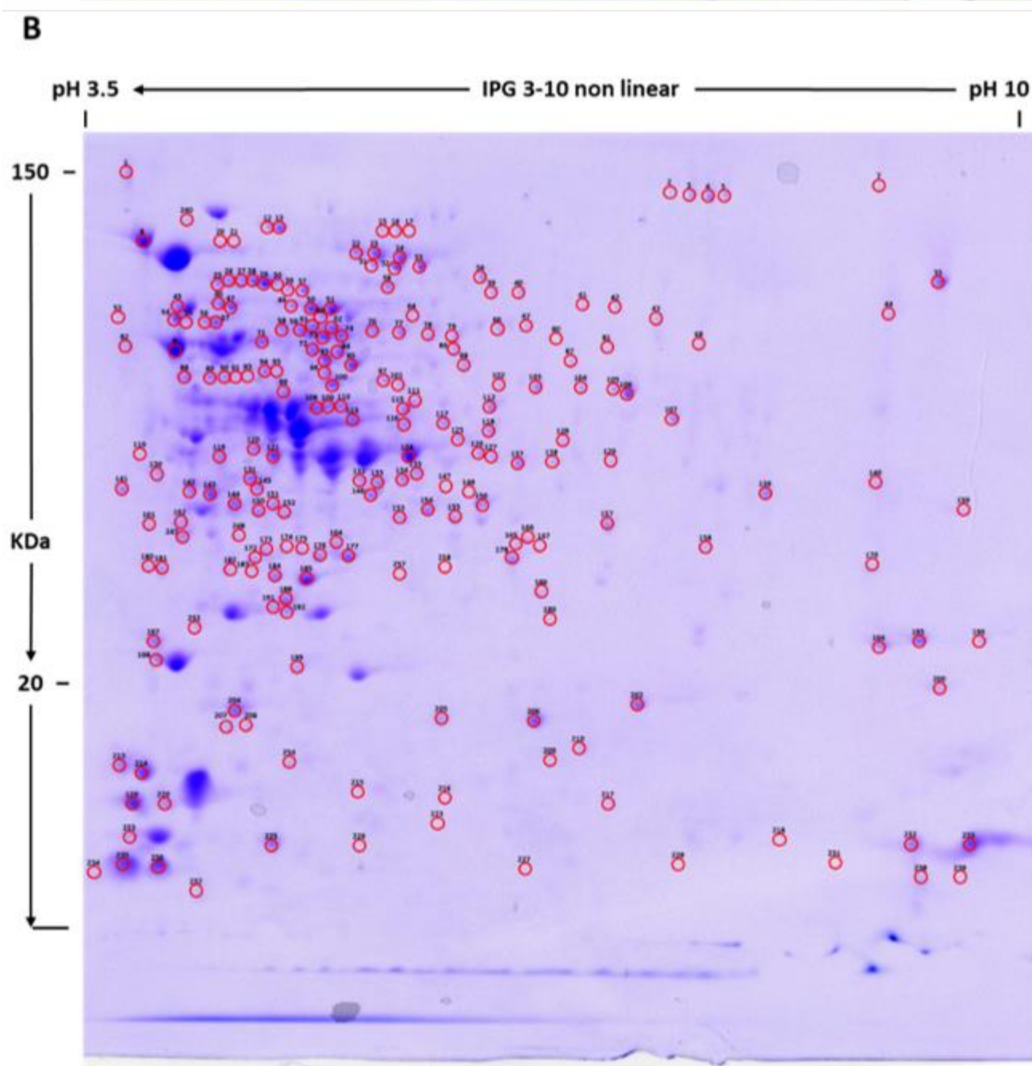
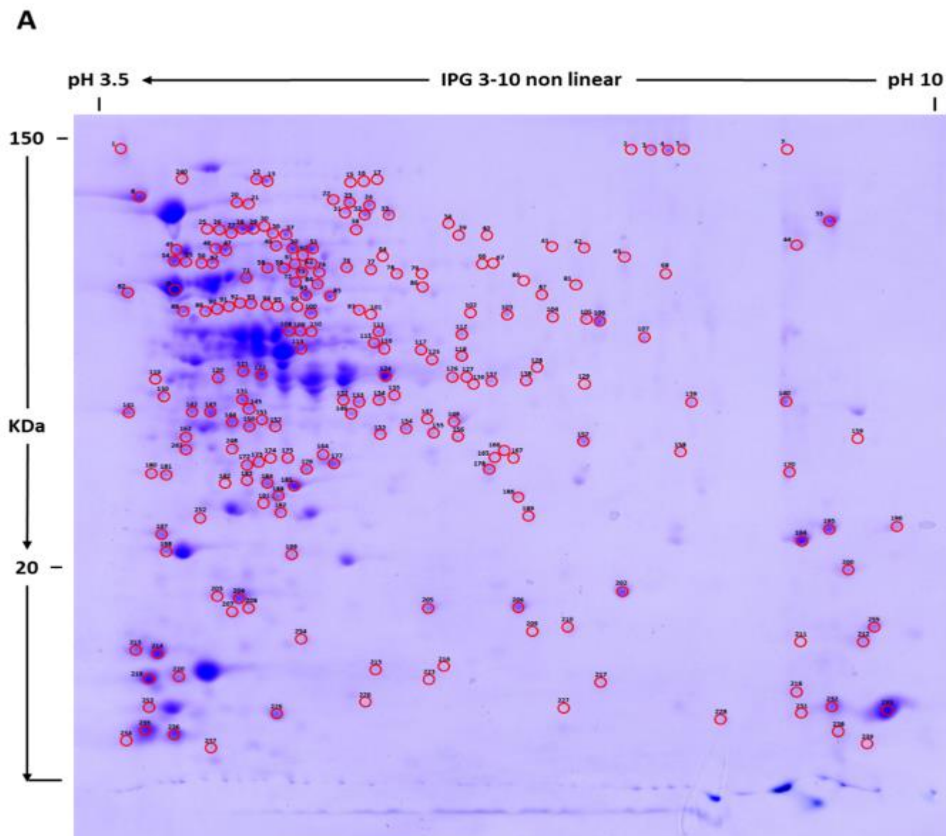


Figure 4: Two-dimensional electrophoresis analysis of intracellular proteins synthesized by Listeria monocytogenes Scott A cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached and treated for one hour to ethanol (1% v/v) and citral at 85 mg/mL (panel A) or 125 mg/L (panel B). The numbered circles refer to proteins with decreased or increased amount compared to un-treated cells. Spot designation corresponds to that of the proteins in Table 2.



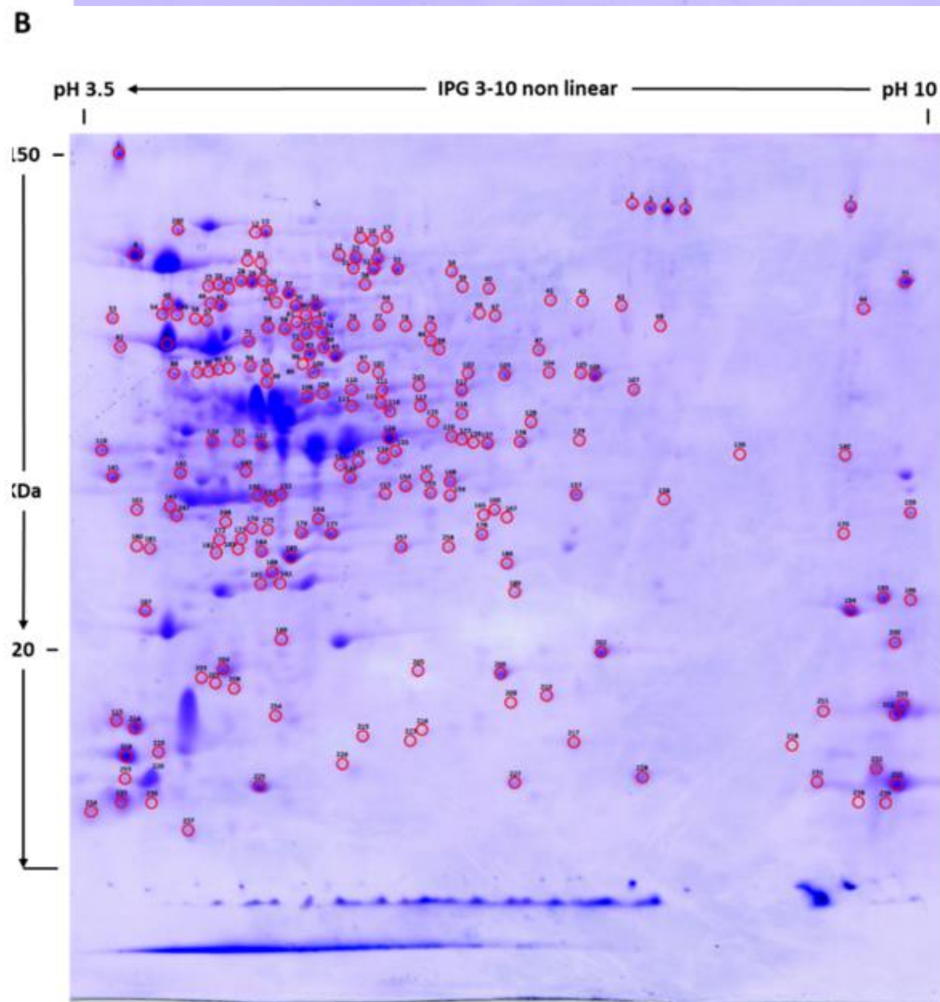
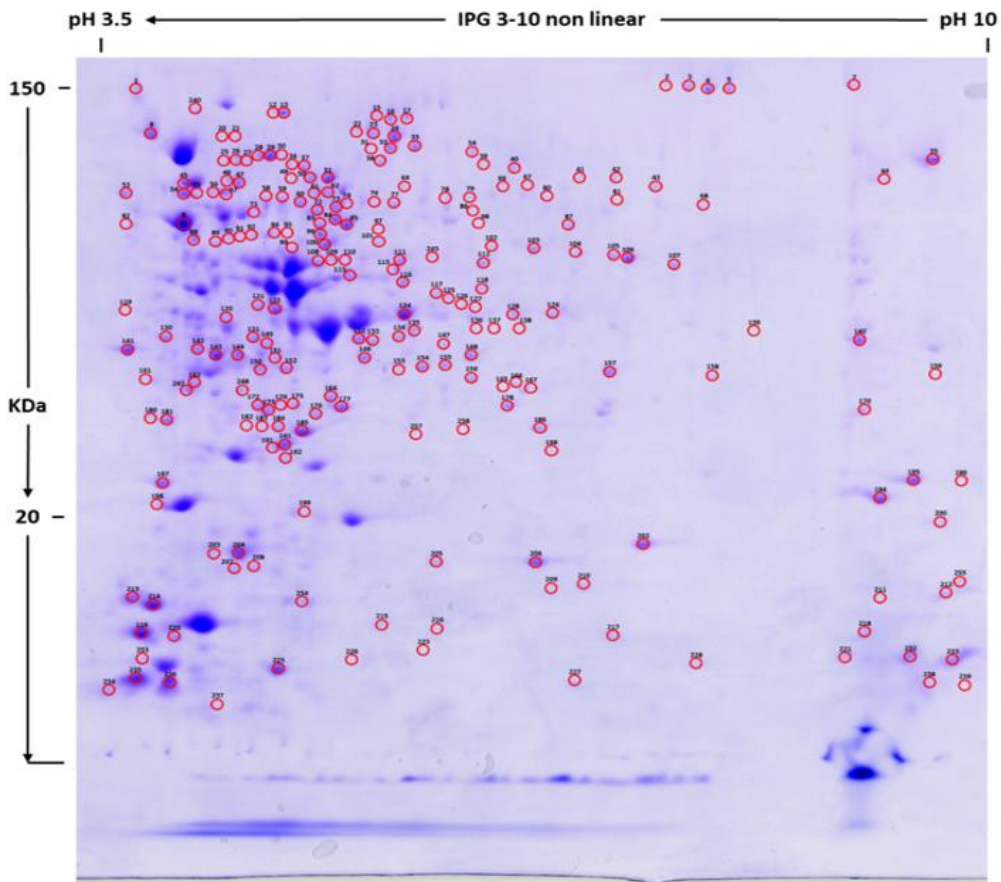


Figure 5: Two-dimensional electrophoresis analysis of intracellular proteins synthesized by Listeria monocytogenes Scott A cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached and treated for one hour to ethanol (1% v/v) and carvacrol at 20 mg/mL (panel A), 35 mg/L (panel B) or 50 mg/L (panel C). The numbered circles refer to proteins with decreased or increased amount compared to un-treated cells. Spot designation corresponds to that of the proteins in Table 2.

C



A

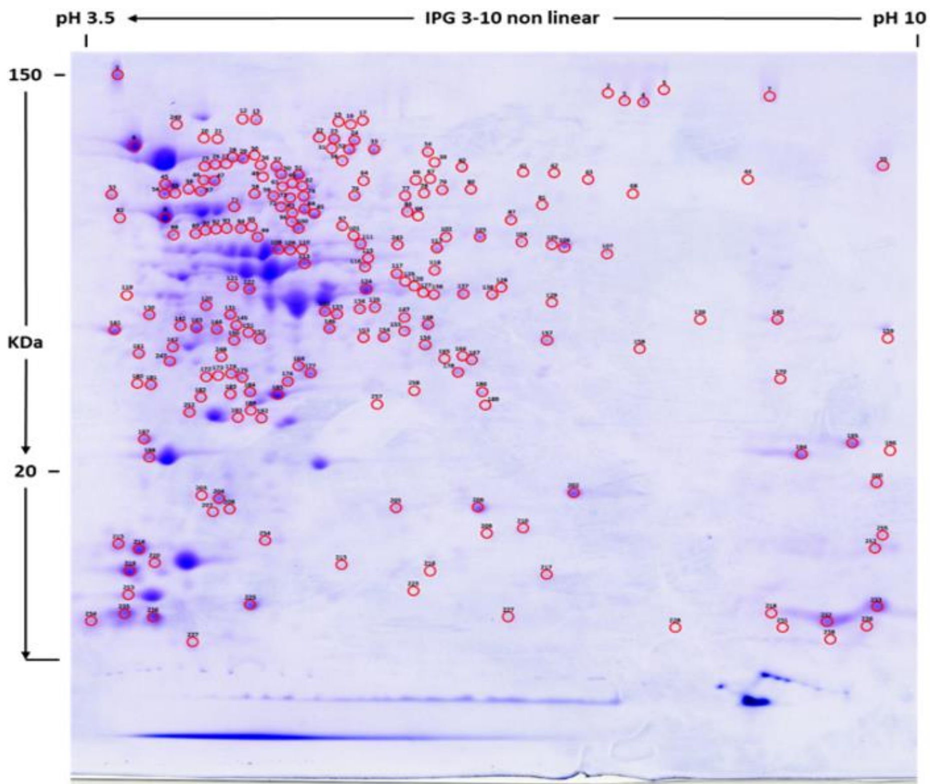
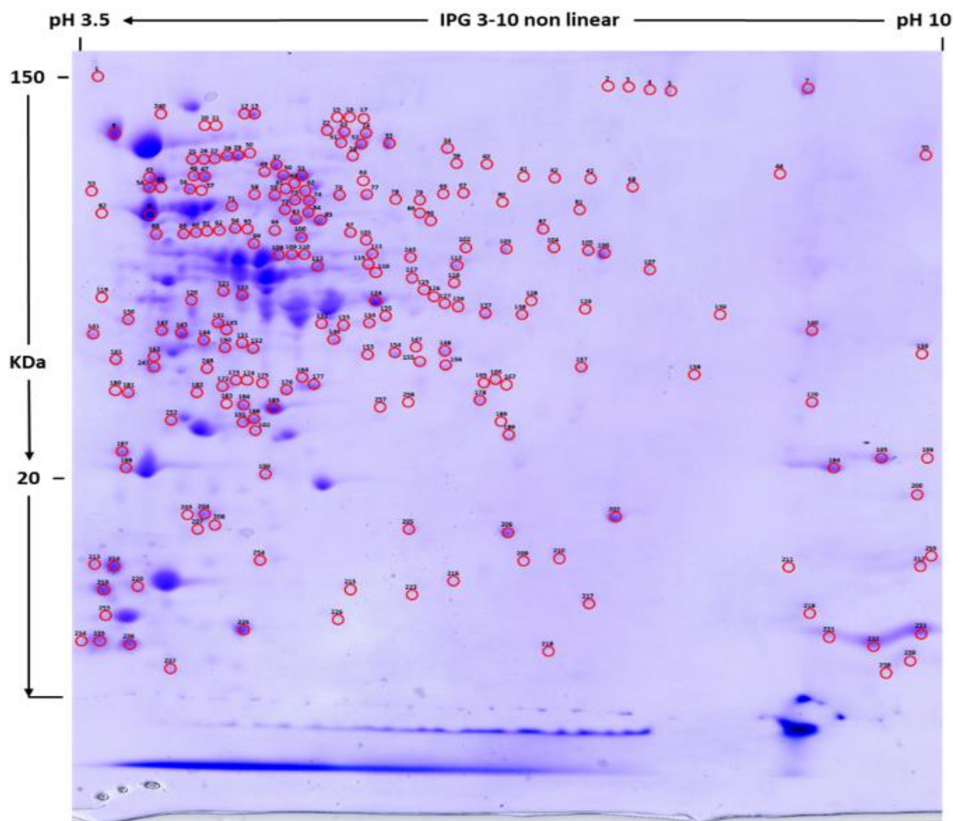
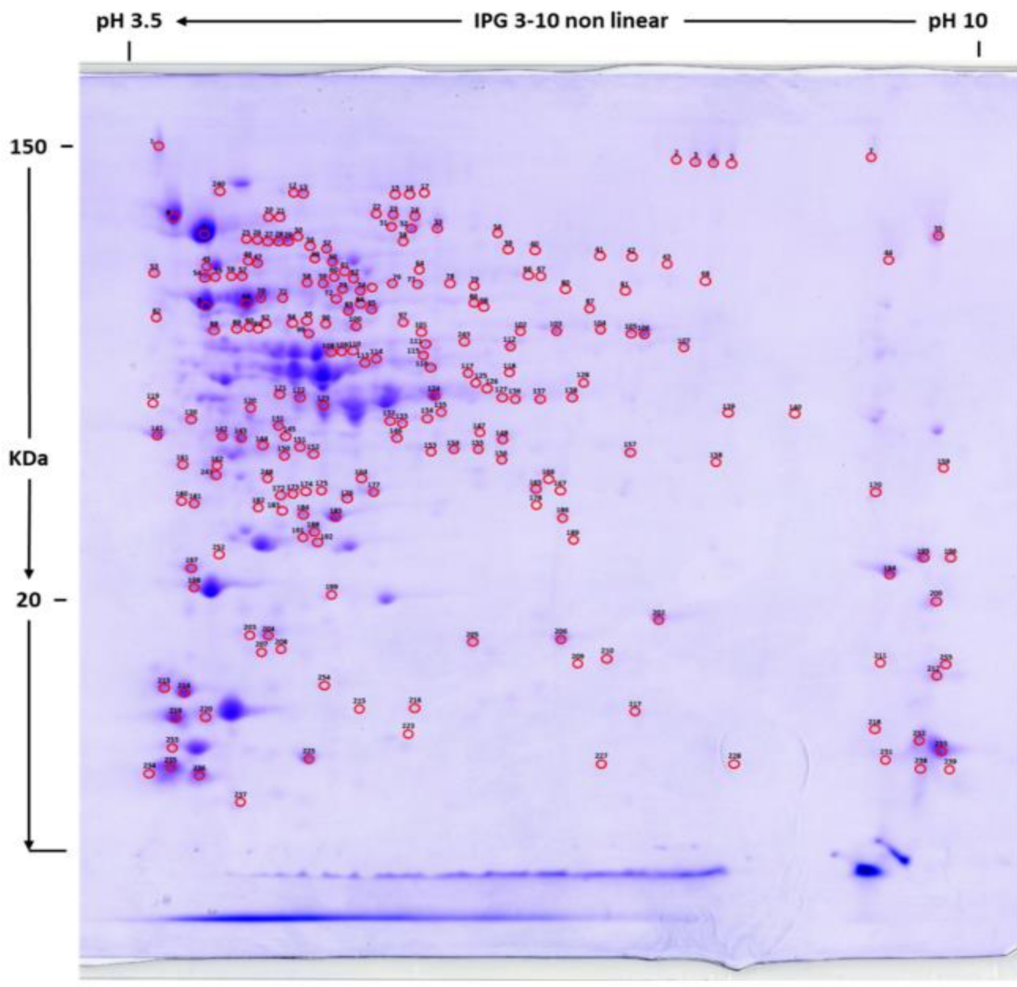


Figure 6: Two-dimensional electrophoresis analysis of intracellular proteins synthesized by *Listeria monocytogenes Scott A* cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached and treated for one hour to ethanol (1% v/v) and (E)-2-hexenal at 150 mg/mL (panel A), 250 mg/L (panel B) or 400 mg/L (panel C). The numbered circles refer to proteins with decreased or increased amount compared to un-treated cells. Spot designation corresponds to that of the proteins in Table 2.

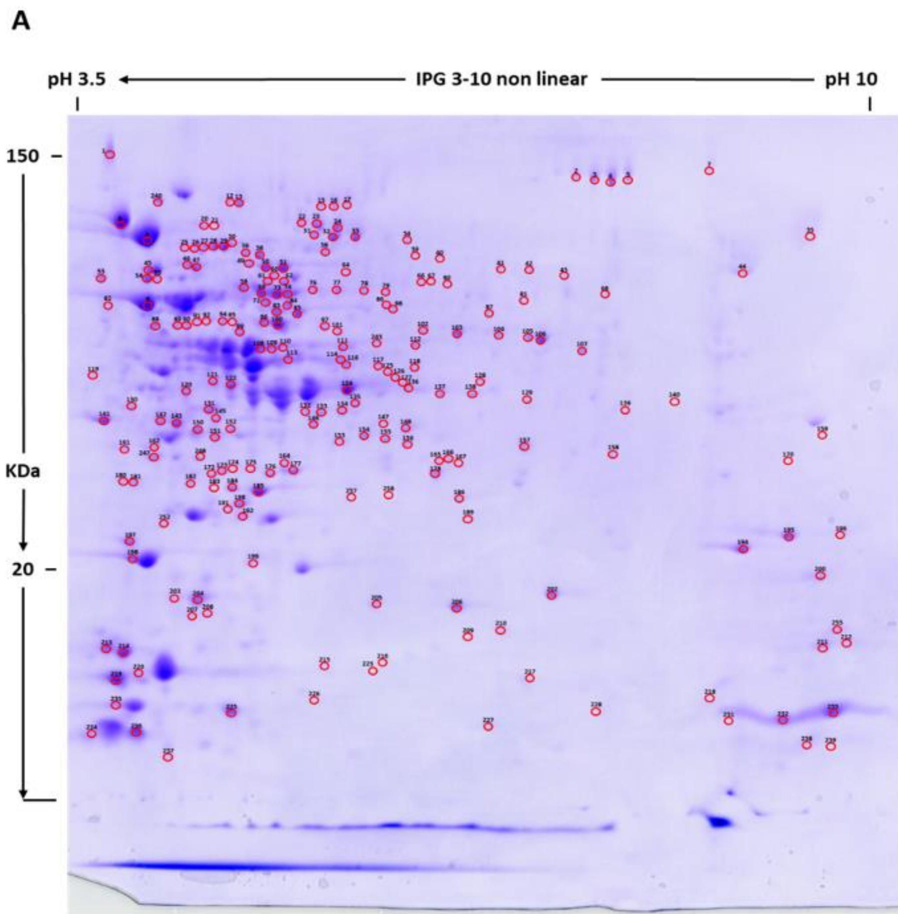
B



C



A



B

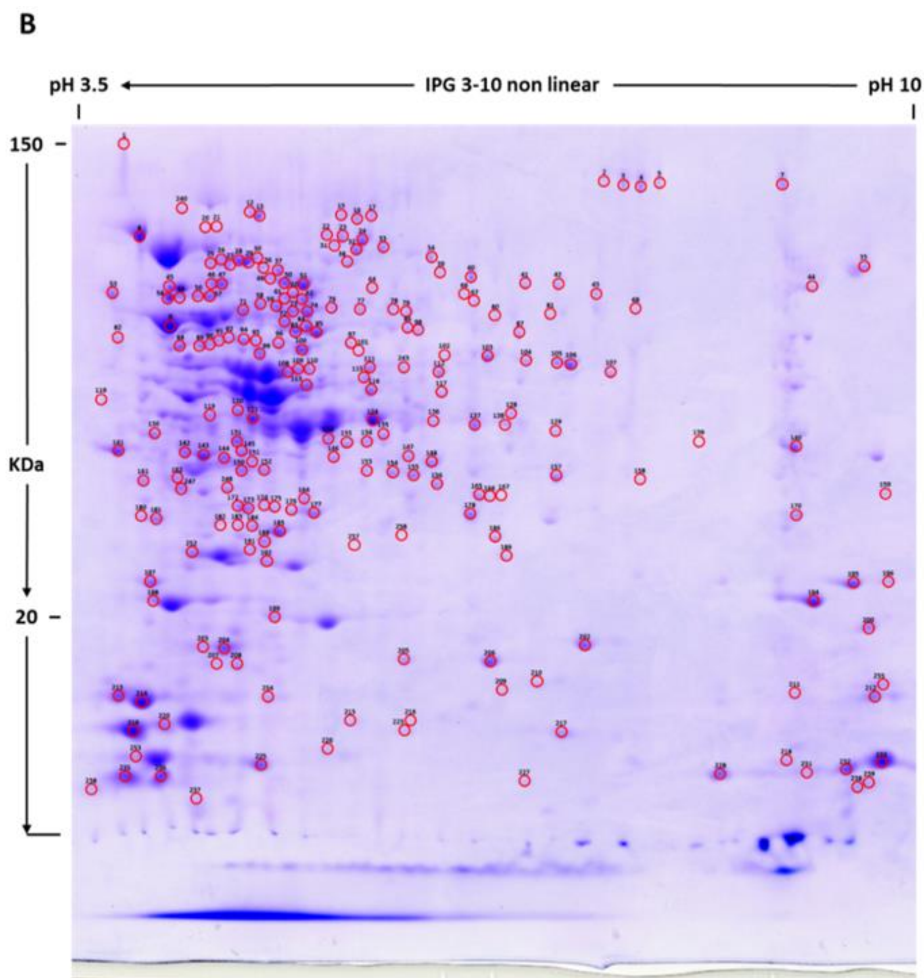
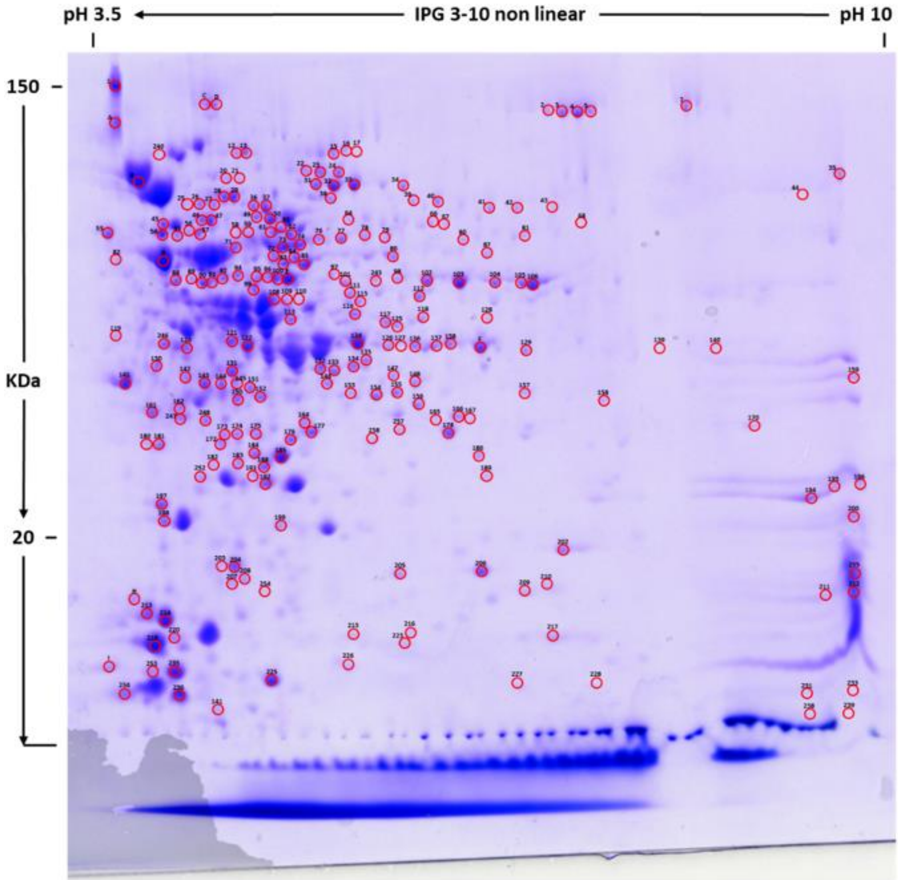


Figure 7: Two-dimensional electrophoresis analysis of intracellular proteins synthesized by Listeria monocytogenes Scott A cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached and treated for one hour to ethanol (1% v/v) and Thyme EO at 40 mg/mL (panel A), 70 mg/L (panel B) or 100 mg/L (panel C). The numbered circles refer to proteins with decreased or increased amount compared to un-treated cells. Spot designation corresponds to that of the proteins in Table 2.

C



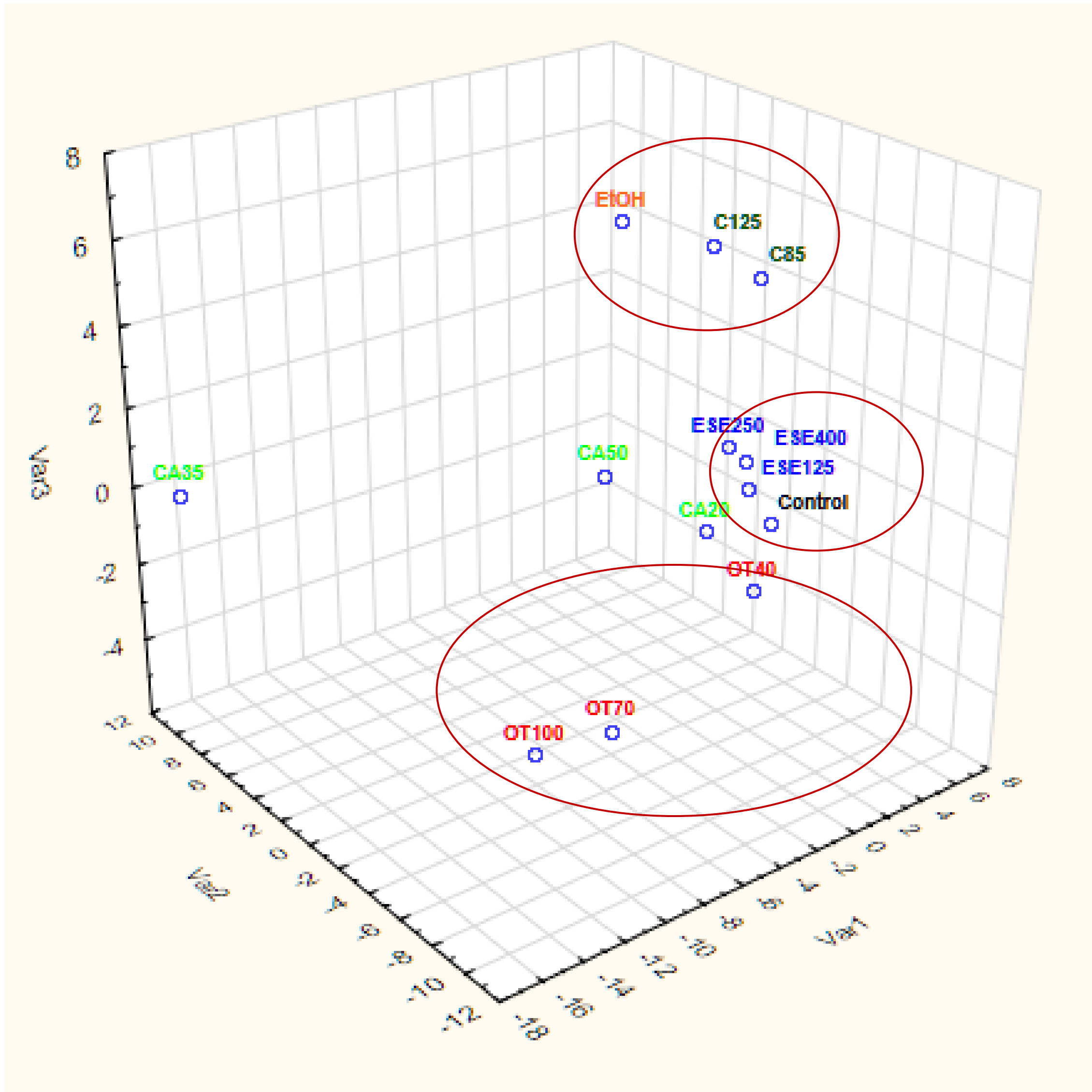


Figure 8: Principal component analysis loading plot of the first three components of the proteins showing different relative amounts of Listeria monocytogenes Scott A *in relation to the stress condition applied.*

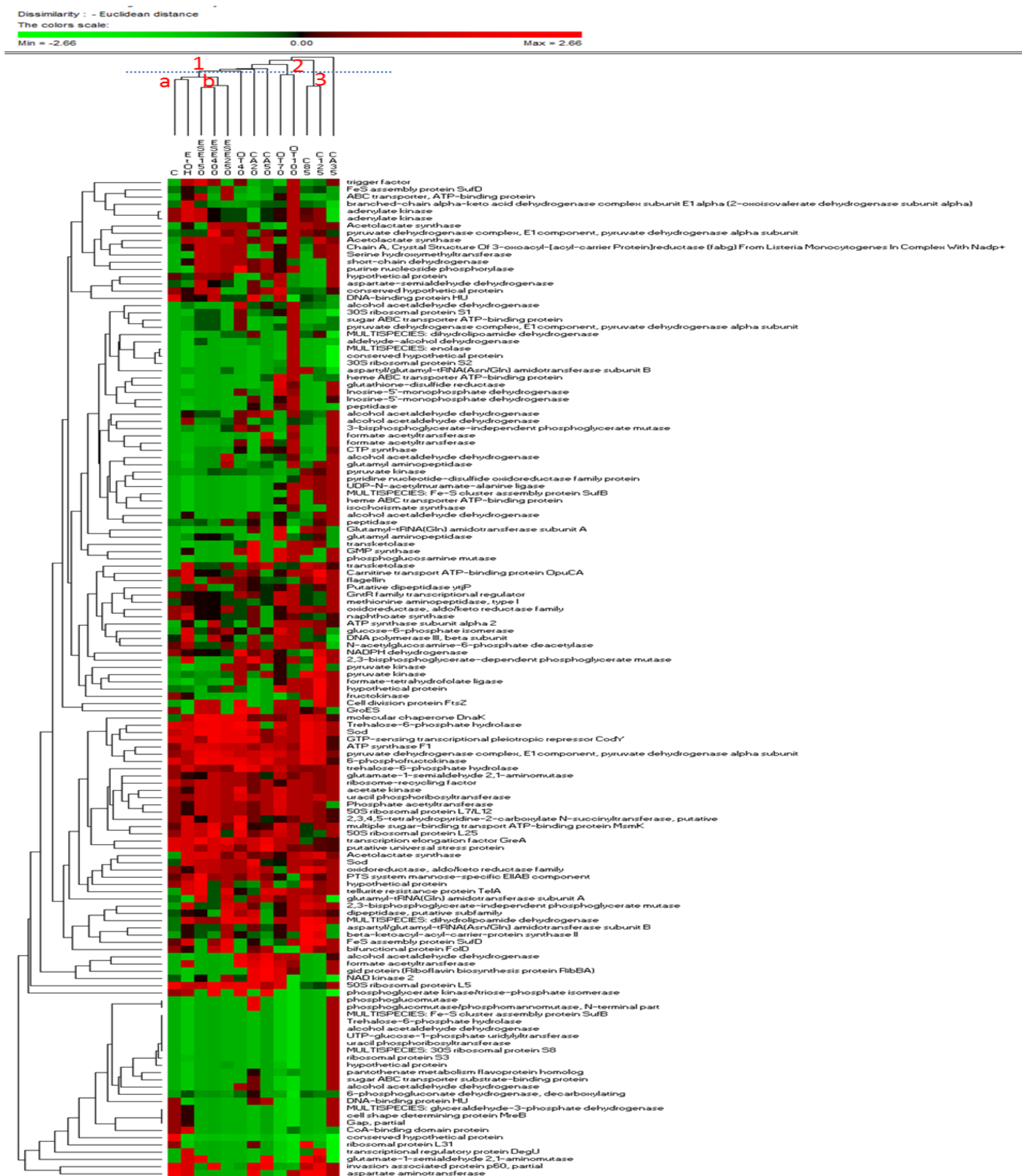


Figure 9: Sample clusterization on the base of the proteins identified and differentially expressed. Three cluster were identified: Cluster 1 formed by control samples with or without ethanol supplementation (1a) and samples treated with sublethal concentrations of (E)-2-hexenal (2b); Cluster 2 formed by samples treated with thyme EO (70-100 mg/L); Cluster 3 formed by samples treated with citral (85-125 mg/L).

Table 1: Protein spots and standardized relative abundance found in Listeria monocytogenes Scott A cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached. Cells were further incubated for 1 h at 37°C in fresh medium alone (untreated, C) or added of ethanol (1% v/v, EtOH) and antimicrobial compounds. The antimicrobial compounds were used: citral at 85 (C85) and 125 (C125)mg/L; carvacrol at 20 (CA20), 35 (CA35) or 50 (CA50); (E)-2-hexenal at 150 (ESE1), 250 (ESE2) or 400 (ESE4) mg/L; and Thyme EO at 40 (OT40), 70 (OT70) or 100 (OT100) mg/L. Only spots showing an increased or decreased (≤ or ≥ of 2-fold, p <0.05) level of synthesis in L. monocytogenes Scott A during at least one treatment were reported.

Spot number	C	EtOH	C85	C125	CA20	CA35	CA50	ESE150	ESE250	ESE400	OT40	OT70	OT100
1	-0.856	0.869	-0.657	-0.446	-0.846	0.444	-0.581	1.514	-0.876	0.616	0.395	-1.076	0.846
2	-0.856	-1.121	-0.841	-0.933	1.250	0.154	1.874	-0.835	-0.876	-0.798	1.844	0.929	0.846
3	-0.856	-0.458	-0.288	-0.446	-0.247	-0.718	0.120	-0.835	-0.876	-0.798	1.119	-0.217	0.846
4	-0.856	0.073	-0.288	-0.446	0.651	0.735	1.172	-0.194	-0.876	-0.192	0.395	-0.790	0.604
5	-0.856	0.603	-0.288	-0.770	-0.846	0.735	-0.230	-0.621	-0.323	-0.596	0.395	-0.790	-0.125
7	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.581	-0.621	1.152	-0.596	-0.692	-0.217	0.361
8	-0.376	0.869	1.557	1.178	0.352	0.154	0.471	2.368	1.889	2.232	1.844	0.356	0.846
12	-0.856	-1.121	0.819	0.529	1.849	-0.718	-0.756	-0.835	-0.876	-0.798	-0.148	-0.933	0.846
13	-0.856	1.399	1.188	0.853	0.651	0.154	1.172	-0.194	0.414	-0.192	-0.330	-0.217	-0.125
15	-0.856	-0.989	-0.841	-0.933	-0.995	0.735	1.523	-0.835	-0.876	-0.798	-0.692	-1.076	0.604
16	-0.856	-0.989	-0.841	-0.933	-0.995	0.735	0.822	-0.835	-0.876	-0.798	0.395	-0.217	0.846
17	-0.856	1.134	-0.841	-0.933	1.549	0.735	2.225	-0.835	-0.876	-0.798	2.206	1.215	0.604
20	-0.856	-1.121	-0.841	-0.933	1.849	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
21	-0.856	-1.121	-0.841	-0.933	1.849	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
22	-0.856	-1.121	-0.657	2.152	0.352	-0.427	-0.756	-0.835	0.783	-0.798	0.757	0.069	-0.368
23	-0.696	-0.723	0.450	0.204	-0.546	0.735	-0.581	-0.621	-0.323	-0.192	-0.330	-0.790	-0.611
24	-0.696	-0.458	1.926	1.503	-0.546	0.735	0.822	-0.621	-0.323	-0.596	0.757	0.069	-0.368
25	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
26	-0.856	-1.121	-0.841	0.529	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	0.846
27	-0.856	0.073	0.819	-0.933	1.849	0.735	-0.756	-0.835	-0.876	-0.798	0.757	1.502	0.846
28	1.544	1.399	1.926	1.503	1.849	0.735	-0.756	1.941	1.889	2.232	2.568	1.502	0.846
29	0.584	1.399	0.450	0.529	1.849	0.735	0.822	0.660	0.414	0.616	2.206	1.215	0.604
30	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461

31	-0.856	0.073	0.819	0.529	0.352	0.444	-0.756	-0.835	0.783	1.020	0.757	-1.048	0.846
32	-0.696	1.399	1.188	1.503	0.352	0.444	0.471	1.514	0.414	1.424	1.119	0.069	0.846
33	-0.696	0.869	-0.288	1.178	0.352	0.444	0.822	1.087	1.152	1.020	0.757	-0.790	0.846
34	-0.856	-0.989	-0.657	-0.933	-0.995	0.444	0.120	-0.621	-0.692	-0.596	0.033	0.069	0.846
35	1.864	1.399	1.004	1.827	1.549	0.735	2.225	-0.621	-0.876	2.232	-0.692	-0.790	-0.854
36	-0.856	-0.591	-0.841	-0.933	0.352	0.735	-0.756	-0.835	-0.139	-0.798	0.757	-0.790	0.846
37	-0.696	1.399	1.557	1.178	0.352	0.735	-0.581	-0.194	1.520	-0.192	1.119	0.069	0.846
38	-0.856	-1.121	0.819	2.152	-0.995	0.735	-0.756	-0.621	-0.876	-0.798	0.757	0.069	0.846
39	-0.856	-1.121	-0.841	0.529	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	0.069	0.846
40	-0.856	-1.121	-0.841	-0.446	-0.995	-0.427	-0.230	-0.835	-0.876	-0.798	-0.909	1.502	0.846
41	-0.856	-0.989	-0.841	-0.933	0.352	0.154	-0.756	-0.835	-0.876	-0.798	-0.873	0.069	0.846
42	-0.856	-0.723	-0.841	-0.933	-0.546	0.735	-0.756	-0.835	-0.876	-0.798	0.395	1.215	0.604
43	-0.856	0.073	-0.657	0.529	0.352	0.735	-0.581	-0.835	-0.876	-0.798	-0.873	0.069	-0.368
44	-0.856	-1.121	-0.841	-0.933	0.352	0.735	-0.756	-0.835	-0.876	-0.798	0.757	-1.220	-1.461
45	-0.696	-1.121	-0.657	1.827	-0.995	0.735	-0.756	1.087	-0.876	1.020	1.844	0.929	0.846
46	-0.216	0.073	0.819	0.529	1.250	0.154	-0.756	0.019	1.889	-0.798	0.757	0.069	0.846
47	-0.376	-0.193	-0.103	0.204	0.052	0.735	-0.055	0.660	-0.692	-0.596	0.395	0.069	0.846
49	-0.856	-1.121	-0.288	0.204	0.352	-1.589	-0.756	-0.835	-0.323	-0.798	-0.330	-0.933	0.846
50	-0.376	1.399	2.295	1.827	1.549	0.735	-0.756	0.660	1.889	0.616	1.844	0.929	0.846
53	-0.856	-0.989	-0.841	-0.933	-0.546	-1.589	-0.230	-0.194	-0.876	-0.192	0.395	0.069	0.846
54	0.424	1.399	1.926	1.503	0.651	0.154	1.172	1.514	1.152	1.424	1.119	0.929	0.604
55	-0.856	-1.121	-0.841	-0.933	1.849	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	0.846
56	-0.856	-1.121	0.819	0.529	0.352	-0.718	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	-1.461
57	-0.376	-1.121	1.188	0.853	0.651	-1.734	1.172	1.514	1.152	-0.192	-0.330	0.356	0.846
58	-0.696	-0.193	-0.288	-0.446	0.052	-0.427	-0.756	-0.621	-0.692	-0.596	-0.330	-0.790	-1.583
59	-0.376	0.073	2.664	2.152	0.352	-0.718	-0.230	-0.194	0.783	-0.192	-0.330	0.069	0.846
60	-0.856	0.073	0.819	0.529	0.352	0.154	-0.756	-0.835	-0.139	1.020	-0.873	0.929	0.846
61	-0.856	-0.723	0.819	-0.446	-0.995	-1.298	-0.756	-0.835	-0.323	-0.798	-0.873	-0.504	0.846

62	-0.376	-0.458	0.819	2.152	-0.995	0.735	-0.756	-0.835	0.783	-0.192	-0.330	1.502	0.846
64	-0.856	-1.121	-0.841	-0.933	1.849	0.735	2.575	-0.835	-0.876	-0.798	-0.873	1.502	0.361
66	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	0.361
67	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	0.361
68	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
71	0.584	1.399	0.819	2.152	0.352	0.735	-0.756	0.019	-0.139	0.010	0.757	0.069	0.846
72	-0.696	0.073	2.664	1.503	-0.247	-0.718	-0.581	-0.194	0.783	-0.596	0.033	0.069	-0.368
73	-0.696	1.399	0.819	0.529	-0.247	0.735	1.523	1.941	1.520	-0.192	0.033	1.502	0.846
74	-0.696	1.399	-0.288	0.529	-0.546	-0.718	0.120	-0.194	0.783	0.212	0.033	1.502	0.846
76	0.264	1.399	2.664	2.152	0.052	0.735	-0.756	-0.835	1.889	0.616	-0.873	-0.217	0.846
77	-0.376	0.073	-0.288	-0.121	-0.995	-0.718	-0.581	-0.194	0.783	-0.596	-0.692	-0.217	0.846
78	-0.856	-1.121	0.819	0.529	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	-0.125
79	-0.856	-1.121	0.819	0.529	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	0.069	0.846
80	-0.856	-1.121	-0.841	-0.933	-0.546	-1.008	-0.756	-0.194	-0.876	-0.798	-0.873	-0.933	0.846
81	-0.856	-1.121	-0.841	-0.933	0.052	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	-0.611
82	-0.856	-1.121	-0.841	-0.933	0.352	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
83	0.584	0.073	0.819	0.529	0.352	0.735	-0.055	0.019	0.783	1.020	0.757	0.929	0.361
84	0.584	0.073	0.081	0.529	0.352	0.735	0.822	1.087	0.783	0.010	0.757	0.929	0.361
85	0.584	0.073	0.819	0.529	0.352	0.735	0.822	1.087	0.783	1.020	1.844	1.444	0.846
86	-0.856	-0.989	-0.841	0.529	-0.995	0.735	-0.756	1.087	0.783	1.020	0.757	0.069	0.846
87	0.264	-0.193	-0.841	-0.933	-0.995	0.735	0.471	0.660	0.414	0.616	-0.873	0.929	-0.611
88	-0.056	1.399	-0.288	-0.121	0.651	-1.298	-0.581	-0.621	0.045	1.020	-0.692	0.929	0.846
89	-0.376	1.399	-0.288	-0.446	-0.546	-1.298	-0.230	-0.194	-0.692	-0.596	-0.692	-0.217	0.846
90	-0.696	0.869	-0.288	-0.770	-0.546	-1.298	-0.756	-0.621	-0.323	-0.596	-0.692	-0.790	0.846
91	-0.856	-1.121	-0.841	-0.933	0.052	-1.008	-0.756	-0.835	-0.876	-0.798	-0.873	0.356	0.846
92	-0.856	-1.121	-0.841	-0.933	0.052	-1.008	-0.756	-0.835	-0.876	-0.798	-0.873	0.356	0.846
94	-0.376	-0.458	-0.472	0.204	0.052	0.735	-0.756	-0.194	0.414	-0.596	-0.330	0.069	-0.854
95	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.692	-0.798	-0.873	-1.048	-1.461

96	-0.856	1.399	2.664	1.178	-0.995	-2.024	2.575	1.941	-0.876	-0.798	-0.873	-1.220	-1.461
97	2.184	1.399	2.664	2.152	1.849	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	0.846
98	-0.056	1.399	0.081	0.204	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	0.356	0.846
99	2.184	0.603	-0.288	-0.446	-0.546	-0.137	-0.756	-0.194	0.783	-0.192	-0.330	1.502	0.846
100	2.184	-1.254	-1.026	-1.095	-1.145	-2.170	-0.932	-1.048	-1.061	-1.000	-1.054	-1.363	-1.583
101	0.264	0.869	0.450	0.853	0.651	-1.008	-0.230	-0.194	0.414	-0.192	-0.330	0.929	0.846
102	-0.856	-0.723	-0.841	-0.933	-0.995	-0.427	-0.756	-0.194	-0.876	-0.192	0.395	-0.933	0.846
103	0.264	1.399	-0.103	0.529	0.052	0.735	0.120	1.087	0.414	1.828	2.568	1.502	0.846
104	-0.856	-0.723	-0.620	-0.446	-0.995	-0.427	-0.756	-0.194	-0.876	-0.192	0.395	-0.217	0.846
105	-0.376	-0.193	0.450	0.204	-0.546	-0.137	0.471	-0.194	0.414	1.828	0.757	0.069	0.846
106	0.584	0.869	1.742	1.503	1.250	0.444	1.523	1.941	1.520	1.828	1.119	1.358	0.846
107	0.584	0.073	-0.841	1.503	0.352	0.735	0.822	0.019	-0.139	0.010	1.844	0.069	-0.368
108	0.584	0.073	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
109	0.584	0.073	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
110	0.584	0.073	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
111	-0.216	-0.591	-0.841	-0.933	0.352	0.735	-0.581	1.087	-0.139	0.010	-0.873	0.929	-0.611
112	-0.696	-0.989	-0.472	0.529	-0.546	0.735	-0.230	-0.621	0.783	-0.596	-0.692	-0.790	0.846
113	0.584	0.073	0.819	0.529	0.352	-0.718	0.822	1.087	0.783	1.020	0.757	1.502	0.846
115	0.584	0.073	1.926	0.529	0.352	0.735	0.822	-0.835	1.889	1.020	-0.873	-1.220	-1.340
116	0.584	0.073	-0.288	-0.446	0.352	0.154	0.822	1.087	-0.876	1.020	-0.873	1.502	-0.368
117	-0.856	-0.591	-0.103	0.204	-0.995	-0.718	-0.756	0.019	-0.139	0.010	-0.873	1.502	-0.368
118	0.584	0.073	0.819	2.152	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-0.368
119	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
120	-0.376	1.399	0.450	1.503	0.052	0.735	-0.230	-0.621	0.414	0.616	0.395	-0.217	-0.611
121	0.584	0.869	0.819	0.529	0.352	0.735	-0.756	0.019	0.783	0.010	-0.148	0.069	0.846
122	0.584	0.073	1.926	1.503	1.250	0.735	0.822	1.087	0.783	1.020	0.757	-0.532	0.846
124	1.544	0.869	1.926	1.503	1.250	0.154	1.874	2.368	1.889	1.828	1.844	0.929	0.846
125	-0.856	-1.121	-0.841	2.152	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461

126	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
127	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
128	-0.856	1.399	-0.841	-0.933	-0.995	0.735	0.822	-0.835	0.783	-0.798	0.757	0.069	-1.461
129	1.544	1.399	-0.841	1.503	-0.995	0.735	-0.581	-0.835	-0.692	-0.798	-0.692	-1.220	-1.461
130	1.544	1.399	0.450	0.204	1.250	0.154	0.471	0.660	0.414	0.616	0.395	0.929	0.846
131	1.544	1.399	1.926	1.503	1.849	0.154	0.471	0.660	1.889	2.232	1.844	0.929	0.846
132	-0.856	-0.591	-0.841	-0.933	-0.397	0.154	0.822	2.368	-0.139	1.020	-0.873	-0.933	0.846
133	0.584	0.073	-0.103	-0.283	0.352	0.735	-0.055	0.019	-0.139	0.010	0.757	1.072	0.846
134	-0.216	0.073	-0.103	-0.283	-0.397	0.735	-0.055	0.019	-0.139	0.010	-0.148	1.072	0.846
135	-0.216	0.073	-0.103	-0.283	-0.397	0.735	-0.055	0.019	-0.139	0.010	-0.148	0.069	0.846
136	-0.856	-1.121	-0.103	0.529	0.352	0.735	-0.756	0.019	-0.876	0.010	-0.873	-1.076	0.846
137	-0.216	0.073	-0.103	-0.933	-0.397	0.735	-0.756	0.019	0.783	-0.798	-0.148	0.929	0.361
138	-0.856	0.338	-0.841	0.853	-0.995	0.154	-0.756	-0.194	-0.876	-0.798	-0.330	-0.933	0.846
139	2.184	0.869	-0.103	0.529	-0.397	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
140	-0.216	-1.121	-0.841	-0.933	1.849	-1.734	2.575	0.019	0.783	-0.798	-0.148	1.502	-1.461
141	0.584	1.399	-0.103	-0.283	0.352	0.735	0.822	1.087	-0.139	2.232	1.844	1.272	0.846
142	0.584	0.869	0.266	0.529	0.352	0.735	-0.055	0.019	-0.139	1.020	0.757	-0.647	-0.368
143	0.584	1.399	0.819	0.529	0.352	0.735	1.874	1.087	1.889	1.020	0.757	0.069	0.361
144	0.584	1.399	0.819	0.529	0.352	0.154	-0.055	0.019	0.783	1.020	1.844	0.069	0.361
145	0.584	1.399	-0.103	-0.283	0.352	0.154	-0.756	0.019	-0.139	0.010	-0.148	0.069	0.361
146	0.584	1.399	2.664	0.529	-0.546	0.735	0.822	1.941	0.783	-0.192	-0.330	-0.790	-0.368
147	0.584	1.399	-0.841	-0.933	-0.995	-0.718	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-0.368
148	-0.216	0.073	-0.841	-0.933	-0.995	0.735	0.822	1.087	-0.876	0.010	-0.148	0.069	-0.368
150	0.584	1.399	0.192	0.529	-0.995	-1.444	-0.055	1.087	-0.139	0.010	-0.148	0.069	0.846
151	0.584	1.399	0.081	0.529	-0.995	-1.444	-0.055	1.087	-0.139	0.010	-0.148	0.069	0.846
152	0.584	1.399	0.302	0.529	-0.995	-1.444	-0.055	1.087	-0.139	0.010	-0.148	0.069	0.846
153	0.584	-1.121	0.819	-0.933	0.352	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
154	-0.696	1.399	-0.288	2.152	1.849	0.735	0.822	-0.835	-0.692	-0.798	0.757	0.069	0.846

155	2.184	1.399	1.742	2.152	0.352	0.735	0.822	1.087	0.783	1.020	0.757	1.502	0.846
156	-0.856	0.073	-0.657	0.529	-0.846	0.735	0.822	1.087	0.783	1.020	0.757	0.069	0.846
157	1.224	0.073	1.557	1.178	1.849	0.735	1.523	1.941	0.783	1.020	0.757	1.502	0.118
158	-0.856	1.399	-0.472	2.152	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
159	-0.856	-0.989	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
161	-0.216	0.073	-0.472	-0.608	-0.995	-0.718	-0.756	0.019	-0.139	0.010	-0.148	0.069	0.846
162	0.584	0.869	-0.841	-0.933	-0.995	-2.024	2.575	-0.835	-0.876	2.232	1.844	-1.220	-1.461
164	-0.696	1.399	-0.288	-0.283	-0.696	-0.718	0.822	0.019	-0.876	-0.798	-0.873	-0.647	-0.976
165	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
166	-0.856	0.073	-0.841	-0.933	-0.995	-0.718	-0.756	1.087	0.783	-0.798	-0.873	0.069	0.846
167	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
170	-0.856	0.073	-0.657	0.529	1.849	-2.024	0.822	-0.835	0.783	-0.798	-0.873	1.502	-1.461
172	0.584	0.073	0.819	0.529	-0.995	0.735	2.575	-0.835	0.783	1.020	0.757	1.502	0.846
173	0.584	-1.121	0.819	0.529	0.352	0.735	2.575	-0.835	0.783	1.020	0.757	1.502	0.846
174	-0.856	-1.121	-0.841	-0.933	0.352	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	0.846
175	-0.856	0.073	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	0.846
176	1.224	0.603	0.819	0.529	0.352	0.735	0.822	1.087	0.783	1.020	0.757	0.069	0.846
177	1.224	0.603	1.557	1.178	0.950	0.735	1.523	1.941	1.520	1.828	1.481	0.642	0.846
178	0.584	0.603	0.819	1.178	0.950	0.735	0.822	1.087	0.783	1.020	1.481	1.502	0.846
180	2.184	1.399	2.664	2.152	1.849	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
181	0.264	0.869	-0.657	-0.770	0.052	-1.008	2.575	0.660	0.414	0.616	-0.330	1.502	0.846
182	2.184	-1.121	-0.841	-0.446	-0.995	0.154	-0.756	-0.835	-0.323	-0.192	-0.330	-1.220	-1.461
183	-0.856	-1.121	-0.841	-0.933	-0.995	-0.718	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	0.846
184	-0.216	1.399	0.819	0.529	1.849	0.735	-0.055	1.087	0.783	1.020	0.757	-0.017	0.846
185	1.064	1.399	1.926	1.503	1.250	0.735	0.471	2.368	1.889	2.232	1.844	1.215	0.846
186	-0.856	0.073	-0.841	-0.933	0.352	0.735	0.822	1.087	0.783	1.020	0.757	0.069	-0.368
188	-0.216	0.869	0.819	0.529	1.849	0.735	0.822	1.087	0.783	1.020	0.757	0.098	0.846
189	-0.856	-1.121	-0.841	-0.933	0.352	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	0.069	-0.368

191	2.184	-1.121	-0.841	-0.933	-0.995	0.735	2.575	-0.835	2.627	-0.798	-0.873	-1.220	-1.461
192	-0.376	-0.723	-0.288	-0.446	-0.546	-1.298	-0.756	-0.621	-0.692	-0.192	-0.330	0.012	0.846
194	2.184	1.399	-0.288	-0.446	1.849	0.735	0.822	1.087	2.258	2.636	2.206	1.502	-1.461
195	2.184	1.134	-0.288	-0.121	1.849	0.735	0.822	1.087	1.889	2.636	2.206	1.502	-1.461
196	0.584	0.073	-0.841	-0.770	1.849	0.735	-0.756	-0.835	-0.876	1.020	-0.873	0.069	-1.461
197	1.544	1.399	-0.288	0.204	1.250	0.154	1.874	2.368	1.889	1.424	1.119	0.929	0.846
198	1.544	0.869	0.635	1.503	1.250	-1.008	-0.756	2.368	1.889	2.232	1.844	-1.220	0.846
199	0.584	-1.121	0.819	0.529	0.352	0.735	-0.756	1.087	0.783	1.020	0.757	1.502	0.846
200	1.544	0.869	0.635	0.204	1.250	0.735	-0.756	0.660	-0.876	0.616	0.395	0.929	-1.461
202	0.264	0.869	0.819	0.204	1.250	0.735	0.471	2.368	0.783	0.616	0.395	0.069	-0.854
203	2.184	-1.121	-0.841	-0.933	1.849	-2.024	2.575	-0.835	2.627	-0.798	-0.873	-1.220	-1.461
204	1.544	1.399	0.450	0.204	1.250	0.154	2.575	0.660	1.152	1.424	0.395	0.929	0.361
205	0.584	0.073	0.819	0.529	1.849	-1.444	-0.055	0.019	-0.139	0.010	-0.148	-0.647	-0.733
206	1.544	0.869	0.635	1.503	1.250	0.735	1.874	1.087	1.889	1.020	0.757	0.929	0.361
207	0.584	1.399	0.819	-0.933	0.352	0.735	0.822	1.087	0.783	-0.798	-0.873	-1.220	-0.368
208	0.584	1.399	0.819	-0.933	0.352	0.735	0.822	1.087	0.783	-0.798	-0.873	1.502	0.846
209	0.584	0.073	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	0.069	-1.340
210	0.584	0.073	-0.841	-0.933	0.352	0.735	0.822	1.087	0.783	-0.798	0.757	0.069	-1.340
211	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
212	0.264	0.869	-0.841	-0.770	-0.247	0.735	-0.756	-0.621	-0.876	-0.596	-0.692	-0.504	-1.218
213	1.544	0.869	0.450	0.204	1.250	0.154	0.471	-0.194	-0.323	2.232	1.844	1.502	0.846
214	0.584	0.073	0.081	0.204	0.352	-0.718	0.822	1.087	0.783	1.020	0.757	1.502	0.846
215	-0.856	1.399	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	-1.461
216	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	-1.461
217	0.584	0.073	-0.841	-0.933	-0.846	0.735	-0.055	0.019	-0.139	-0.798	-0.873	0.929	0.361
218	0.584	-1.121	2.664	-0.933	0.352	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
219	0.584	0.073	0.819	0.529	0.352	0.154	0.822	1.087	0.783	1.020	0.757	1.358	0.846
220	2.184	0.869	0.450	-0.933	-0.995	-2.024	2.575	0.660	0.414	0.616	-0.873	-1.220	0.846

223	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	-1.461
225	0.584	1.134	0.081	0.529	0.352	-0.718	0.822	1.087	1.889	1.020	0.757	0.069	0.846
226	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	-1.461
227	-0.856	1.399	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	1.502	-1.461
228	-0.376	0.073	-0.841	-0.608	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.076	-0.854
231	0.584	-1.121	-0.841	-0.933	0.352	0.735	0.822	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
232	-0.696	-1.121	-0.657	-0.933	-0.995	-2.024	-0.756	2.368	1.889	-0.798	2.206	1.502	-1.340
233	1.544	0.073	-0.103	-0.283	1.849	-0.718	-0.581	0.019	-0.139	1.020	-0.148	0.929	-1.461
234	-0.856	-1.121	-0.841	-0.933	-0.995	-0.718	-0.055	0.019	-0.876	-0.798	-0.692	-1.076	0.846
235	2.184	1.399	1.926	1.503	1.849	-0.718	1.874	2.368	1.889	2.232	1.844	0.929	0.846
236	2.184	1.399	2.664	2.152	1.849	-2.024	2.575	3.222	2.627	3.041	2.568	1.502	0.846
237	2.184	0.073	0.819	0.529	0.352	0.735	-0.756	1.087	0.783	1.020	0.757	-1.220	0.846
238	2.184	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
239	2.184	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
240	-0.856	1.399	-0.841	-0.933	-0.397	-2.024	-0.756	-0.835	-0.876	-0.798	0.757	-1.220	-1.461
243	-0.856	-1.121	-0.103	-0.933	-0.995	-0.718	-0.756	0.019	-0.876	-0.798	-0.873	1.502	-1.461
247	0.584	1.399	-0.103	-0.121	0.352	-0.718	-0.055	1.087	1.889	1.020	-0.148	0.069	-0.368
248	-0.856	-1.121	-0.841	-0.933	0.352	0.735	0.822	1.087	0.783	1.020	2.568	0.069	0.846
252	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	2.627	3.041	-0.873	-1.220	-1.461
253	0.584	-1.121	0.081	0.529	1.849	-0.718	-0.756	1.087	-0.876	1.020	2.568	0.757	0.846
254	2.184	0.073	-0.841	-0.933	-0.995	0.735	0.822	1.087	0.783	-0.798	-0.873	0.069	-1.461
255	2.184	-0.193	-0.841	-0.933	1.849	-2.170	-0.756	0.660	-0.323	0.616	-0.330	0.356	-1.583
257	-0.856	-0.193	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
258	-0.856	-1.121	-0.841	-0.933	-0.995	0.735	-0.756	-0.835	-0.876	-0.798	-0.873	-1.220	-1.461
A ot100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-0.876	0.846
Bot100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	0.846
Cot100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.076	0.846
Dot100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-0.675	0.846

Eot100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-0.991	0.846
Fot100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-0.647	0.846
H 0t100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-0.933	0.846
I ot100	-0.856	-1.121	-0.841	-0.933	-0.995	-2.024	-0.756	-0.835	-0.876	-0.798	-0.873	-1.076	0.846

*Table 2**:** Identified proteins synthesized by Listeria monocytogenes Scott A cells grown on BHI broth until the middle exponential phase of growth (OD= 0.4, λ=600 nm) was reached. Only spots showing an increased or decreased (≤or ≥of 2-fold, p <0.05) level of synthesis in L. monocytogenes Scott A during at least one antimicrobial treatments were reported.*

Spot	Protein	Microrganism	MR	PI	SCORE	COVERAGE (%)	Peptides
1	Trigger Factor	*L. monocytogenes*	47824	4.50	498	28	7(7)
2	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	387	10	7(7)
3	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	640	11	8(8)
4	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	633	11	8(8)
5	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	491	8	6(6)
7	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	609	10	8(8)
8	Molecular Chaperone Dnak	*L. monocytogenes*	66092	4.57	623	12	6(6)
12	Transketolase	*L. monocytogenes*	71831	5.11	393	10	6(6)
13	Transketolase	*L. monocytogenes*	71854	5.11	384	8	5(5)
15	Formate Acetyltransferase	*L. monocytogenes HCC23*	84046	5.49	661	16	9(9)
16	Formate Acetyltransferase	*L. monocytogenes HCC23*	84046	5.49	585	14	8(8)
17	Formate Acetyltransferase	*L. monocytogenes HCC23*	84046	5.49	638	16	9(9)
20	Phosphoglucomutase	*L. monocytogenes*	64232	5.03	222	8	4(4)
21	Chaperone Protein Groel	*L. monocytogenes str. 1/2a F6854*	47082	4.64	779	22	7(7)
22	Pyruvate Kinase	*L. monocytogenes serotype 4b str. H7858*	59821	5.38	812	19	8(8)
23	Pyruvate Kinase	*L. monocytogenes serotype 4b str. H7858*	59821	5.38	826	19	8(8)
24	Pyruvate Kinase	*L. monocytogenes serotype 4b str. H7858*	59821	5.38	808	21	8(8)
25	Multispecies: Fe-S Cluster Assembly Protein Sufb	*L. monocytogenes*	52711	4.87	366	12	5(5)
26	Multispecies: Fe-S Cluster Assembly Protein Sufb	*L. monocytogenes*	52711	4.87	299	9	6(6)

27	Gmp Synthase	*L. monocytogenes L99*	58238	5.02	700	20	9(9)
28	Trehalose-6-Phosphate Hydrolase	*L. monocytogenes*	62040	4.86	534	14	6(6)
29	Trehalose-6-Phosphate Hydrolase	*L. monocytogenes*	63746	5.02	698	18	7(7)
30	Trehalose-6-Phosphate Hydrolase	*L. monocytogenes*	62040	4.86	397	11	5(5)
31	Acetolactate Synthase	*L. monocytogenes serotype 4b str. LL195*	61991	5.47	511	14	7(7)
32	Acetolactate Synthase	*L. monocytogenes serotype 4b str. LL195*	61991	5.47	530	15	8(8)
33	Acetolactate Synthase	*L. monocytogenes serotype 4b str. LL195*	61991	5.47	596	17	9(9)
34	Ctp Synthase	*L. monocytogenes serotype 4b str. LL195*	62694	5.46	393	12	7(7)
35	Invasion Associated Protein P60, Partial	*L. monocytogenes*	47381	9.15	758	25	9(9)
36	3-Bisphosphoglycerate-Independent Phosphoglycerate Mutase	*L. monocytogenes*	56063	5.10	434	11	4(4)
37	2,3-Bisphosphoglycerate-Independent Phosphoglycerate Mutase	*L. monocytogenes*	56130	5.18	574	18	8(8)
38	Formate--Tetrahydrofolate Ligase	*L. monocytogenes 07PF0776*	59945	5.28	366	9	4(4)
39	Heme Abc Transporter Atp-Binding Protein	*L. monocytogenes*	56649	5.78	362	11	5(5)
40	Heme Abc Transporter Atp-Binding Protein	*L. monocytogenes*	56649	5.78	424	12	6(6)
41	Inosine-5'-Monophosphate Dehydrogenase	*L. monocytogenes serotype 4b str. LL195*	55258	7.18	486	15	6(6)
42	Inosine-5'-Monophosphate Dehydrogenase	*L. monocytogenes serotype 4b str. LL195*	55258	7.18	440	14	6(6)
43	Hypothetical Protein Lm5578_1722	*L. monocytogenes 08-5578*	49331	6.10	449	18	7(7)
	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	41	1	1(1)
44	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	132	2	2(2)
45	Cell Division Protein Ftsz	*L. monocytogenes serotype 4b str. LL195*	43612	4.88	405	14	6(6)
46	Dipeptidase, Putative Subfamily	*L. monocytogenes str. 1/2a F6854*	27710	5.50	504	25	6(6)
47	Putative Dipeptidase Ytjp	*L. monocytogenes serotype 4b str. LL195*	53330	4.95	753	19	8(8)
49	Multispecies: Dihydrolipoamide Dehydrogenase	*L. monocytogenes*	49571	5.24	377	12	4(4)
50	Multispecies: Dihydrolipoamide Dehydrogenase	*L. monocytogenes*	49571	5.24	479	15	6(6)
53	30s Ribosomal Protein S1	*L. monocytogenes*	41317	4.50	791	29	9(9)
54	Atp Synthase F1	*L. monocytogenes J2818*	52408	4.80	1191	36	10(10)
55	Phosphoglucosamine Mutase	*L. monocytogenes*	48600	4.72	449	16	7(7)
	Cell Division Protein Ftsz	*L. monocytogenes serotype 4b str. LL195*	43612	4.88	192	9	3(3)
56	Glutamyl-Trna(Gln) Amidotransferase Subunit A	*L. monocytogenes serotype 4b str. LL195*	53586	5.03	281	7	3(3)
57	Glutamyl-Trna(Gln) Amidotransferase Subunit A	*L. monocytogenes*	52434	4.92	327	9	4(4)
	Dnak	*L. monocytogenes*	66164	4.57	143	4	2(2)

58	6-Phosphogluconate Dehydrogenase, Decarboxylating	*L. monocytogenes str. 4b H7858*	51107	5.10	431	16	6(6)
	Glutamate Decarboxylase	*L. monocytogenes FSL F2-208*	46398	5.13	165	5	2(2)
59	Aspartyl/Glutamyl-Trna(Asn/Gln) Amidotransferase Subunit B	*L. monocytogenes FSL F2-208*	53287	5.42	394	12	5(5)
60	Atp Synthase Subunit Alpha 2	*L. monocytogenes serotype 4b str. LL195*	55447	5.34	625	15	6(6)
61	Aspartyl/Glutamyl-Trna(Asn/Gln) Amidotransferase Subunit B	*L. monocytogenes FSL F2-208*	53287	5.42	444	12	5(5)
	Atp Synthase Subunit Alpha 2	*L. monocytogenes FSL F2-208*	25547	6.36	196	12	2(2)
62	Atp Synthase Subunit Alpha 2	*L. monocytogenes serotype 4b str. LL195*	55447	5.34	693	15	7(7)
	Glucose-6-Phosphate 1-Dehydrogenase	*L. monocytogenes*	56221	5.24	198	5	3(3)
64	Gid Protein	*L. monocytogenes J2818*	40314	5.25	97	6	3(3)
	Glycine Cleavage System P Protein, Subunit 2	*L. monocytogenes serotype 4b str. F2365*	53562	5.61	88	2	1(1)
	Atp Synthase Subunit Alpha 2	*L. monocytogenes FSL F2-208*	25547	6.36	79	5	1(1)
66	Isochorismate Synthase	*L. monocytogenes*	51763	5.61	164	9	4(4)
	Glutathione-Disulfide Reductase	*L. monocytogenes FSL F2-208*	49277	5.66	52	3	1(1)
67	Glutathione-Disulfide Reductase	*L. monocytogenes FSL F2-208*	49277	5.66	79	7	2(2)
68	Alcohol Acetaldehyde Dehydrogenase	*L. monocytogenes*	95036	6.58	36	1	1(1)
71	Carnitine Transport Atp-Binding Protein Opuca	*L. monocytogenes serotype 4b str. LL195*	51456	5.94	132	8	3(3)
72	Beta-Ketoacyl-Acyl-Carrier-Protein Synthase Ii	*L. monocytogenes FSL F2-208*	18913	5.17	40	6	1(1)
73	Hypothetical Protein Lmosa_4530	*L. monocytogenes str. Scott A*	47451	5.44	296	16	4(4)
	Tellurite Resistance Protein Tela	*L. monocytogenes*	45464	5.32	54	5	2(2)
74	Hypothetical Protein Lmosa_4530	*L. monocytogenes str. Scott A*	47451	5.44	110	7	2(2)
76	Fes Assembly Protein Sufd	*L. monocytogenes serotype 4b str. F2365*	47587	5.61	59	5	2(2)
77	Fes Assembly Protein Sufd	*L. monocytogenes serotype 4b str. F2365*	47587	5.61	40	2	1(1)
78	Pyridine Nucleotide-Disulfide Oxidoreductase Family Protein	*L. monocytogenes FSL F2-208*	48588	5.39	54	3	1(1)
79	Udp-N-Acetylmuramate--Alanine Ligase	*L. monocytogenes FSL F2-208*	42673	5.55	73	5	2(2)
80	Aldehyde-Alcohol Dehydrogenase	*L. monocytogenes str. 1/2a F6854*	68004	6.31	171	7	4(4)
81	Pantothenate Metabolism Flavoprotein Homolog	*L. monocytogenes*	43426	6.10	31	2	1(1)
82	Sugar Abc Transporter Substrate-Binding Protein	*L. monocytogenes*	46603	4.60	267	15	5(5)
83	Glutamate-1-Semialdehyde 2,1-Aminomutase	*L. monocytogenes*	46658	5.47	65	1	1(1)
85	Acetate Kinase	*L. monocytogenes str. 1/2a F6854*	44794	5.33	241	15	5(5)
86	Serine Hydroxymethyltransferase	*L. monocytogenes serotype 4b str. LL195*	47287	5.76	72	3	1(1)
88	Dna Polymerase Iii, Beta Subunit	*L. monocytogenes FSL F2-208*	28610	5.00	75	9	2(2)

89	Branched-Chain Alpha-Keto Acid Dehydrogenase Complex Subunit E1 Alpha	*L. monocytogenes*	36603	4.96	66	4	1(1)
92	Peptidase	*L. monocytogenes FSL R2-503*	40757	5.07	30	4	1(1)
94	Peptidase	*L. monocytogenes FSL R2-503*	40757	5.07	46	2	1(1)
96	Glutamate-1-Semialdehyde 2,1-Aminomutase	*L. monocytogenes*	46658	5.47	97	6	3(3)
97	Aspartate Aminotransferase	*L. monocytogenes FSL J2-071*	24034	5.21	114	13	2(2)
100	Conserved Hypothetical Protein	*L. monocytogenes FSL J2-071*	36334	6.23	159	8	3(3)
101	N-Acetylglucosamine-6-Phosphate Deacetylase	*L. monocytogenes FSL F2-208*	41619	5.43	98	3	1(1)
102	Sugar Abc Transporter Atp-Binding Protein	*L. monocytogenes*	41179	5.82	223	10	4(4)
103	Multiple Sugar-Binding Transport Atp-Binding Protein Msmk	*L. monocytogenes FSL F2-208*	34636	5.97	216	9	3(3)
104	Pyruvate Dehydrogenase Complex, E1 Component, Pyruvate Dehydrogenase Alpha Subunit	*L. monocytogenes str. 4b H7858*	36682	5.65	162	11	4(4)
105	Pyruvate Dehydrogenase Complex, E1 Component, Pyruvate Dehydrogenase Alpha Subunit	*L. monocytogenes str. 4b H7858*	36682	5.65	132	11	3(3)
106	Pyruvate Dehydrogenase Complex, E1 Component, Pyruvate Dehydrogenase Alpha Subunit	*L. monocytogenes str. 4b H7858*	36682	5.65	192	11	4(4)
107	Nadph Dehydrogenase	*L. monocytogenes serotype 4b str. LL195*	38368	8.30	140	10	3(3)
108	Multispecies: Glyceraldehyde-3-Phosphate Dehydrogenase	*L. monocyotogenes*	36421	5.12	131	15	3(3)
109	Cell Shape Determining Protein Mreb	*L. monocytogenes FSL R2-503*	36988	5.23	158	10	3(3)
110	Gap, Partial	*L. innocua*	22226	5.25	98	8	1(1)
111	Aspartate-Semialdehyde Dehydrogenase	*L. monocytogenes FSL R2-503*	25748	6.34	42	5	1(1)
112	Glutamyl Aminopeptidase	*L. monocytogenes FSL F2-208*	38709	5.69	289	12	3(3)
113	Phosphate Acetyltransferase	*L. monocytogenes*	15308	6.85	47	7	1(1)
115	Bifunctional Protein Fold	*L. monocytogenes*	31002	5.44	54	5	1(1)
116	Conserved Hypothetical Protein	*L. monocytogenes serotype 1/2a str. F6854*	36925	5.36	111	7	2(2)
117	Glutamyl Aminopeptidase	*L. monocytogenes FSL F2-208*	38709	5.69	174	8	2(2)
118	Fructokinase	*L. monocytogenes FSL F2-208*	31579	5.65	161	12	3(3)
120	Flagellin	*L. monocytogenes*	30409	4.91	134	9	2(2)
121	Oxidoreductase, Aldo/Keto Reductase Family	*L. monocytogenes str. 4b H7858*	31717	4.87	86	8	2(2)
	Nad+ Synthetase	*L. monocytogenes FSL F2-208*	30626	5.00	68	4	1(1)
122	Oxidoreductase, Aldo/Keto Reductase Family	*L. monocytogenes str. 4b H7858*	31717	4.87	203	16	4(4)
124	6-Phosphofructokinase	*L. monocytogenes str. 4b H7858*	27165	5.40	65	7	2(2)
127	Utp-Glucose-1-Phosphate Uridylyltransferase	*L. monocytogenes F6900*	37183	5.45	113	5	1(1)
131	Gtp-Sensing Transcriptional Pleiotropic Repressor Cody	*L. monocytogenes FSL F2-208*	28495	4.90	97	10	2(2)
133	Gntr Family Transcriptional Regulator	*L. monocytogenes*	28008	5.13	71	9	2(2)

134	Methionine Aminopeptidase, Type I	*L. monocytogenes FSL F2-208*	22426	5.21	39	6	1(1)
140	Multispecies: Nad Kinase 2	*L. monocytogenes*	30594	6.54	46	4	1(1)
141	50s Ribosomal Protein L25	*L. monocytogenes*	22641	4.44	171	28	5(5)
144	2,3,4,5-Tetrahydropyridine-2-Carboxylate N-Succinyltransferase, Putative	*L. monocytogenes str. 4b H7858*	10942	5.74	50	12	1(1)
145	Naphthoate Synthase	*L. monocytogenes HPB2262*	27681	5.72	233	19	3(3)
147	Coa-Binding Domain Protein	*L. monocytogenes str. 4b H7858*	19366	5.56	32	12	1(1)
150	Adenylate Kinase	*L. monocytogenes str. 4b H7858*	23804	5.00	43	4	1(1)
152	Adenylate Kinase	*L. monocytogenes str. 4b H7858*	23804	5.00	88	13	2(2)
154	2,3-Bisphosphoglycerate-Dependent Phosphoglycerate Mutase	*L. monocytogenes serotype 4b str. LL195*	27629	5.62	85	6	1(1)
156	Chain A, Crystal Structure Of 3-Oxoacyl-[Acyl-Carrier Protein] Reductase (Fabg) From *Listeria monocytogenes* In Complex With Nadp+	*L. monocytogenes*	29025	5.82	86	9	2(2)
158	Transcriptional Regulatory Protein Degu	*L. monocytogenes FSL F2-208*	14527	9.24	41	9	1(1)
159	Ribosomal Protein S3	*L. monocytogenes str. 1/2a F6854*	17247	9.89	31	9	1(1)
162	Phosphoglycerate Kinase/Triose-Phosphate Isomerase	*L. monocytogenes FSL N3-165*	27608	4.78	80	3	1(1)
165	Uracil Phosphoribosyltransferase	*L. monocytogenes FSL R2-561*	16944	5.70	33	10	1(1)
166	Abc Transporter, Atp-Binding Protein	*L. monocytogenes str. 4b H7858*	12617	4.97	67	31	2(2)
176	Ribosome-Recycling Factor	*L. monocytogenes*	20743	5.25	40	12	1(1)
178	Uracil Phosphoribosyltransferase	*L. monocytogenes FSL R2-561*	16944	5.70	88	18	2(2)
184	Sod	*L. monocytogenes*	14658	4.95	34	12	1(1)
185	Sod	*L. monocytogenes*	14658	4.95	79	12	1(1)
186	Short-Chain Dehydrogenase	*L. monocytogenes*	20932	5.91	30	9	1(1)
195	50s Ribosomal Protein L5	*L. monocytogenes serotype 4b str. LL195*	21249	9.17	84	13	2(2)
197	Transcription Elongation Factor Grea	*L. monocytogenes str. 4b H7858*	16570	4.58	67	15	2(2)
202	Pts System Mannose-Specific Eiiab Component	*L. monocytogenes serotype 4b str. LL195*	19655	9.33	50	17	2(2)
204	Putative Universal Stress Protein	*L. monocytogenes FSL F2-208*	17555	4.98	83	16	2(2)
212	Multispecies: 30s Ribosomal Protein S8	*L. monocytogenes*	14635	9.48	71	19	2(2)
213	Hypothetical Protein	*L. monocytogenes*	11250	4.46	38	10	2(2)
218	Ribosomal Protein L31	*L. monocytogenes*	9241	8.93	36	13	2(2)
219	50s Ribosomal Protein L7/L12	*L. monocytogenes*	12462	4.54	47	10	2(2)
231	Dna-Binding Protein Hu	*L. monocytogenes serotype 4b str. LL195*	13590	9.10	32	12	2(2)
233	Dna-Binding Protein Hu	*L. monocytogenes serotype 4b str. LL195*	13590	9.10	90	22	2(2)

248	Purine Nucleoside Phosphorylase	*L. monocytogenes*	25341	4.87	39	6	1(1)
253	Groes	*L. monocytogenes*	10042	4.60	31	15	2(2)
B	Multispecies: Enolase	*L. monocytogenes*	46458	4.70	84	7	2(2)
E	Conserved Hypothetical Protein	*L. monocytogenes str. 4b H7858*	11284	4.79	39	14	2(2)
F	30s Ribosomal Protein S2	*L. monocytogenes serotype 4b str. LL195*	30581	6.28	38	3	1(1)

Cell morphology and motility related proteins

FtsZ, an essential cell division protein that forms a contractile Z ring structure, was under-synthetized in *L. monocytogenes* Scott A cells treated with ethanol compared to untreated cells. However, the treatments with ethanol and citral, carvacrol, (E)-2-hexenal or thyme EO differently affected the relative amount of FtsZ protein which showed the highest levels in citral (125 mg/L) and thyme (40 mg/L). Cell shape determining protein MreB decreased in treated cells, especially for thyme EO. Compared to untreated cells, *L. monocytogenes* Scott A increased ($p<0.05$) the level of flagellin A (FlaA) under sub-lethal level of ethanol. The addition of other antimicrobial compounds differently affected the inductive effect of ethanol on FlaA synthesis. FlaA remained similar ($p>0.05$) to the control when high concentrations of carvacrol or thymol were used.

Synthesis of ribosomal and regulation system proteins

Compared to control, cells treated with ethanol increased the level of 30S ribosomal S1, S3 and 50S ribosomal L25. On the contrary, 50S ribosomal L31 decreased. Citral at 85 mg/L specifically induced the synthesis of L31 protein. Cells treated with ethanol and other antimicrobials further increased the levels of S1 (except forcitral), 30S ribosomal S2 (only thyme EO) and S3 (carvacrol 35 mg/L). Compared to ethanol alone, 30S ribosomal L5 and L25 decreased in citral and (E)-2-hexenal treated cells. The treatment of *L. monocytogenes* Scott A with carvacrol 35 mg/L specifically induced the level of 30S ribosomal protein S8. Ribosome recycling factor (Frr) was found at the highest level in cells treated with carvacrol (35 mg/L) or thyme EO at 100 mg/L.

Compared to control, cells treated with ethanol increased the level of a peptydil-prolyl cis-trans isomerase (trigger factor, TF). The addition of the other antimicrobials to the medium leads a decrease of TF compared to cells treated by ethanol alone. The only exceptions were found for carvacrol (35 mg/L) or thyme EO at 100 mg/L.

Compared to control, cells treated with ethanol and carvacrol (50 mg/L) or (E)-2-hexenal (150 mg/L) showed lower relative abundances of GTP-sensing transcriptional pleiotropic repressor CodY. Transcription elongation factor GreA was found at the lowest levels in citral treated cells. Ethanol strongly induced the relative amount of transcriptional regulatory protein DegU compared to control *L. monocytogenes* Scott A cells. Compared to ethanol alone, the addition of other antimicrobials to the media resulted in a reduces level of DegU protein. The only exception was for citral at 125 mg/L. GntR family transcriptional regulator was found at the highest levels in cells treated with ethanol added of carvacrol (35 mg/L) or thyme EO at 100 mg/L. On the contrary, citral

and (E)-2-hexenal caused a decreased of the relative amount of GntR family transcriptional regulator compared to control cells.

Carbohydrate transport and metabolism and energy production

Proteins related to carbohydrate transport (sugar ABC transporter ATP-binding proteins; multiple sugar-binding transport ATP-binding protein MsmK; PTS system mannose-specific EIIAB component ManX) were over-synthesized in *L. monocytogenes* Scott A cells treated with ethanol (MsmK, ManX) and especially, in ethanol plus carvacrol (35 mg/L) or thyme EO at 100 mg/L (except for ManX). With few exceptions, carbohydrate transport proteins were down-synthesized in cells treated with ethanol plus citral or (E)-2-hexenal compared to ethanol alone. Proteins related to carbohydrate metabolism (3-bisphosphoglycerate-independent phosphoglycerate mutase, GpmA; glucose-6-phosphate isomerase, Pgi; phosphoglucosamine mutase, Pgm; pyruvate kinase, Pyk; Trehalose-6-phosphate hydrolase, TpiA; transketolase, TktB; formate acetyltransferase, PflB) were found over-synthesized in *L. monocytogenes* Scott A cells treated with ethanol (GpmA, Pgi) and especially, in ethanol plus carvacrol (35 mg/L) or thyme EO at 100 mg/L. Compared to ethanol alone, Pyk were over-synthetized also in cells treated with ethanol plus citral.

Compared to control, 6-phosphofructokinase (pfkA), enolase (Eno), glyceraldehyde-3-phosphate dehydrogenase (Gap) and fructokinase (CscK) resulted specifically over-synthesized in ethanol plus thyme EO at 100 mg/L (pfkA and Eno) and ethanol plus citral at 100 mg/L or ethanol plus carvacrol (35 mg/L) (Gap, CscK). With few exceptions, carbohydrate metabolism proteins were down-synthesized in cells treated with ethanol plus (E)-2-hexenal compared to ethanol alone.
Proteins related to energy production and conversion (acetate kinase,) were found over-synthesized in *L. monocytogenes* Scott A cells treated with ethanol plus carvacrol (35 mg/L) or thyme EO at 100 mg/L.
Alcohol acetaldehyde dehydrogenase, also involved in heat shock protein binding interacting selectively and non-covalently with any protein synthesized or activated in response to heat shock, was induced in stressed cells compared to control. The highest level of alcohol acetaldehyde dehydrogenase was found in cells treated with ethanol plus carvacrol at 35 mg/mL.
Similarly, F-type H+-transporting ATPase subunit alpha (ATP synthase F1) [EC:3.6.3.14], involved in oxidative phosphorylation (ko00190) and also in environmental stress response, was induced in

stressed cells compared to control. The highest level of alcohol acetaldehyde dehydrogenase was found in cells treated with ethanol alone or plus Thyme EO at 100 mg/mL.

Nucleotide and nitrogen metabolism

GMP synthase [GuaA, EC:6.3.5.2], involved in purine metabolism (ko00230), was found at the highest levels in cells treated with ethanol alone and, especially, with ethanol plus carvacrol (at 20 and 35 mg/L) or thyme EO (at 70 and 100 mg/L). Adenylate kinase [EC:2.7.4.3], involved in both purine and thiamine metabolism (ko00730) was specifically induced during cell treated with ethanol alone. The level of adenylate kinase decreased in all conditions compared to ethanol stressed cells. The only exception was for cells treated with ethanol plus thyme EO (100 mg/L). CTP synthase, related to pyrimidine metabolism (ko00240), was detected at the highest levels in cells treated with ethanol plus carvacrol (35 mg/L) and thyme EO (100 mg/L). Uracil phosphoribosyltransferase [Upp, EC:2.4.2.9], related to pyrimidine metabolism, was found at the highest levels in cells treated with ethanol alone and, especially, with ethanol plus carvacrol (at 20 and 35 mg/L) or thyme EO (at 70 and 100 mg/L). Purine-nucleoside phosphorylase [PpnP, EC:2.4.2.1], involved in purine and pyrimidine metabolism, nicotinate and nicotinamide metabolism (ko00760), was induced during the cell treatment with ethanol plus carvacrol (maxum level at 35 mg/L), E)-2-hexenal and thyme EO (max at 40 and 100 mg/L). Compared to control, peptidase enzymes were found over-synthesized in *L. monocytogenes* Scott A cells treated with in ethanol plus carvacrol (20 and 35 mg/L) or thyme EO (70 and 100 mg/L). Compared to control, aspartate aminotransferase (Dat) was over-synthesized in ethanol plus carvacrol at 50 mg/L, (E)-2-hexenal (all concentrations tested) or thyme EO at 40 and 70 mg/L. Enzymes involved in aminoacid transport and metabolism were also variously affected during cell treatments with ethanol alone or in combinations with the other antimicrobial compounds.

Cofactors and vitamins metabolism

Transketolase enzyme acting in the acyloin condensation reaction between C2 and C3 of pyruvate and glyceraldehyde 3-phosphate producing 1-deoxy-D-xylulose-5-phosphate (DXP) plays a key role in the thiamine metabolism (ko00730). It was induced during ethanol stress and remained at higher level in all treated cells compared to control. Formate-tetrahydrofolate ligase [EC:6.3.4.3],

involved in the tetrahydrofolate interconversion of one carbon pool by folate (ko00670), increased during cell treatments with ethanol plus citral (all concentrations), carvacrol (35 mg/L) and thyme EO (all concentrations) compared to control. Isochorismate synthase (MenF) and naphthoate synthase (MenB), involved in ubiquinone and other terpenoid-quinone biosynthesis (ko00130), were specifically induced during cell treatment with ethanol plus carvacrol (35 mg/L) and thyme EO at 70 and 100 mg/L (MenF) and ethanol alone (MenB).

Stress response

Compared to control, molecular chaperone DnaK increased in stressed cells showing the highest value during the treatment with ethanol plus thyme EO at 100 mg/L. Cell treatment with ethanol plus thyme EO strongly increased GroES levels independently on the concentration of thyme EO used. Compared to control, cell treatment with ethanol strongly increased superoxide dismutase (Sod). The level of Sod was retained at the maximum level in cells treated by ethanol plus carvacrol (35 mg/L) or thyme EO at 100 mg/L. Glutathione-disulfide reductase and oxidoreductase, aldo/keto reductase family were specifically induced during cell treatment with ethanol plus carvacrol (35 mg/L) or thyme EO at 70 and 100 mg/L. Bifunctional protein FolD (glutathionylspermidine amidase/synthetase [EC:3.5.1.78 6.3.1.8]) from glutathione metabolism (ko00480) was found at the highest level in cells treated with ethanol plus carvacrol (35 mg/L). FeS assembly proteins SufB and SufD related to post-translational modification, protein turnover, and chaperones, were induced in cells treated by ethanol alone (SufD) or ethanol added of carvacrol (35 mg/L) or thyme EO (100 mg/L). Proteins (FabG and FabY) involved in both fatty acid biosynthetic process and membrane repair, were induced under ethanol stress and, especially in cells treated by ethanol plus citral (FabY) and ethanol plus carvacrol (35 mg/L) or thyme EO (100 mg/L) (FabG).

Discussion

Based on environmental conditions, bacteria modify cell morphology and protein synthesis to optimize growth, survival, and propagation (Woldemeskel and Goley, 2017). Sub-lethal level of ethanol stress reduced the cell division protein FtsZ while the relative amount of cell shape determining protein MreB was unaffected. In rod-shaped bacilli, MreB put out the cell length and

arrange elongation of the cell- wall whereas FtsZ, a tubulin-like protein, give the arrangement of the cell division septum and the resulting cell poles (Margolin, 2009). Both proteins were also involved in the synthesize the glycan strands of peptidoglycan (Uehara and Park, 2008). Interestingly, ethanol stress down-regulated the synthesis of FtsZ in other Gram positive bacteria (e.g., *Lactobacillus plantarum*) (van Bokhorst-van de Veen et al., 2011). Overall, the co-occurrence of ethanol and other antimicrobials increased FtsZ levels but decreased MreB protein supporting the hypothesis that plant essential oils (EOs) interacted with bacteria cell-wall proteins other than the cytoplasmic membrane (Zengin and Baysal, 2014). Guevara et al., (2015) showed that the level of MreB increased during moderate heat, carvacrol and thymol treatments. Previously, it was described that monoterpenes (e.g., citral, carvacrol and thymol) mainly affect the membrane structures increasing membrane fluidity and permeability, and leading disturbances in the respiration chain with and subsequently dissipation of the proton-motive force (Ultee et al., 1999;Lambert et al., 2001;Bakkali et al., 2008;Somolinos et al., 2008;Hyldgaard et al., 2012) Nikbakht et al., (2014). However, further mechanisms involving cell-wall and cytoplasmic proteins could be involved during stress adaptation of *L. monocytogenes* to citral, carvacrol, (E)-2-hexenal or thyme EO.

Previously, it was shown that *L. monocytogenes* strains regulate flagellar motility according to temperature (Raengpradub et al., 2008;Cordero et al., 2016) and salt (Durack et al., 2013) stresses. First, it was found that sub-lethal level of natural antimicrobials also affected the level of flagellin (FlaA) in dose-dependent manner. Except for (E)-2-hexenal, high concentrations of natural antimicrobials was not correlated with high level of FlaA. The decreased level of flagellar proteins could be related to an energy saving mechanism under critical stress conditions (low temperature, osmotic stress) (Shen and Higgins, 2006;Hingston et al., 2015). However, persistence and the ability to form biofilm were not decreased in mutant strains for some flagellar genes (Todhanakasem and Young, 2008). Overall, adaptation to different antimicrobials leads to modification of the amount of some ribosomal proteins involved in translation, ribosomal structure and biogenesis (30S ribosomal protein S1, S2, S3 and S8, 50S ribosomal protein L5, L7/L12, L25 and L31). Previously, *L. monocytogenes* cells decreased the level of S1 protein under triple stresses (low pH, high salinity and low temperature) (He et al., 2015). Guevara et al., (2015) showed that the level of L7/L12 increased during moderate heat, carvacrol and thymol treatments. *L. monocytogenes* cells increased the levels of some ribosomal proteins (S1, L25) in presence of ethanol alone. In *Bacillus* genus, L25 (Ctc) works as general stress protein, showing a

σ^B-dependent promoter and it was induced during osmotic, heat, oxidative and starvation stresses (Hecker and Volker, 1998). In *L. monocytogenes*, the expression of Ctc is dependent by σ^B, an alternative factor encoded by sigB (Gardan et al., 2003;Cacace et al., 2010). The transcription of σ^B-dependent genes which encode for proteins related to transport, general stress response and metabolism, increased in *L. monocytogenes* under environmental stresses (Chan and Wiedmann, 2008). Ctc protein was up-synthetized in *L. monocytogenes* under osmotic stress (Gardan et al., 2003) and cold adaptation (Cacace et al., 2010). Consequently, ethanol stressed cells could better survival during osmotic stress. On the contrary, *L. monocytogenes* cells treated with sub-lethal dose of (E)-2-hexenal and, especially, citral inhibited the synthesis of Ctc which could cause a decrease survival under osmotic and cold stresses (Gardan et al., 2003;Cacace et al., 2010).

L. monocytogenes cells treated with specific concentration of ethanol (1%) and carvacrol (35 mg/L) or thyme EO (100 mg/L) increased the level of ribosome recycling factor, which plays a key role in bacterial growth (Janosi et al., 1994). Trigger factor (TF), a ribosome-associated protein playing a kay role in protein synthesis and folding also cooperating with DnaK and GroEL chaperones (Kandror and Goldberg, 1997) was induced in *L. monocytogenes* cells under ethanol (this study) and cold stresses (Cacace et al., 2010). In *Streptococcus suis*, TF-deficient strain showed attenuated pathogenicity in mouse peritonitis model (Wu et al., 2011). Interestingly, the use of plant antimicrobials (especially, citral and (E)-2-hexenal) seems to decreased the inductive effect of ethanol.

Overall, ribosome is one cellular target for antibiotics and microbes pointed out a panel of adaptation responses to increase resistance (Wilson, 2014). Based on the number of ribosomal proteins, a ribosomal response to antimicrobial treatments with ethanol and citral, carvacrol or thyme EO in *L. monocytogenes* SCOTT A can be also hypothesized.

As found in sequenced genomes, *L. monocytogenes* has regulatory proteins which play an important role in the cell adaptation to different niches. First, this study showed that natural antimicrobials differently affected the level of synthesis of proteins involved in transcription (GTP-sensing transcriptional pleiotropic repressor CodY, GntR family transcriptional regulator, transcription elongation factor GreA and transcriptional regulatory protein DegU). CodY is one of the major cellular global regulator, serving as a repressor and activator of metabolic and virulence genes in Gram positive bacteria. Recently, it was shown that CodY regulates carbon and nitrogen metabolisms, bacterial motility, stress related and virulence functions and metabolic adaptations in *L. monocytogenes* strains (Lobel and Herskovits, 2016). Interestingly, specific combinations of

ethanol and carvacrol or (E)-2-hexenal reduced the relative abundance of CodY. In addition, cells treated with ethanol and citral inhibited the synthesis of the transcription elongation factor GreA. *L. monocytogenes* strains increased the level of GreA under heat and antimicrobial adaptation (55 °C, carvacrol 0.3 mM and thymol 0.3mM for 30 minutes) (Guevara et al., 2015), during treatment with lactic acid (Omori et al., 2017). GreA is essential factor in the RNA polymerase elongation complex and protect proteins against aggregation. It was found that the over-expression of GreA increased the bacterial resistance to heat and oxidative stress (Li et al., 2012;Omori et al., 2017). Consequently, cells treated with ethanol and citral, showing the lowest level of GreA, could decrease the adaptability to heat and oxidative stress. Ethanol strongly increased the transcriptional regulatory protein DegU which play a role in motility, chemotaxis, biofilm formation and virulence of *L. monocytogenes* (Gueriri et al., 2008). Probably due to the modulation of DegU phosphorylation by acetyl phosphate, the inductive effect of ethanol was reduced adding carvacrol, (E)-2-hexenal and thyme EO.

Overall, the use of ethanol alone and, especially, ethanol added of carvacrol at 35 mg/L or thyme EO at 100 mg/L strongly increased the relative amount of several proteins related to carbohydrate transport and metabolism. Some of them (PTS mannose transporter subunit IIAB, glyceraldehyde-3-phosphate dehydrogenase, bisphosphoglycerate-independent phosphoglycerate mutase and triosephosphate isomerase) were also found over-synthetized in *L. monocytogenes* CECT 4031 cells adapted at 55°C alone or in presence of EO (55°C and carvacrol 0.3 mM and thymol 0.3 mM) for 30 min (Guevara et al., 2015). This study also showed that sub-lethal ethanol stress in *L. monocytogenes* SCOTT A variously affected the level of synthesis of many proteins involved in energy production, nucleotide and nitrogen metabolism, cofactors and vitamins metabolism and stress response. If other natural antimicrobial compounds were added to ethanol containing medium, *L. monocytogenes* SCOTT A adapt the proteome profile depending on the type and the specific concentrations of antimicrobials. Overall, carvacrol at 35 mg/L and thyme EO at 100 mg/L produced the highest induction in protein synthesis compared to citral and, especially, (E)-2-hexenal. Over-synthesis of proteins involved in energy metabolism could be useful to compensate for partially impaired energy generation caused by antimicrobial treatments interacting with the bacterial cytoplasmic membrane.

The sublethal concentrations of the natural antimicrobials used induced an over expression of the transporters, enzymes and cofactors involved in the less efficient energy generation mechanisms adopted by L. monocytogenes cells in response to the stress exposure and in the maintenance of

the cell oxidoreductive potential. Otherwise the shift toward less efficient, in terms of energy yield, metabolic pathways due to the exposure to natural antimicrobials is widely documented in Gram positive and Gram negative bacteria. Picone et al., (2013) showed that the exposure to different concentrations of cavacrol induced in *Escherichia coli* 555 an intracellular accumulation of glucose and a shift from aerobic metabolism fermentation. In addition a wide literature showed that EOs and their bioactive compounds negatively affected the respiration chain and the generation of the proton-motive force in several spoilage and pathogenic microorganisms (Ultee et al., 1999;Lambert et al., 2001;Bakkali et al., 2008;Somolinos et al., 2008;Hyldgaard et al., 2012). In addition also Nilsson et al., (2013) highlithed a clear energy generation shift to fermentation processes in *L. monocytogenes* in response to adverse (alkalinisation) environmental conditions. Otherwise, it is known that bacterial metabolism is a complex systems of interconnected metabolic pathways, and the negative modulation on one pathway often lead to compensatory adjustments into others. However, the compensation mechanisms are directly dependent on the microorganism, the kind of stress applied and the exposure medium and time (Goh et al., 2002). Also Lungu et al., (2009) demonstrated that different stress sources can generate in *L. monocytogenes* the same general metabolic switch (from respiration to fermentation) through different coordinate changes in gene expression and protein expression, demonstrating its ability to differently modulate the energy generation pathway in response to different growth conditions. The stress proteins such as glutathione-disulfide reductase, induced in cells treated with carvacrol at 35 mg/L and thyme EO at 100 mg/L, plays a key role to maintain the low intracellular redox potential required to have proteins in their reduced form.

Conclusions

The findings presented in this works contribute to understand the mechanisms of action of natural antimicrobials and the strategies put in place by *Listeria monocytogenes* Scott A to survive after one hour exposure to sublethal concentration of citral, carvacriol, (E)-2-hexenal and thyme EO. The detailed knowledge of the effects of natural antimicrobials on the microbial cell physiology is mandatory for their exploitation in the food industries as alternatives to traditional preservatives in order to maximize their efficacy even when used at sublethal concentrations, due to their low sensory threshold, and in combination with other hurdles.

For these reasons their application, requires the identification of combined strategies able to counteract the microorganism defense strategies highlighted in this paper in order increase food safety.

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Flow cytometric evaluation of *Listeria monocytogenes Scott A* and *Escherichia coli* K12 MG1655 cell membrane and esterase activity changes in response to natural antimicrobial exposure

Introduction

Consumer demand for minimally processed, and ready-to-eat foods with a reduced content of synthetic preservatives has stimulated the research of alternative preservation strategies. Essential oils (EOs) or their components represent one of the most promising natural feasible alternatives to improve food safety, shelf-life and quality. Recognized as safe from internationals food authority, they are traditionally used in food industry as flavor and taste enhancers (Newberne et al., 2000). Their antimicrobial activity and the wide action spectra against several pathogenic and spoilage microorganisms are well documented and several reviews are available (Burt, 2004;Hyldgaard et al., 2012;Tongnuanchan and Benjakul, 2014;Patel, 2015;Pandey et al., 2017). A wide literature documents their application as natural preservatives also in different food matrices, such as meat (Fratianni et al., 2010;Barbosa et al., 2015;Radha krishnan et al., 2015), dairy products (Amatiste et al., 2014;Ehsani et al., 2016;Ben Jemaa et al., 2017), minimally processed fruits and vegetables (Patrignani et al., 2015;Siroli et al., 2015b;Siroli et al., 2015c) and beverages (Kiskó and Roller, 2005;Chueca et al., 2016). Among the natural antimicrobials, thyme EO, and some components of citrus and officinal Eos, such as citral, carvacrol, and (E)-2-hexenal, are very promising alternatives to traditional preservatives (Ivanovic et al., 2012). In fact, they are widely reported to be able to improve safety and shelf-life of several foods also when used at concentrations lower than their bactericidal ones and compatible with the product sensory properties (Zanini et al., 2014a;Zanini et al., 2014b;Silva-Angulo et al., 2015). In general cell wall, the cytoplasm membrane and membrane proteins have been considered the main targets of EOs and their components (Burt, 2004). In fact, due their hydrophobic properties EOs and their components interfere with cell membrane integrity and functionality. Also the cytosol coagulation and the depletion of the microbial cell proton-motive force have been identified as action mechanisms of EOs (Burt, 2004;Hyldgaard et al., 2012;Patel, 2015). However, the EO action

mechanisms are reported to vary according the natural antimicrobial and its concentration, the target species or strain, the food matrix, the storage conditions, etc. (Valero and Francés, 2006;Somolinos et al., 2008;Somolinos et al., 2010;Patrignani et al., 2013). In general, cell wall and membrane are the primary site of toxic action of terpenes like citral and carvacrol (Burt, 2004;Hyldgaard et al., 2012). As reported by Somolinos et al., (2010) the exposure to different citral concentration cause on *Escherichia coli* BJ4 and BJ4L1 the cytoplasmic cell membrane disruption. A similar permeabilization effect was also observed on different *Listeria monocytogenes* strains. As reported by Zanini et al., (2014a) and Zanini et al., (2014b), the exposure to citral and carvacrol, even at sublethal concentrations, increases the permeabilization of the cytoplasmic cell membrane and potentiates the activity of various antibiotics. Aldehydes such as hexanal and 2-(E)-hexenal, antimicrobial produced by plants and vegetable tissues damaged by biotic or abiotic stresses throughout the lipoxygenase pathway to prevent and or inhibit the growth of plant pathogens (Lanciotti et al. 2004), have been demonstrated to possess a noticeable activity against several yeast, mould, Gram-positive and gram-negative bacterial strains of food interest (Nakamura and Hatanaka, 2002;Trombetta et al., 2002;Zhang et al., 2017) both in model and real food systems (Lanciotti et al., 1999;Lanciotti et al., 2003;Siroli et al., 2014). As reported by Patrignani et al., (2008), (E)-2-hexenal acts as a surfactant and permeates by passive diffusion across the plasma membrane of many microorganisms. Reached the cytoplasm the α,β-unsaturated aldehyde is able to reacts with different nucleophilic groups (Kubo and Fujita, 2001;Lanciotti et al., 2004). Moreover, (E)-2-hexenal may cause cytoplasm coagulation as the result of thiol containing enzyme inhibition (Aiemsaard et al., 2011).The antimicrobial properties of thyme essential depend on its chemical composition and the target microorganism (Kim et al., 1995;Nevas et al., 2004;Monika et al., 2011;Picone et al., 2013;Boskovic et al., 2015;Siroli et al., 2015a;Siroli et al., 2015c;Swamy et al., 2016). Thyme essential oil is constituted by numerous different compounds but its antimicrobial activity is mainly attributed to carvacrol and thymol. Thymol is structurally similar to carvacrol and they share their cellular targets. Studies have shown that thymol interacts with cell membrane permeability, leading to depletion of membrane potential, cellular uptake of ethidium bromide, and leakage of potassium ions, ATP, and carboxyfluorescein (Helander et al., 1998;Lambert et al., 2001;Xu et al., 2008). Although the literature on action mechanisms of citral, carvacrol, (E)-2-hexenal and thyme EO have been dramatically increased in the last years, the knowledge on their mechanisms on *Listeria monocytogenes* and *Escherichia coli* is still fragmentary since it is affected by several factors such

as concentration, strains, cell physiological state, treatment conditions, microbial interaction with exposure systems, etc.. In addition, antimicrobial activity of EOs and their components are not attributable to a specific mechanism but to the actions towards several cell targets . Moreover, for EOs an holist approach should be considered, since synergistic actions among components present also at very low concentrations greatly affects their antimicrobial activities (Caccioni et al., 1997), and consequently the comprehension of their action mechanisms is more complex. In addition, heterogeneity in microbial population resistance to stresses is reported to occur as a monomodal Gaussian with a narrow or broad distribution, or as a multimodal distribution comprising subpopulations of similar or vastly different numbers of individuals (Dhar and McKinney, 2007). However, the literature on the behavior of *L. monocytogenes* and *E. coli* cell populations exposed to natural antimicrobials is still scarce (Burt, 2004;Bakkali et al., 2008;Hyldgaard et al., 2012). Flow cytometry represents a reliable and fast tool in food microbiology, for the measurements of the changes on physiological single cell properties. By the use of the appropriate fluorescents dyes is possible classify cells into three different categories: metabolically active, intact, or permeabilized cell mixtures (Hewitt and Nebe-Von-Caron, 2004;Johnson et al., 2013). Most common fluorescent dyes used in flow cytometry are fluorescent immune-conjugates and probes for fluorescence *in situ* hybridization and nucleic acid stains. In addition, several probes able to measure the, membrane potential as well as cell enzymatic activity, viability, organelles, phagocytosis, development, and other properties are available (Haugland, 1994). Various authors demonstrated the suitability of flow cytometry to study the microbial cell responses even after the exposure to sub-lethal stress conditions (Luscher et al., 2004;Ananta et al., 2005;Berney et al., 2007;Mathys et al., 2007;Sunny-Roberts and Knorr, 2008;Da Silveira and Abee, 2009;Mols et al., 2010;Fröhling et al., 2012;Tamburini et al., 2013;Fröhling and Schlüter, 2015). In fact, this technique provides several information on the whole cell population and its changes during the exposure to stresses and the following recovery during storage. The comprehension of behavior of the different population fractions after the exposure to natural antimicrobials is fundamental for their further application at industrial level as alternative to traditional preservatives also to avoid resistance phenomena.

In this framework, the main aims of this research is to investigate on the potential of flow cytometry to study the changes of morphological and physiological properties of selected food-borne pathogens, i.e. *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655, after one hour exposure to different sub-lethal and lethal concentrations of citral, carvacrol, (E)-2-hexenal

and thyme essential oil in order to clarify their specific action mechanisms and the responses of the whole cell population. For this purpose different cell viability parameters, such as membrane integrity, esterase activity and cytoplasmic cell membrane potential were measured by flow cytometry.

Material and methods

Natural antimicrobials

Essential oils used in these experiments were purchased from Sigma-Aldrich (Milano, Italy) Citral, carvacrol, and (E)-2-hexenal, while thyme essential oil (EO) was obtained from Flora s.r.l. (Pisa, Italy) Before the experiments, essential oils were proper diluted using absolute ethanol (Sigma-Aldrich, Milano, Italy) to prepare 100X essential oils stock solutions for each concentration tested.

Bacterial strains

L. monocytogenes Scott A and *Escherichia coli* K12 MG1655

Listeria monocytogenes Scott A and *Escherichia coli* K12 MG1655 were stored as glass bead cultures at −80 °C for long-term preservation. To acclimatize cultures to the experimental conditions, one glass bead of each strain was given to 5 ml of Brain Heart Infusion broth (BHI) (Thermo-fisher, Milano, Italy) and incubated for 24 h without shaking at 37 °C. After the growth, cells were sub-cultured at 37°C for 24 h in BHI broth.

Exposure to natural antimicrobials

In each assay, 250 mL of fresh BHI broth were inoculated with 2.5 mL of bacteria suspension (corresponding to the 1% of the final volume) to reach a 4 log CFU/mL concentration and incubated without stirring at 37°C. The growth was monitored by the optical density (OD) at λ=600 nm. For *L. monocytogenes* Scott A the exposure was performed in the middle of the exponential growth phase while for *E.coli* k12 the exposure was performed in the stationary growth phase (OD=2, λ= 600 nm). For both microbial strains, 200 µL of natural antimicrobial hydro alcoholic stock solutions were added to 20 mL of liquid cultures in order to obtain the concentrations

reported in Table 1 and 2. Cultures were incubated for 1 h at 37°C. Natural antimicrobials operative concentration were determined according to the minimal inhibitory and bactericidal concentrations (Siroli et al., 2015a). For both strains, the tested exposure regarded three sublethal concentration corresponding to the 1/5, 1/3, 1/2 of the MIC and biocide concentrations (MIC and/or MBC) depending on the microorganism tested.

Table 1: Essential oils, their components, and relative concentrations, used for the treatments of L. monocytogenes Scott A.

Natural antimicrobial	**Concentration tested (mg/L)**
Citral	50 mg/L[1], 85 mg/L, 125 mg/L, 250 mg/L[1]
Carvacrol	20 mg/L[1], 35 mg/L, 50 mg/L, 100 mg/L[1]
(E)-2-hexenal	150 mg/L[1], 250 mg/L, 400 mg/L, 800 mg/L[1]
Thyme essential oil	40 mg/L[1], 70 mg/L, 100 mg/L, 200 mg/L[1]

[1]MIC value tested for *Listeria monocytogenes* Scott A

Table 2: Essential oils, their components, and relative concentrations, used for the treatments of Escherichia coli K12 MG1655

Natural antimicrobial	**Concentration tested (mg/L)**
Citral	200 mg/L, 330 mg/L, 500 mg/L, 1000 mg/L[1], 3000 mg/L[2]
Carvacrol	25 mg/L, 40 mg/L, 60 mg/L, 120 mg/L[1], 250 mg/L[2]
(E)-2-hexenal	80 mg/L, 135 mg/L, 200 mg/L, 400 mg/L[1], 425 mg/L[2]
Thyme essential oil	50 mg/L, 86 mg/L, 125 mg/L, 250 mg/L[1], 300 mg/L[2]

[1]MIC value tested for *Escherichia coli* K12 MG1655; [2]MBC value tested for *Escherichia coli* K12 MG1655

After the exposure, *L. monocytogenes* and *E.coli* MG1655 the total viable cell count was immediately performed (see chapter below). Afterwards, bacterial cells were harvested by centrifugation at 3214 x *g* at 4 °C for 15 min, resuspended in 250 µl of phosphate buffered saline PBS (50 mM) and again centrifuged at 7000 x *g* and 4 °C for 5 min. For the subsequent staining procedures and flow cytometric analysis, *L. monocytogenes* pellets were resuspended in 100 µl (50 mM) PBS, while *E.coli* samples were resuspended in 100 µL (50 mM) Tris buffer. For both microorganisms the final cell of each samples was about 10^9 cells/mL.

Total Viable Cell Count

The viable cell count of bacteria after exposure to essential oils or their components was determined by plate count methods in duplicate. Samples were serially diluted in microtest plates (96er U-profile, Carl Roth GmbH & Co KG, Germany) using, as dilution solution, physiological saline buffer (9 g/L NaCl). 100 µl of each dilution was spread on BHI agar (Thermo-fisher, Milano, Italy) and the growth (colony forming units) was evaluated after 24 h at 37°C.

Flow Cytometric Analysis

All experiments were performed using a CyFlow ML flow cytometer (Sysmex Partec GmbH) equipped, among others, with a 50 mW blue solid state laser emitting at a wavelength of 488 nm. A photomultiplier with a band pass filter of 536 ± 20 nm was used for collect fluorescence data of thiazole orange (TO), carboxyfluorescein (cF), and green $DiOC_2(3)$, while the fluorescence of propidium iodide and red $DiOC_2(3)$ was recorded in the photomultiplier with a band pass filter of 620 ± 11 nm. To correct the overlap of one dye's emission into another dye's detector fluorescence signal compensation was performed. Data obtained from each photomultiplier channels were collected as logarithmic signals and analyzed using the FloMax software 3.0 (Sysmex Partec GmbH). For each sample, one hundred thousand events were measured at a flow rate of approximately 3000 events/sec. The density plots obtained by flow cytometric analyses were divided into four regions. Each region is associated with cells revealing different physiological or morphological properties. The average of the percentage values obtained from three density plots was calculated and illustrated as diagrams where the x-coordinate displays the treatment concentration and the y-coordinate the percentage of fluorescent cells. The different cell parameters investigated during this experimentation were: the cell membrane integrity (TO-PI stain), the cell membrane potential ($DiOC_2(3)$ stain) and the cell membrane integrity and esterase activity (cF-PI stain) as indicator of the microbial population viability. Staining procedures were performed as described earlier by Fröhling and Schlüter (2015) with some adaptations to the bacteria strains used.

Membrane Integrity

Cell membrane integrity, was evaluated after the treatments using a combination of thiazole orange (TO) and propidium iodide (PI) dyes (Sigma-Aldrich, Germany). Staining procedures were performed in dark with some differences depending on the microorganism tested. For *L. monocytogenes,* 20 µL of resuspended pellets were diluted in PBS (50 mM) to a cell concentration of approximately 10^6 cells/mL. 0.2 µM thiazole orange was added to the samples and incubated for 10 minutes at room temperature. After the incubation, 30 µM propidium iodide was added and samples were analyzed after 5 minutes. As described above, *E. coli* resuspended pellets were diluted in Tris buffer (50 mM) to a cell concentration of approximately 10^6 cells/mL. Thiazole orange was added with a final concentration of 2.5 µM and samples were incubated for 15 minutes at room temperature. Propidium iodide straining was performed as described for *Listeria monocytogenes* samples.

Esterase Activity and Membrane Permeabilization

The cell esterase activity and the membrane integrity were evaluated after the exposure to natural antimicrobials using 5(6)-carboxyfluorescein diacetate mixed isomers (cFDA) (Sigma-Aldrich, Germany) and propidium iodide. 60 µL of concentrated *L. monocytogenes* samples were stained with an equal volume of cFDA (200 µM) stock solution to obtain a final cFDA concentration of 100 µM, incubated at 37 °C in a water bath for 5 min and centrifuged at 7000 x *g* at 4 °C for 5 min. Cells pellets were diluted 1:1 in PBS (50 mM). PI staining procedure was performed as previously described. cFDA staining procedure for *E. coli* followed the protocol described for *L. monocytogenes* with some differences. Cells were incubated with 833 µM cFDA (5 mM, in Tris) at 37 °C in a water bath for 45 min then centrifuged at 7000 x *g* at 4 °C for 5 min. Pellets were resuspended in 60 µL Tris (50 mM) and stained with 30 µM PI. Samples were analyzed after 10 minutes of incubation.

Membrane Potential

The measure the membrane potential of bacteria cells was obtained using 3,3' -diethyloxacarbocyanine iodide [$DiOC_2(3)$] provided by Sigma-Aldrich, Germany. The protocol

applied was in agreement with (Novo et al., 1999) and (Fröhling and Schlüter, 2015) with further modification. *Listeria monocytogenes* bacteria suspensions were diluted in 50 mM PBS containing 10 mM D-Glucose and 30 µM $DiOC_2(3)$ and then incubated for 15 minutes at room temperature in the dark. Afterwards the suspension was centrifuged at 7000 × g and 4°C for 5 min and the pellet resuspended in 1 mL PBS (50 mM). *Escherichia coli* staining procedure required a different staining buffer due to the higher complexity of the outer and inner cell membrane. First, samples were suspended in 10 mM D-Glucose, 30 µM $DiOC_2(3)$ and EDTA (0.5 mM) Tris buffer (50 mM). After the centrifugation and the incubation time as previously described, *E. coli* samples were resuspended in 1 mL of Tris (50 mM). After the staining procedure samples were immediately analyzed to detect shifts in the cell membrane potential. The ratio of the mean red to the mean green $DiOC_2(3)$- fluorescence channel value was calculated to investigate changes in the membrane potential. Due to the chosen cytometer settings the red/green $DiOC_2(3)$-fluorescence ratio of depolarized cells was ≤1. It was assumed that the red/green ratio of untreated cells represents the relative membrane potential of intact cells (Novo et al., 1999). A reduction of the red/green ratio stands for the loss cell membrane potential.

Statistical analysis

Statistical analysis to evaluate significant differences between samples were performed using the R software (R Core Development Team, 2017). OneWay-ANOVA with Tukey test with a significance level of 0.05 were used.

Results

Treatment Effects on Total Viable Count *Listeria monocytogenes* Scott A

The exposure of *Listeria monocytogenes* to the natural antimicrobials at different concentrations was performed, in all the experiments, at the reach of the middle of the exponential growth phase (OD= 0.4; λ=600 nm). In all the trials the cell loads before the treatments were about 8.8 log CFU/mL (Table 3-4). After 1 h exposure the untreated controls and the samples exposed to 1% ethanol showed the same cell counts. Only the one hour exposure to carvacrol and thyme

essential oil, at the highest concentration tested, provoked a significant reduction of the total viable counts (Table 3-4). More specifically, the exposure to 100 mg/L of carvacrol, corresponding to the MIC value, reduced the viable cell load of one logarithmic cycle (7.59 log CFU/mL) (Table 3B). Even more severe effect were observed after the exposure to thyme EO, reducing the viable counts to 7.45 log CFU/mL and 5.23 CFU/mL, after the exposure to 100 and 200 mg/L, respectively (Table 4D). By contrast, 1 hour exposure to citral and (E)-2-hexenal had no significant effect on the *Listeria* cell loads (Table 3A-4C).

Table 3: Total viable counts of Listeria monocytogenes Scott A after one hour exposure to different concentrations of Citral (A) and Carvacrol (B).

A	**log CFU/mL**		**SD**
Colture before treatments	8.84	±	0.33[a]
untreated control	8.79	±	0.10[a]
EtOH 1%	8.95	±	0.23[a]
Citral 50 mg/L	8.86	±	0.11[a]
Citral 85 mg/L	8.59	±	0.10[a]
Citral 125 mg/L	8.88	±	0.14[a]
Citral 250 mg/L (MIC)	8.69	±	0.08[a]

B	**log CFU/mL**		**SD**
Colture before treatments	8.62	±	0.01[a]
untreated control	8.81	±	0.17[a]
EtOH 1%	8.7	±	0.18[a]
Carvacrol 20 mg/L	8.75	±	0.23[a]
Carvacrol 35 mg/L	8.79	±	0.09[a]
Carvacrol 50 mg/L	8.79	±	0.16[a]
Carvacrol 100 mg/L (MIC)	7.59	±	0.14[b]

Different letters mean data significantly different (p<0.05).

Table 4: Total viable counts of Listeria monocytogenes Scott A after one hour exposure to different concentrations of (E)-2-hexenal (C) and Thyme EO (D)

C	**log CFU/mL**		**SD**
Colture before treatments	9.16	±	0.05[a]
untreated control	8.87	±	0.22[a]
EtOH 1%	8.99	±	0.10[a]
(E)-2-Hexenal 150 mg/L	8.99	±	0.23[a]
(E)-2-Hexenal 250 mg/L	9.13	±	0.11[a]
(E)-2-Hexenal 400 mg/L	9.10	±	0.09[a]
(E)-2-Hexenal 800 mg/L (MIC)	8.49	±	0.10[a]

D	**log CFU/mL**		**SD**
Colture before treatments	8.78	±	0.16[a]
untreated control	8.71	±	0.17[a]
EtOH 1%	8.59	±	0.14[a]
Thyme EO 40 mg/L	8.72	±	0.07[a]
Thyme EO 70 mg/L	8.69	±	0.28[a]
Thyme EO 100 mg/L	7.45	±	0.72[b]
Thyme EO 200 mg/L (MIC)	5.23	±	0.15[c]

Different letters mean data significantly different (p<0.05).

Treatment Effects on Total Viable Count *Escherichia coli* K12 MG1655

Escherichia coli samples were exposed to essential oil or their bioactive compounds at the beginning of the stationary growth phase (OD= 2; λ=600 nm). The cell loads before the exposure to the natural antimicrobials ranged between 8.5 and 9.0 log CFU/mL (Table 5-6). Analogously to *Listeria monocytogenes*, no differences on the cell loads were highlighted between the untreated controls and the samples exposed to 1% EtOH (Table 5-6). The highest effect on the *E.coli* growth was observed after the exposure to citral, also at lowest concentration tested (Table 5A). The exposure to 200 mg/L reduced the viable cell load to 7.22 log CFU/mL. Increasing concentrations reduced the total viable counts to values ranging between 6.60 and 6.12 CFU/mL. The exposure to the citral MBC concentration caused a reductions of cell loads under the detection limit (Table 5A). Also thyme EO and carvacrol treatments significantly decreased the total viable cell loads. A reduction of three logarithmic cycles were observed after the exposure to carvacrol MBC concentration (250 mg/L), while thyme essential oil MIC and MBC concentrations provoked cell load reductions of 6.52 and 5.59 log CFU/mL, respectively (Table 5B-6D). The (E)-2-hexenal exposure did not affect the *Escherichia coli* cell loads (Table 6C).

Table 5: Total viable count of Escherichia coli K12 MG1655 *after one hour exposure to different concentrations of Citral (A) and Carvacrol (B).*

A	**log CFU/mL**		**SD**
Colture before treatment	8.5	±	0.13^{a}
untreated control	8.97	±	0.24^{a}
EtOH 1%	8.96	±	0.15^{a}
Citral 200 mg/L	7.22	±	0.20^{b}
Citral 330 mg/L	6.6	±	0.13^{c}
Citral 500 mg/L	6.28	±	0.18^{c}
Citral 1000 mg/L (MIC)	6.12	±	0.20^{d}
Citral 3000 mg/L (MBC)	-*		-

B	**log CFU/mL**		**SD**
Culture before treatment	7.95	±	0.12^{a}
untreated control	8.9	±	0.16^{a}
EtOH 1%	8.98	±	0.21^{a}
Carvacrol 25 mg/L	8.65	±	0.09^{a}
Carvacrol 40 mg/L	9.02	±	0.11^{a}
Carvacrol 60 mg/L	8.75	±	0.25^{a}
Carvacrol 125 mg/L (MIC)	8.79	±	0.22^{b}
Carvacrol 250 (MBC)	4.85	±	0.47^{c}

**Detection limit 1 log CFU/mL*

Different letters mean data significantly different ($p<0.05$).

Table 6: Total viable count of Escherichia coli K12 MG1655 *after one hour exposure to different concentrations of (E)-2-hexenal (C) and Thyme EO (D).*

C	log CFU/mL		SD
Colture before treatment	8.92	±	0.06[a]
untreated control	8.88	±	0.26[a]
EtOH 1%	9	±	0.23[a]
(E)-2-Hexenal 80 mg/L	8.93	±	0.20[a]
(E)-2-Hexenal 135 mg/L	8.78	±	0.20[a]
(E)-2-Hexenal 200 mg/L	8.89	±	0.22[a]
(E)-2-Hexenal 400 mg/L (MIC)	8.83	±	0.09[a]
(E)-2-Hexenal 425 mg/L (MBC)	8.64	±	0.23[a]

D	log CFU/mL		SD
Colture before treatment	8.74	±	0.16[a]
Untreated control	8.76	±	0.24[a]
EtOH 1%	8.63	±	0.22[a]
Thyme EO 50 mg/L	8.53	±	0.32[a]
Thyme EO 85 mg/L	8.63	±	0.35[a]
Thyme EO 125 mg/L	8.86	±	0.53[a]
Thyme EO 250 mg/L (MIC)	6.52	±	0.04[c]
Thyme EO 300 mg/L (MBC)	5.59	±	0.26[d]

Different letters mean data significantly different ($p<0.05$).

Treatment Effects on the membrane integrity of *Listeria monocytogenes* Scott A

The exposure of *Listeria monocytogenes* Scott A to the different natural antimicrobial provoked an augment of the population fractions with cells having slightly damaged and damaged membranes. The distribution of stained cells varied according to different treatments and concentrations used. The percentage of *L. monocytogenes* Scott A stained only with Thiazole orange (TO), having intact cell membranes, remained almost constant (above 80%) after the exposure to 1% ethanol in all the trials performed (Figure 1). The different concentration of citral (Figure 1A) increased the percentages of cells with slightly damaged membranes. The magnitude of the damaging effect raised increasing the citral concentrations. In fact, at the highest concentration (250 mg/L) the 60% of stained cells showed a slightly damaged membrane. A value of about 3% of permeabilized membrane cells was observed in all the conditions, independently on the severity of chemical stress applied (citral concentration). A similar pattern was observed for carvacrol (Figure 1B). All the concentrations tested induced a significant reduction of the population with intact cell membrane. In particular, while in the control samples the population with intact membrane was about 80%, in the samples exposed to 100 mg/L (MIC value) this value decreased to 25%. Simultaneously the percentage of slightly membrane cells increased augmenting the carvacrol concentrations (from 12 to 50%). The exposure to the MIC carvacrol concentration also raised the percentage of permeabilized membrane cells up to 15% of all fluorescent cells. Compared to the

untreated control, the exposure to (E)-2-hexenal had no significant effect on the cell membrane integrity of *Listeria monocytogenes* Scott A (Figure 1C). Thyme essential oil had the highest effect on the cell membrane integrity compared to the other natural antimicrobials tested. The percentage of slightly and permeabilized cells increased with the treatment concentrations, and the exposure to 200 mg/L of thyme EO (MIC concentration) induced a complete membrane permeabilization in over 90% of cell population (Figure 1D). Except for Thyme EO exposure, the percentage of unstained cells/cell fragments were lower than 3% independently on the antimicrobial and its concentrations.

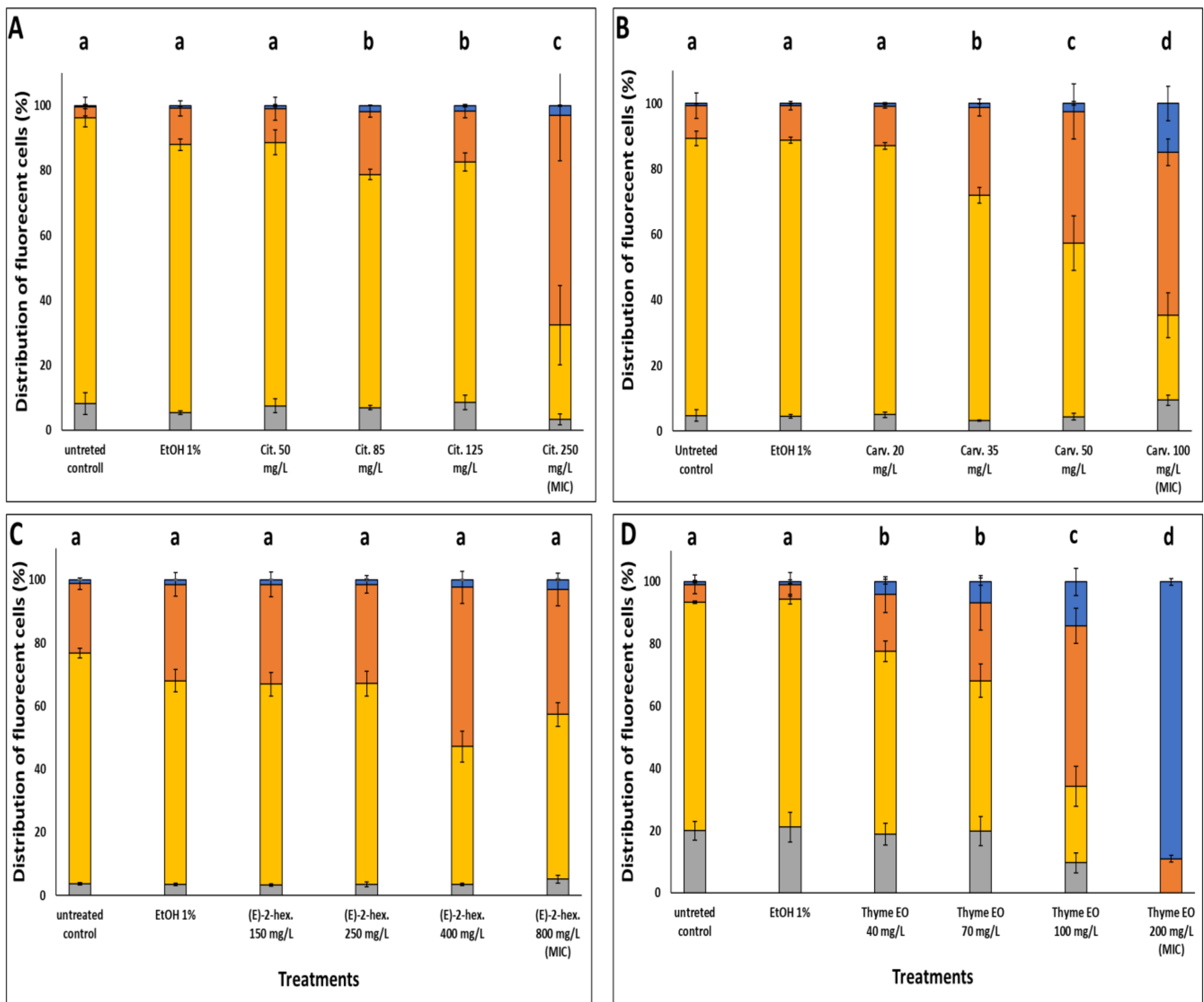

Figure 1: membrane integrity of Listeria monocytogenes Scott A after 1 h exposure to different concentrations natural antimicrobials: Citral (A), Carvacrol (B), (2)-hexenal (C) and thyme EO (D). Grey bars represent cell fragments or unstained cells; Yellow bars intact cell membrane; orange bars slight cell membrane permeabilization; Blue bars (complete) cell membrane permeabilization. Different letters mean data significantly different ($p<0.05$).

Treatment effects on the esterase activity and membrane integrity of *Listeria monocytogenes* *Scott A*

No effects on the esterase activity of *Listeria monocytogenes* were evidenced after the exposure to natural antimicrobials or their bioactive compounds. As showed in Figure2, the percentage of fluorescent cells with intact cell membranes and esterase activity was constant between treatments and above the 80% with the only exception of thyme EO 200 mg/L exposure. The MIC thyme EO provoke a complete permeabilization of the cells. Only without a lack in the esterase activity (Figure 2D).

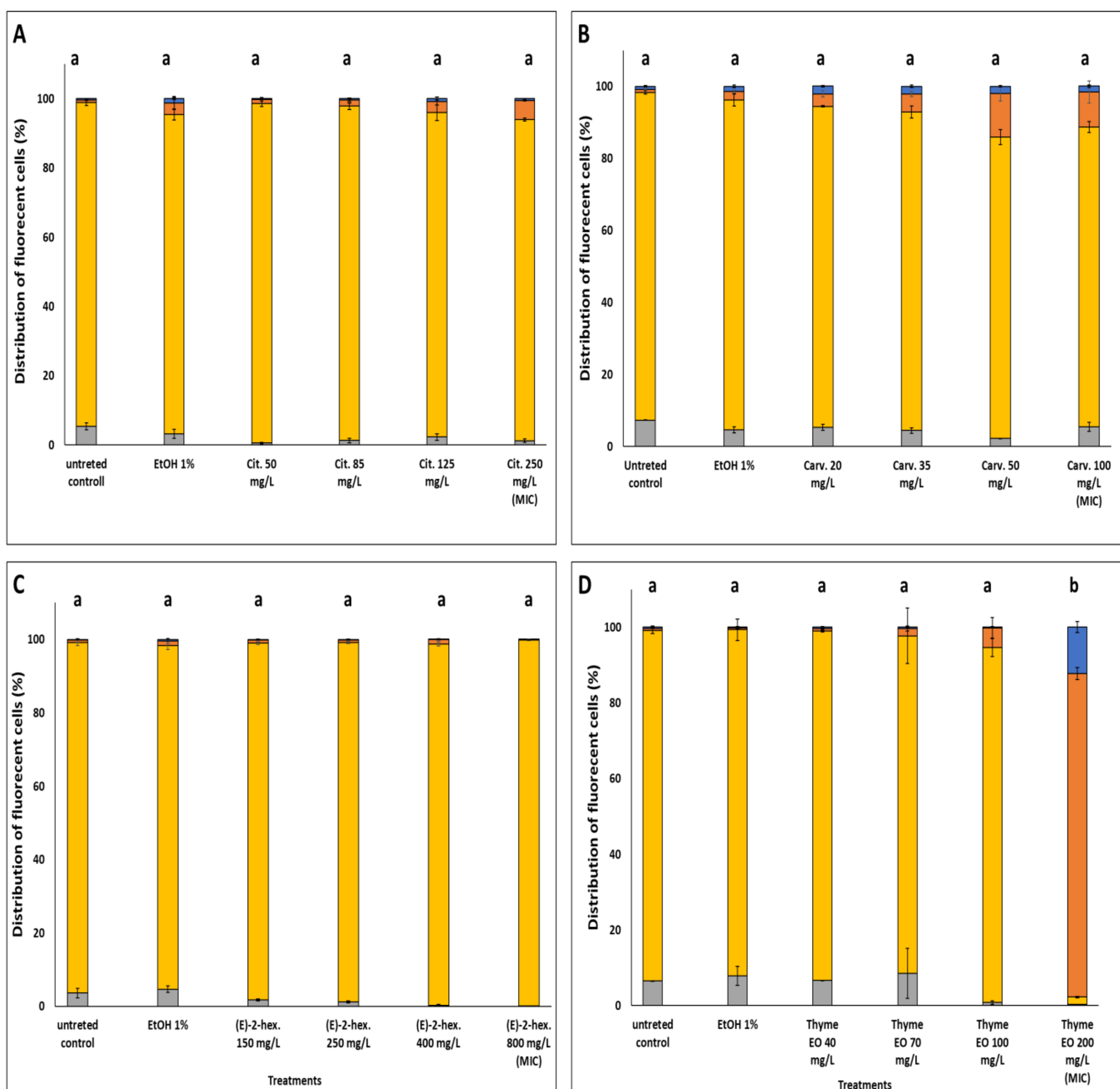

Figure 2: esterase activity and membrane integrity of Listeria monocytogenes Scott A after the exposure to natural antimicrobials: Citral (A), Carvacrol (B), (2)-hexenal (C) and thyme EO (D). Grey bars represent cell fragments or unstained cells; Yellow bars intact

cell membranes and esterase activity; orange bars cell membrane permeabilization but still esterase activity; Blue bars cell membrane permeabilization and no esterase activity. Different letters mean data significantly different (p<0.05).

Treatment effects on the cell membrane potential of *Listeria monocytogenes* Scott A

The measurement of relative membrane potential using 3,3'-Diethyloxacarbocyanine iodide DiOC2(3) showed no significant differences compared to the untreated cells of *Listeria monocytogens* Scott A. In fact, red/green ratios were always lower than 1 independently on the treatments and concentrations used. These data showed that the natural antimicrobials and the concentrations used at our experimental conditions were unable to significantly modify the membrane potential of *L. monocytogenes* Scott A (Figure 3).

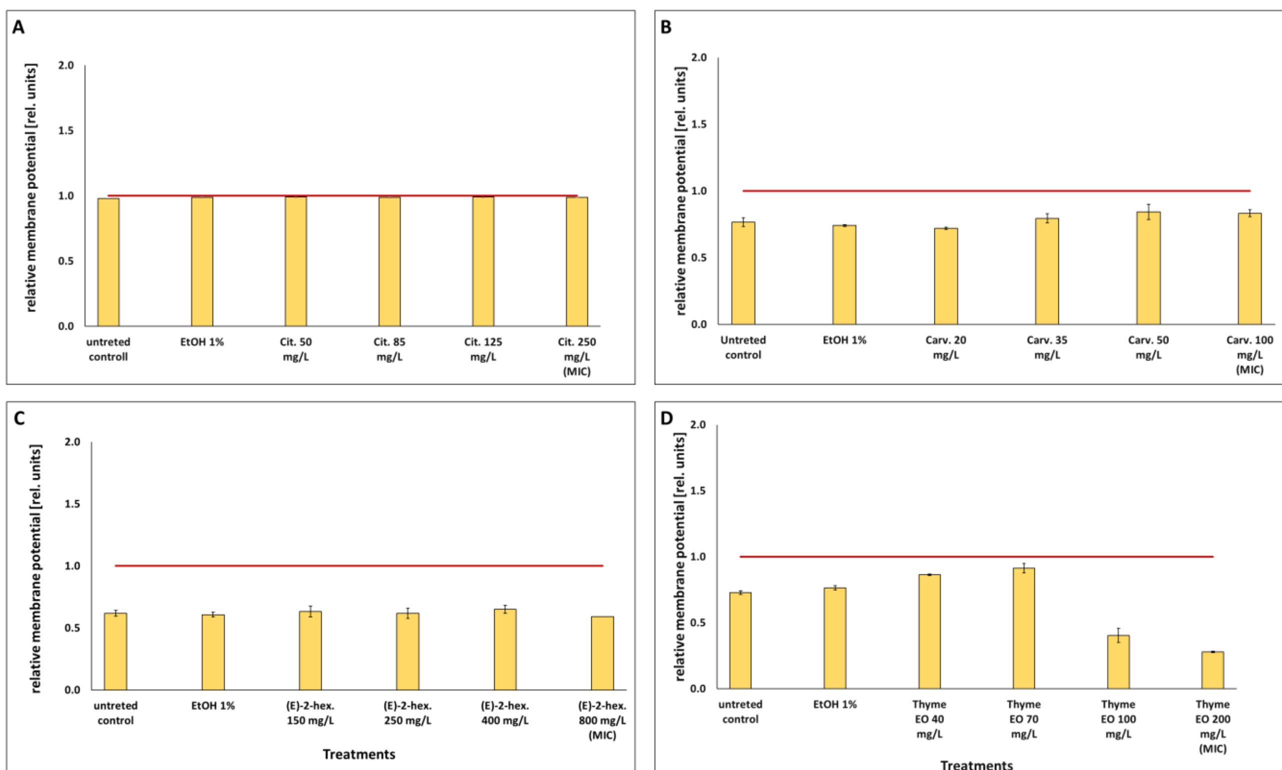

Figure 3: relative membrane potential of Listeria monocytogenes Scott A, expressed as red/green ratio of DiOC2(3)-fluorescence intensity, after the exposure to natural antimicrobials: Citral (A), Carvacrol (B), (2)-hexenal (C) and thyme EO (D).

Treatment effects on the membrane integrity of *Escherichia coli* K12 MG1655

In all the trials performed, the percentage of *E. coli* stained only with TO (cells with intact cell membrane) remained almost constant (above 80%) after the exposure to 1% ethanol (Figure 4). Citral showed a significant effect on cell membrane integrity even at sublethal concentrations

(Figure 4A). After the exposure to 200-300 mg/L of citral the percentages of slightly membrane damaged cells of were higher than 80%. No membrane intact cells were found after the exposure to the citral MIC and MBC values (Figure 4A). Minor impacts on *Escherichia coli* cell membrane integrity were evidenced after the exposure to carvacrol and (E)-2-hexenal, independently on their sub-lethal concentrations (Figure 4B-C). Only the exposure to carvacrol inhibent and bactericidal concentrations (120 – 250 mg/L) provoked the cell membrane permeabilization (Figure 4B). Thyme EO effect on the cell membrane was concentration dependent. The fluorescence signal of the intact cells decreased with the treatment concentration increase (80 – 20%), while the percentage of damaged membrane cells simultaneously increased (Figure 4D).

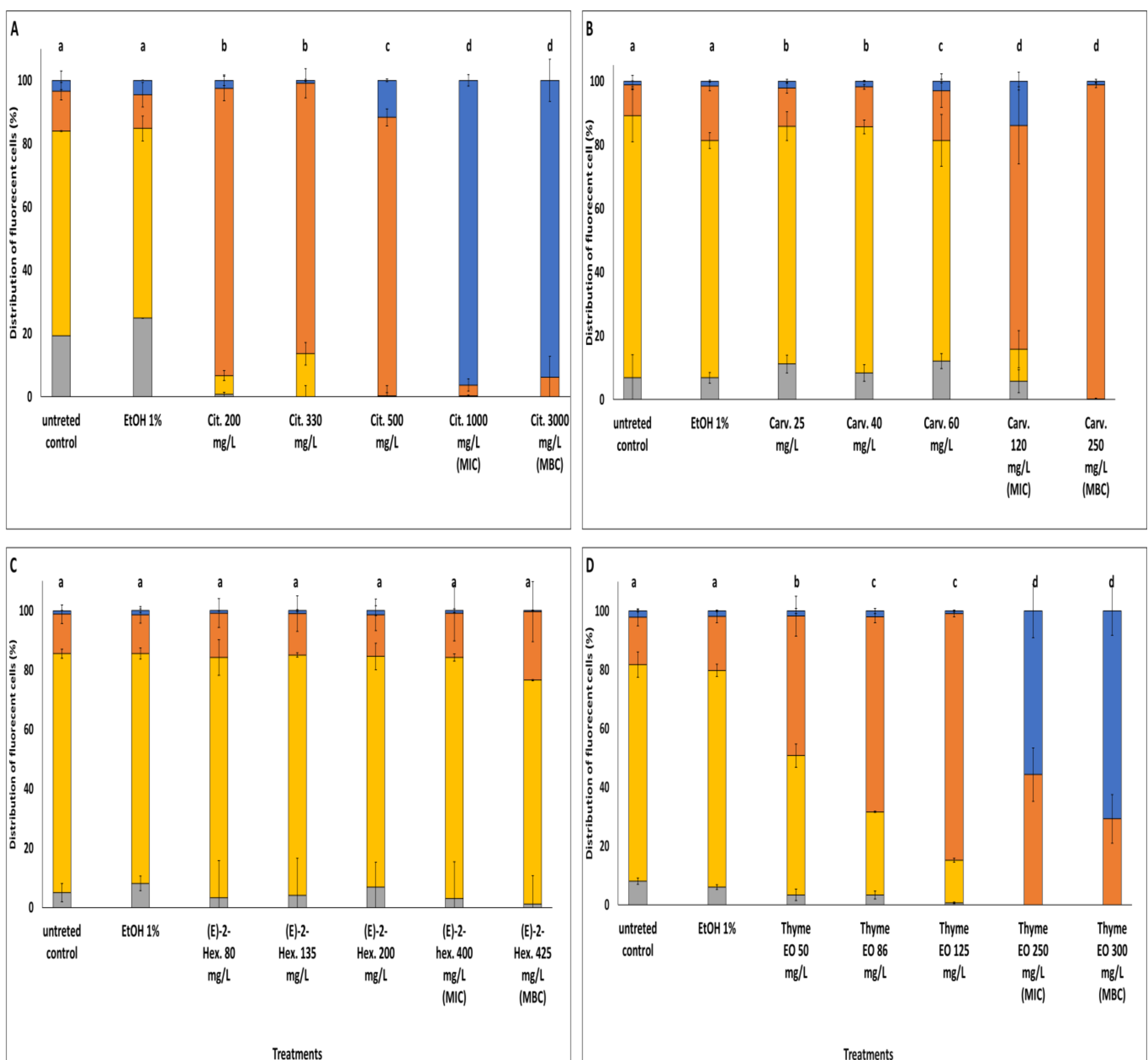

Figure 4: membrane integrity of Escherichia coli K12 MG1655 *after 1 h exposure to different concentrations of natural antimicrobials: Citral (A), Carvacrol (B), (2)-hexenal (C) and thyme EO (D). Grey bars represent cell fragments or unstained cells;*

Yellow bars intact membrane cells; orange bars slight membrane permeabilized cells ; Blue bars (complete) membrane permeabilized cells. Different letters mean data significantly different (p<0.05).

Treatment effects on the esterase activity and membrane integrity of *Escherichia coli* K12 MG1655

The exposure to citral induced on *Escherichia coli*, the cell membrane permeabilization without a loss of the esterase activity. The percentage of fluorescent cells with permeabilized cell membranes and esterase activity maintenance was higher than 80% independently on the natural antimicrobial concentrations. Only the exposure to bactericidal concentrations (1000 and 3000 mg/L) increased (10%) the amounts of the populations with permeabilized cell membrane without esterase activity (Figure 5A). Minor impacts on *Escherichia coli* cell membrane integrity and esterase activity were evidenced after the exposure of carvacrol and (E)-2-hexenal. *Escherichia coli* samples treated with the bactericidal concentrations (120 mg/L and 250 mg/L) of carvacrol showed a cell membrane permeabilization without a loss in the esterase activity (Figure 5B). The exposure to (E)-2-hexenal increased the percentage of cell fragments or unstained cells, independently on the concentration used. They represented in all the conditions tested about 15% of the whole population (Figure 5C). Thyme EO effects on the cell membrane and esterase activity were related to the concentration tested. The fluorescence signal of the intact cells decreased with the exposure to the sublethal concentration tested (80– 30%) while the percentage of cell populations with damaged membrane and esterase activity raised (Figure 5D). A significant loss in the cell esterase activity was observed only after the exposure to 250 mg/L and 300 mg/L concentrations.

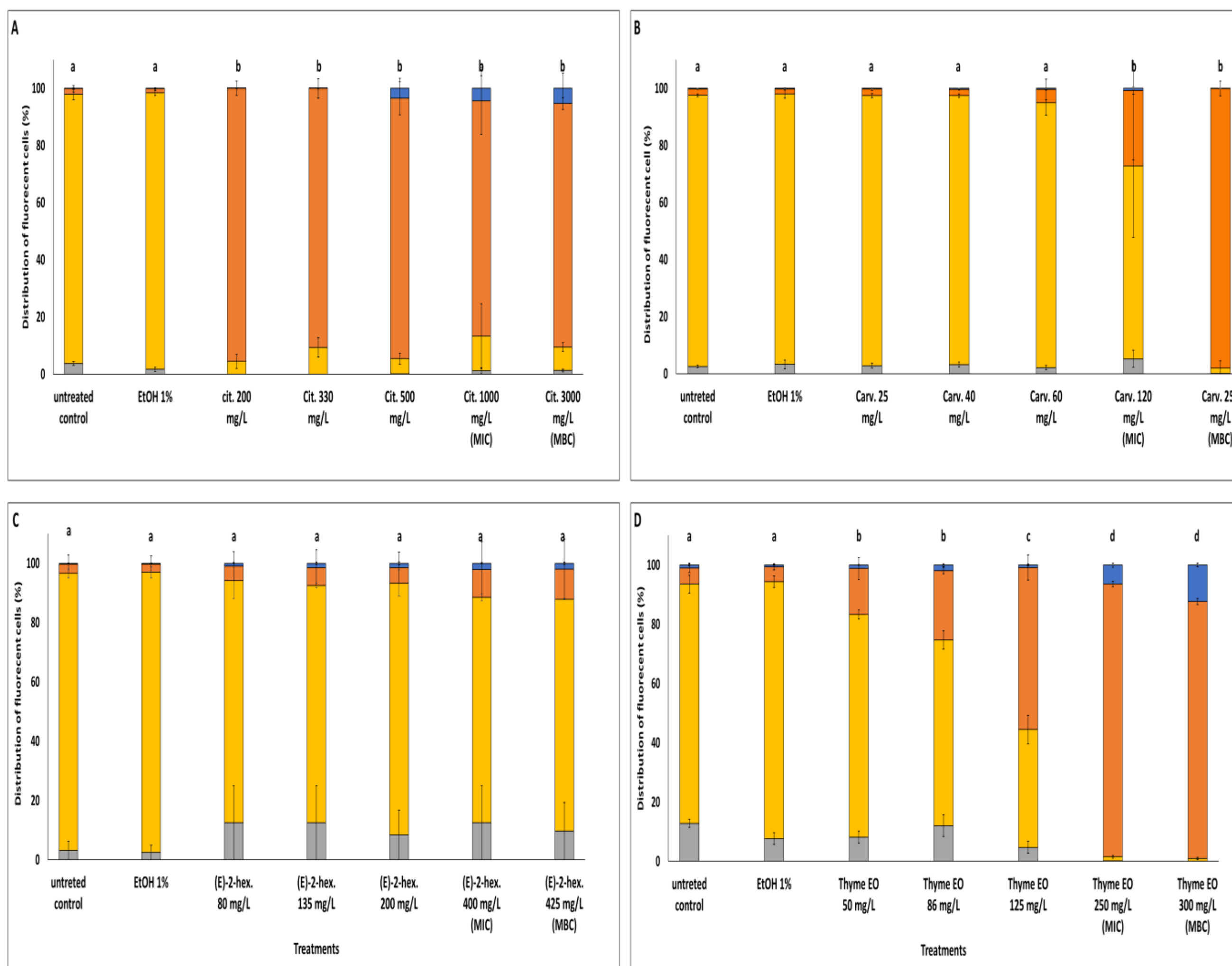

Figure 5: esterase activity and membrane integrity of Eschierichia coli k12 1655 after the exposure to natural antimicrobials: Citral (A), Carvacrol (B), (2)-hexenal (C) and thyme EO (D). Grey bars represent cell fragments or unstained cells; Yellow bars intact cell membranes and esterase activity; orange bars cell membrane permeabilization but still esterase activity; blue bars cell membrane permeabilization and no esterase activity. Different letters mean data significantly different (p<0.05).

Treatment effects on the cell membrane potential of *Escherichia coli* K12 MG1655

The measurement of relative membrane potential using DiOC2(3) showed that the untreated *E.coli* cells had a red/green ratio of 1.69 before the exposure to citral (Figure 6A). The value was reduced below 1 independently on the concentration used, suggesting the capability of citral to depolarize the cell membrane of *E. coli*. A membrane depolarization was also observed for the MIC and MBC values of thyme EO (Figure 6D). No cell membrane depolarization were observed after the exposure to carvacrol and (E)-2-hexenal (Figure 6B,C) but (E)-2-hexenal determined a concentration dependent reduction of the cell membrane potential.

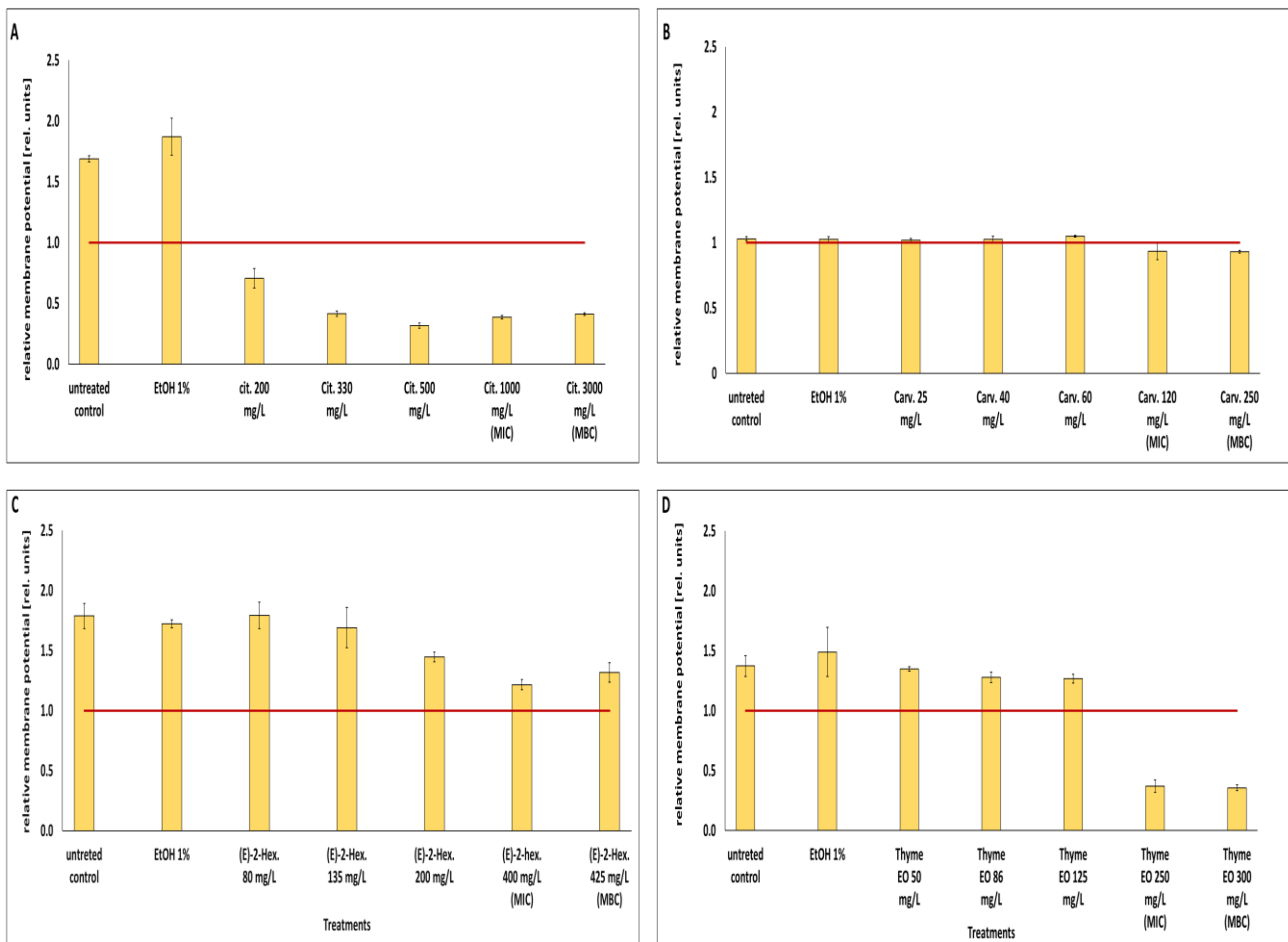

Figure 6: Relative membrane potential of Escherichia coli K12 MG1655*, expressed as red/green ratio of DiOC2(3)-fluorescence intensity, after the exposure to natural antimicrobials: Citral (A), Carvacrol (B), (2)-hexenal (C) and thyme EO (D).*

Discussion

As described by many authors, essential oils and their bioactive compounds are characterized by an antimicrobial activity both *in vitro* and real food systems. Although their antimicrobial properties are well documented, only few and fragmented information are available about their mechanisms of action on *Escherichia coli* and *Listeria monocytogenes* (Lambert et al., 2001;Burt, 2004;Bakkali et al., 2008;Xu et al., 2008;Hyldgaard et al., 2012;Picone et al., 2013). Moreover, the literature available reports on the cell targets of their lethal concentrations, that are not generally compatible with the sensory properties of foods (Gill and Holley, 2006;Paparella et al., 2008). Also the literature on the responses of the whole cell populations of these two pathogenic species to EO is still scarce (Xu et al., 2008). However, cell wall, membrane and energetic pathways are generally considered as the main EOs microbial cell targets (Burt, 2004;Bakkali et al.,

2008;Hyldgaard et al., 2012). In this framework multiparametric flow cytometric analyses were performed in order to assess the effects of citral, carvacrol, (E)-2-hexenal and thyme EO on the whole microbial populations of the two target microorganisms considered. Consequently, their effects on membrane integrity, esterase activity and cell membrane potential were investigated.

Flow cytometry data of *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655 populations, after 1 hour stress exposure reveled specific response patterns in relation to the natural antimicrobials and their concentrations. Concerning the membrane permeabilization, expect for the (E)-2-hexenal, the percentage of damaged cells raised with the antimicrobial concentration applied. The membrane integrity was analyzed using dye exclusion methods and thiazole orange (TO) and the divalent propidium iodide (PI) as probes. In fact, TO is able to pass thought lipidic bilayers and to stain both DNA or RNA while PI due to multiple charges can react with nucleic acids only when membrane is disrupted or permeabilized membrane (Kim et al., 2009;Díaz et al., 2010). Both the target strains showed an increased cell membrane permeabilization with the increase of the citral, carvacrol and thyme EO concentrations. However, *E. coli* resulted more sensitive to all the natural antimicrobials. In fact, they induced more severe the membrane permeabilization and cell load reductions of *E. coli* compared to L. monocytogenes. The most effective on *E. coli* resulted citral. In fact, *E. coli* showed the highest cell load reduction and membrane permeabilization after the exposure to citral. The higher sensitiveness of a Gram-negative pathogenic species compared to a Gram-positive one makes the natural antimicrobials used, and mainly citral, particularly interesting as as food preservative alternative to traditional ones. A wide literature shows as the Gram negative bacteria outer membrane, which acts as a barrier against macromolecules and hydrophobic substances, increase their resistance to several antimicrobials including many EO (Nikaido and Vaara, 1985;Helander et al., 1997). However, also Somolinos et al., (2008) demonstrated that citral was more effective on *E.coli* J1 than *L. monocytogenes* NCTC11994 under different experimental conditions, and especially at pH 7. These authors, using florescence microscopy and propidium iodide as probe demonstrated that citral disrupted the *E. coli* outer cell envelope forming pores permitting the cytoplasm entrance of molecules of 660 Da. To destabilize the lipopolysaccharide layer of outer membrane, the use of several chelating agents, such as EDTA, citric acid and other substances, and high pressure homogenization have been proposed (Cutter and Siragusa, 1995;Helander et al., 1997;Vannini et al., 2004;Patrignani et al., 2010). On the other hand some literature reports showed that citral, 6 atoms aldehydes and some ketons, having low molecular masses and sufficiently hydrophilic to

pass, throughout porin proteins to the deeper parts of Gram-negative bacteria without any the destabilization of outer membrane (Helander et al., 1997;Lanciotti et al., 2003;Belletti et al., 2004;Belletti et al., 2008).

Also carvacrol and thyme EO reduced significantly *E. coli* cell loads but only when used at MIC and MBC. Only these concentrations induced a significant membrane permeabilization of *E. coli* cells. These data are in agreement with those of Xu et al. (2008) obtained by flow cytometry on *Escherichia coli* AS1 90 exposed to 200 mg/L of carvacrol and thymol, the major constituent of thyme essential oil. These Authors showed cell membrane permeabilization processes associated to significant reductions of the cell loads (Xu et al., 2008). Also Gill and Holley (2006) using confocal laser scanning microscopy showed a clear membrane disruption of *E. coli* O157:H7 after the 10 minutes exposure to eugenol and carvacrol, but when used concentrations able reduce its viability of about 8 log cycles.

Also in *Listeria monocytogenes* Scott A a concentration dependent permeabilization process was evidenced after the exposure to citral, carvacrol and thyme EO. No literature is available on the effects of such antimicrobials on *L. monocytogenes* membrane permeabilization. However, Ultee et al., (1999) showed in a Gram-positive bacterium, such as *Bacillus cereus*, that carvacrol caused increased membrane permeability to cations such as H^+ and K^+. Also no significant *L. monocytogenes* cell load reductions were highlighted for citral, independently on the concentration used. Also Somolinos et al., (2008) showed a scarce sensitiveness of *L. monocytogenes* ATCC19114 serotype 4a to citral.
More effective compared to citral on *L. monocytogenes* were carvacrol and thyme EO. However, significant cell load reductions were observed only using the MIC of carvacrol and thyme EO concentrations. Friedman et al. (2002) described, using a microplate assay, how carvacrol and thymol had the highest effect on the cell loads of *Listeria monocytogenes* RM2199 and RM2388 compared to other 23 essential oil constituents.

The effects of the natural antimicrobials on the esterase activity of *L. monocytogenes* and *E. coli* was measured because it is considered as reliable way to evaluate the cell damages induced after several antimicrobial treatments (Díaz et al., 2010;Surowsky et al., 2014;Fröhling and Schlüter, 2015;Hong et al., 2015;Combarros et al., 2016;Meng et al., 2016). In fact, Carboxyfluorescein diacetate (cFDA), the probe used to measure esterase activity, is a lipophilic non-fluorescent compound converted in the cytoplasm into the fluorescent carboxyfluorescein (cF) by unspecific

esterases. According to several literature data only cells with integer membrane and active intracellular enzymes remain fluorescent (Haugland, 1999;Hoefel et al., 2003;Fröhling and Schlüter, 2015;Reineke et al., 2015). However, the data obtained in our experimental conditions showed for both target strains that this enzymatic activity *w*as not affected by the exposure to the antimicrobials used independently on their concentrations. This data seem in disagreement with literature showing that membrane permeabilization is generally associated to the losses of esterase activity (Hayouni et al., 2008;Paparella et al., 2008;Xu et al., 2008;Fröhling and Schlüter, 2015). A esterase activity decrease associated to membrane permeabilization was observed also in *L. monocytogenes* and *E. coli* exposed to EOs or their components (Xu et al., 2008). However the literature data evidenced losses of esterase activity only when lethal antimicrobial concentrations were used. For example, Paparella et al., (2008) showed by flow cytometry analyses that L. *monocytogenes* ATCC19114 serotype 4a after one hour exposure to emulsified cinnamon, oregano, thyme essential oils, had significant reductions of both esterase activity and cell loads (Paparella et al., 2008). However, these Authors tested the effects of emulsions having EO concentrations ranging between 0.02 and 0.5%. These concentrations are generally lethal concentrations for *L. monocytogenes* independently on the exposure conditions. However, they are not compatible with any usage in food systems due to the low sensory thresholds of the natural antimicrobials tested. In our experimental conditions, probably due to the use of concentrations significantly lower to those tested in literature, the amount of cFDA hydrolyzed into cF, after the exposure to natural antimicrobials considered, remained constant independently on the cell membrane permeabilization degree and the microorganism considered. Also *L. monocytogens* membrane potential was not affected by the 1 h exposure to the natural antimicrobial considered. On the other hand, the membrane potential, due to the different ion content inside and outside the cell and measured using lipophilic dyes such as the DiOC2(3), is considered as fundamental in numerous processes of the live cell physiology and it is strongly related to bacterial viability (Novo et al., 1999). In fact, according to the literature, only living cells are able to maintain membrane potential (Díaz et al., 2010).

In our experimental conditions, also at MIC values, the antimicrobials tested didn't affect *L. monocytogenes* membrane potentials compared to the control ones. However, also at MIC values of all the tested antimicrobials, *L. monocytogenes* cells showed only a slight or the absence of permeabilization of the membrane. By contrast, *E. coli*, endowed with a higher sensitiveness to almost all the antimicrobials considered compared to *L. monocytogenes*, showed also cell

membrane depolarization, which levels depended on the antimicrobial used and its concentration. A complete depolarization (red/green ratio below 1) of *E. coli* membrane was observed for all the citral concentrations tested and thyme EO MIC and MBC values. Also (Kim and Kang, 2017) observed that cell membrane potential of *E. coli* O157:H7 was significantly reduced after exposure to citral and thymol combined with a ohomic heating treatments, both in model and real food systems.

In our experimental conditions also (E)-2-hexenal exposure caused a reduction of the *E. coli* population relative membrane potential. On the opposite no effect on *E. coli* membrane potential was evidenced after carvacrol treatments. Different studies confirmed that Gram-negative bacteria, due the outer membranes, were characterized by a higher resistance to carvacrol (Kokoska et al., 2002;Okoh et al., 2010).

The reduction of membrane potential after antibacterial treatments is considered fundamental for pathogenic species since live but not culturable cells are reported to be still able to cause diseases (Fröhling and Schlüter, 2015).

In general the data obtained indicated that sublethal treatments had minor impacts on *L. monocytogenes* compared to *E. coli*. In fact, all the antimicrobial tested induced only a slight cell membrane permeabilization (with the exception of (E)-2-hexenal) of *L. monocytogenes*, while only the exposure to carvacrol and thyme essential oil MIC values reduces the cultivability and significant cell loads reductions were observed.

E. coli was more sensitive to all the antimicrobials considered not only in terms of cultivability but also in terms of membrane permeability and potential. However also for this strains, the antimicrobial used were unable to cause irreversible damages. In fact, the percentage of unstained cells of fragments remained constant and below the 3% independently on the strain, natural antimicrobial and the concentration used. As reported by (Booyens and Thantsha, 2014) during an antimicrobial treatment, the increase of unstained population is related to a severe cell lysis or to a decreased staining accuracy due conglomerates formation. In addition, the increase of the unstained fraction subpopulation is reported to be related with highly permeabilized or lysed cells unable to growth (Fröhling and Schlüter, 2015).

Conclusion

The flow cytometry approach used allowed to understand the *Listeria monocytogenes* and *Escherichia coli* cell targets to sub-lethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO. The data showed that the membrane permeabilization as a common action mechanism of the antimicrobials considered on both strains. By contrast they showed that esterase activity was not affected independently on strain, antimicrobial and its concentration. The approach used revealed that some antimicrobials such as citral, carvacrol and thyme EO were more effective against the Gram negative strain used. These results are particularly important since Gram negative are more resistant to many antimicrobials. However, the multiparameter data obtained showed that the natural antimicrobials and the concentrations used caused also on *E. coli* k12 1655, the most sensitive strain tested, reversible damages since the percentage of cell fragments remained constants also when the MIC values were used and when the membrane was depolarized. These data suggests that the levels used of citral, carvacrol and thyme EO can be used as preservatives to control the growth of pathogens such as *Listeria monocytogenes* and *Escherichia coli* only in combinations with other hurdles. In fact, concentrations able to have lethal effects are incompatible with the food sensory features due to their low sensory threshold. Consequently, the detailed knowledge of the action mechanisms of natural antimicrobials considered in relation to the others hurdles applied is absolutely necessary for their implementation at industrial level as preservation strategies. The implementation processes should be also related to the food matrices and production processes.

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Effects of the exposure to sub-lethal concentrations of citral, carvacrol, (E)-2-hexenal on membrane fatty acid composition of *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG 1655.

Introduction

Essential oils (EOs) and some of their components have proved to be a source of natural alternatives to improve food safety and shelf-life. Recognized as safe from different international food authorities, they are commonly used in food industry as flavor and teste enhancers (Newberne et al., 2000). 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 dramatically increasing (Belletti et al., 2010;Siroli et al., 2014;Siroli et al., 2015b;Siroli et al., 2015c). Since their antimicrobial activity is proved both in model and in real foods thyme EO, and some components of EOs like citral (a mixture of monoterpene aldehydes: geranial and neral), carvacrol, and (E)-2-hexenal are promising alternatives to traditional preservatives, (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 (Lanciotti et al., 2004;Di Pasqua et al., 2006;Di Pasqua et al., 2007;Picone et al., 2013;Nazzaro et al., 2017). The antibacterial activity of essential oil and their components is not attributable to one specific mechanism but to the action towards several specific cell targets (Burt, 2004;Lanciotti et al., 2004;Bakkali et al., 2008;Hyldgaard et al., 2012;Patel, 2015). Some authors (Ultee et al., 1999;Lambert et al., 2001;Nazzaro et al., 2013) have proposed the cell wall, the cell membrane, the cytosol coagulation and the depletion of the microbial cell proton motive force as main cellular targets of EOs. Due their hydrophobic properties, EOs and their components are reported to interfere with cell membrane integrity and functionality.

Patrignani et al., (2008) showed that the presence of sub-lethal concentrations of hexenal and (E)-2-hexenal in the growth media and in combination with high pressure homogenization treatments

were able to cause modifications of the volatile profiles and membrane fatty acid composition of various food-borne pathogens including some strains of *L. monocytogenes* and *E.coli.* Also Siroli et al. (2015a) demonstrated that the growth in the presence of citral, carvacrol, (E)-2-hexenal, thymol, thyme and oregano EOs induced significant modification of membrane fatty acid and volatile molecule profiles of *Listeria monocytogenes* and *E.coli*.

When microbial cells are exposed to sub-lethal stresses, their cell membrane are necessarily subjected to changes in fatty acid composition to face with the new environment (Wouters et al., 2001). The modification of membrane fatty acid composition is fundamental for the cells in maintaining of the proper membrane fluidity when exposed to external stresses (Di Pasqua et al., 2006;Tabanelli et al., 2014). The major adaptive response of the cells to keep the fluidity of their membranes at a value compatible with their functionality, is known as homeo-viscous adaptation. This process allows to prevent the loss of the mechano-chemical properties of the lipid bilayer (Russell and Fukunaga, 1990). Homeo-viscous adaptation include alterations of saturation degree, carbon chain length, branching position, cis/ trans isomerization, conversion of unsaturated fatty acids (UFAs) into cyclopropanes and in the protein membrane continent (Guerzoni et al., 2001;Roller, 2003). However, the adaptation mechanisms depend on stress applied, species, strains, cell physiological state, and conditions during the stress exposure (Cronan Jr and Gelmann, 1975;Fulco, 1983;Russell and Fukunaga, 1990). Many factors can influence the fatty acid content of the cell membranes and they include temperature and pH shifts, ethanol concentration, external osmolarity, the presence of substances able to affect the microbial growth, and transition to the stationary phase (Denich et al., 2003). Generally the reduction of the growth temperature increase the fatty acid unsaturation degree in different microorganisms (Suutari et al., 1990;Suutari and Laakso, 1994). In fact, incorporation of unsaturated fatty acids in the cell membrane increase the cell membrane fluidity (Ingram, 1976). However, this adaptation has been regarded as response mechanisms common to many microorganisms exposed to several environmental stresses (Keweloh and Heipieper, 1996). In fact, the introduction of a double bond is reported to reduce the damage of oxygen reactive species generated by the unbalance between anabolic and catabolic pathways under stress conditions, since the desaturase of many microorganisms are oxygen dependent (Dodd et al., 1997;Chatterjee et al., 2000;Guerzoni et al., 2001).

In fact, also natural antimicrobials such as citral, carvacrol, citral, carvacrol, (E)-2-hexenal, thymol, thyme and oregano EOs induced the increase of unsaturation level in many microbial species, including *Listeria monocytogenes* and *Escherichia coli* when grown in their presence at sublethal concentrations (Siroli et al., 2015a).

Nowadays no information on the short time exposure to citral, carvacrol, (E)-2-hexenalof *Listeria monocytogenes* and *Escherichia coli* are available. These information are fundamental to understand the action mechanisms of such antimicrobial since it is well known that the gene over and down regulations by sublethal stress conditions are significantly time dependent varying over time during the cell recovering and adaptation. In fact, the understanding of short time response of pathogens can be fundamental to set up preservation strategies alternative to traditional ones not depleting the safety features of foods.

In this framework, the main aim of this work was the study of the changes in cell membrane fatty acid composition of *Listeria monocytogenes* Scott A and *Escherichia coli* k12 MG 1655 after one hour exposure to sub-lethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme essential oil. This microorganisms were chosen as target ones due to their relevance as food-borne pathogens (European Food Safety et al., 2015). In fact, the exploitation of natural antimicrobials in foods has to be supported by a full comprehension of the short term adaptation of such pathogenic species and the cell membrane is their primary target.

Material and methods

Essential oils

Essential oils and their bioactive compound citral, carvacrol, and (E)-2-hexenal were purchased from Sigma-Aldrich (Milano, Italy), while thyme essential oil (EO) was obtained from Flora s.r.l. (Pisa, Italy) Before the experiments, essential oils were proper diluted using absolute ethanol (Sigma-Aldrich, Milano, Italy) to prepare 100X essential oils stock solutions.

Bacterial strain

Listeria monocytogenes Scott A and *Escherichia coli* k12 MG 1655, belonged to the Department of Agricultural and Food Sciences of Bologna University, were stored as cryo-culture at -80 °C for long-term preservation. To acclimatize cultures to the experimental conditions, the strain was given to 5 ml of Brain Heart Infusion broth (BHI) (Thermo-fisher, Milano, Italy) and incubated for 24 h without shaking at 37 °C. After the growth, cells were sub-cultured at 37°C for 24h in BHI broth.

Exposure to natural antimicrobials

The inoculation of *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG 1655 were performed in 500 mL flasks, containing 250 mL of BHI broth (Oxoid, Milano, Italy) with 2.5 mL (1% of the final volume) to reach a 4 log CFU/mL concentration and incubated without stirring at 37°C. Exposure to natural antimicrobials were performed for *L. monocytogenes* Scott A in the middle of the exponential growth phase (OD=0.4, λ= 600 nm) while for *E.coli* K12 MG1655 the exposure was performed in the stationary growth phase (OD=2, λ= 600 nm). The EOs or their components used were conveyed through ethanol used at 1% in the final solution (v/v) and the concentrations used corresponded to 1/2 of the minimal inhibition concentration (MIC) values as described by (Siroli et al., 2014;Siroli et al., 2015a). 250 µL of natural antimicrobial hydro-alcoholic stock solutions were added to 250 mL of liquid cultures in order to obtain the concentrations reported in Table 1 and Table 2. Cultures were incubated for 1 h at 37°C without stirring.

Table 1: Essential oils, their components, and relative concentrations, used for the treatments of L. monocytogenes Scott A.

Natural antimicrobial	Concentration tested (mg/L)
Citral	125 mg/L
Carvacrol	50 mg/L
(E)-2-hexenal	400 mg/L
Thyme essential oil	100 mg/L

Table 2: Essential oils, their components, and relative concentrations, used for the treatments of Escherichia coli k12 MG1655.

Natural antimicrobial	Concentration tested (mg/L)
Citral	500 mg/L
Carvacrol	60 mg/L

(E)-2-hexenal	200 mg/L
Thyme essential oil	125 mg/L

After the exposure, the total viable cell count for *L. monocytogenes* Scott A and *E. coli* K12 MG1655, was immediately performed (see chapter below). Afterwards, bacterial cells were harvested by centrifugation at 8000 x for 10 min, washed using 250 mL of physiological saline solution (0.9% NaCl) again centrifuged g at 4° C for 10 min.

Total Viable Cell Count

The viable cell count of both bacteria after exposure to essential oils or their components was determined by plate count methods in duplicate. Samples were serially diluted in glass tubes using as dilution solution, physiological saline solution (9 % NaCl). 100 µl of each dilution was spread on BHI agar (Thermo-fisher, Milano, Italy) and the growth (colony forming units) was evaluated after 24 h at 37°C.

Lipids extraction and fatty acids analysis

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;Siroli et al., 2015a). 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.

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 in relation to the antimicrobials used.

Results

Cell fatty acid changes induced by sublethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO

The one hour exposure to the tested molecules did not significantly affect the growth of *L. monocytogenes* Scott A and *E. coli* K12. In fact, no significant cell load reductions were observed after 1 hour of incubation at 37°C with ethanol, citral, carvacrol, 2-(E)-hexenal and thyme EO (data not shown). By contrast, these molecules affected both membrane associated and released fatty acids (FA) of the strains tested. The main fatty acids (FAs) detected in *Listeria monocytogenes* control cells and those exposed to sublethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO were C10:0, C12:0, C13 ante, C13:0, C14:0 iso, C14:1 *cis*11, C14:0, C15 iso, C15 ante, C15:0, C16 iso, C16:1 *trans* 9, C16:1 *cis* 9, C17 iso, C17 ante, C17:0, C18 iso, C18:2 (*cis*,*cis*) 9-12, C18:1 *trans* 9, C18:0, C19:0 and C20:0. However, their relative abundances varied according to exposure conditions (i.e. the presence/absence of ethanol or natural antimicrobials). In fact, each exposure conditions determined significant quali-quantitative modifications in the membrane fatty acid profiles of *L. monocytogenes*. However, the fatty acids subjected to the major and significant changes in relation to the antimicrobial used are reported in Table 3. In general the exposure to the different sublethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO induced, compared to the untreated controls, significant reductions of the of the unsaturation levels (UL) and chain length (CL) values. These reductions were mainly due to significant increases of the relative percentages the C12:0, C14:0, C17:0 iso and C19:0 saturated FAs as well as, to a reduction of the C14:1 *cis*, C16:1 *trans* 9, C18:1 *cis 9*, C18:1 *trans* 9 unsaturated fatty acids (UFAs) levels. Also the cell exposed to ethanol without any other natural antimicrobials showed the same behavior, even if with less marked modifications compared to the others stress conditions. The highest reductions of UL and CL values were observed in the cell exposed to sublethal level (½ MIC value) of thyme EO (Table 3). Moreover, the exposure to the natural antimicrobial considered induced also the reduction of the relative percentages of C15:0 ante of C15:0. The highest reductions of such FAs were observed after the exposure to 2-(E)-hexenal (Table 3).

The data of the *Listeria monocytogenes* Scott A free fatty acids (FFAs) showed that, untreated samples, were mainly characterized by the branched FAs (BFAs) C15 iso and C15 ante, the saturated FAs C16:0, C18:0 and the unsaturated C18:1 *cis* 9, C18:1 *trans* 9 and C18:2 *cis,cis* 9-12

UFAs. The one hour exposure to ethanol significantly decreased the UL also of the released membrane FA, due the reduction the UFAs, such as C18:2 *cis,cis 9*-12, C18:1 *cis* 9, C18:1 *trans* 9, relative abundances, as well as the increase of some saturated and branched FAs. In particular, a marked increase of C14:0, C16:0, C18:0, C15ante and C17ante relative abundances was observed. A similar pattern was observed after the exposure to carvacrol and thyme EO sublethal concentrations (50 – 100 mg/L). The exposure to carvacrol and thyme EOs caused the reduction of UFAs C18:2 *cis,cis* 9-12, while C16:0, C17ante and C18:0 relative percentages showed significant raises. Although the exposure to citral caused a significant reduction of C18:1 *cis* 9, C18:1 *trans* 9 UFA levels, the reduction of the UL was lower compared to the other treatments due an increase of the relative percentage of C18:2 *cis,cis* 9-12. *L. monocytogenes* one hour exposure to (E)-2-hexenal induced a reduction of both UL and CL values mainly due to the significant increase of the C12:0 level (Table 4).

Table 3: Total membrane fatty acid composition of L. monocytogenes Scott A in relation to the stress condition applied.

Total fatty acids (%)																	
	C12:0	**C14:1 cis11**	**C14:0**	**C15 ante**	**C15:0**	**C16:1 *trans* 9**	**C16:0**	**C17 iso**	**C17 ante**	**C17:0**	**C18:2 (cis,cis) 9-12**	**C18:1 *cis* 9**	**C18:1 trans9**	**C18:0**	**C19:0**	**UL[a]**	**CL[b]**
Untreated control	8.50	1.13	0.08	12.85	10.73	2.92	9.38	7.80	5.04	10.55	0.10	3.63	21.54	1.47	4.28	0.29	1629.72
EtOH 1%	65.22	1.45	0.39	5.35	0.85	1.90	1.89	6.66	0.73	2.41	0.13	3.30	6.56	1.10	2.06	0.13	1367.38
Citral 125 mg/L	62.64	0.12	0.80	1.68	2.23	0.10	1.38	16.39	0.19	1.65	0.28	1.27	2.90	0.35	8.03	0.05	1395.61
Carvacrol 50 mg/L	47.22	0.13	0.18	1.06	0.00	0.53	1.18	20.27	0.39	1.89	0.13	1.76	3.87	0.86	20.50	0.07	1506.78
(E)-2-Hexenal 400 mg/L	48.23	0.07	0.26	0.78	0.28	0.22	0.25	19.95	0.25	2.19	0.23	3.31	4.09	0.59	19.31	0.08	1502.12
Thyme EO 100 mg/L	71.46	0.13	0.22	4.53	3.37	0.45	0.80	9.80	0.42	1.36	0.40	0.52	1.03	0.83	4.67	0.03	1336.73

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%.

[a] Unsaturation level calculated as [percentage monoenes + 2(percentage dienes) + 3(percentage trienes)]/100.0

*[b] Mean chain length calculated as (FAP * C) (where FAP is the percentage of fatty acid and C the number of carbon atom*

Table 4: Free membrane fatty acid composition of L. monocytogenes Scott A in relation to the stress condition applied.

Free Fatty Acids (%)

	C12:0	C14:0	C15 iso	C15 ante	C15:0	C16 iso	C16:1 *trans* 9	C16:1 *cis* 9	C16:0	C17 iso	C17 ante	C17:0	C18:2 (*cis,cis*) 9-12	C18:1 *cis* 9	C18:1 *trans* 9	C18:0	U.L[a]	C.L[a]
untreated control	0.67	1.06	2.50	2.56	0.27	0.66	0.02	0.87	17.95	0.61	2.67	0.47	9.08	41.08	3.68	15.84	0.47	1732.95
EtOH 1%	0.87	1.65	2.66	3.71	0.32	1.01	0.24	0.00	36.46	0.55	3.38	0.00	4.73	10.71	0.80	32.91	0.13	1688.76
Citral 125 mg/L	1.38	1.47	1.64	2.57	0.48	0.00	0.14	0.37	32.63	0.44	2.03	0.27	14.36	14.27	1.19	26.75	0.17	1702.71
Carvacrol 50 mg/L	1.93	1.46	2.18	2.88	0.51	0.00	0.30	0.00	37.27	0.74	3.68	0.41	3.24	7.39	0.00	38.00	0.08	1685.91
(E)-2-hexenal 400 mg/L	23.04	1.41	1.00	1.10	0.35	0.36	0.13	0.34	28.82	0.34	1.74	0.33	4.81	7.33	0.81	28.09	0.09	1587.06
Thyme EO 100 mg/L	0.93	1.70	2.05	2.19	0.92	1.60	0.16	0.41	33.94	2.73	6.17	0.45	6.29	10.63	1.05	30.64	0.12	1723.99

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%.

[a] Unsaturation level calculated as [percentage monoenes + 2(percentage dienes) + 3(percentage trienes)]/100.0

*[b] Mean chain length calculated as (FAP * C) (where FAP is the percentage of fatty acid and C the number of carbon atom*

The main fatty acids detected in *Escherichia coli* k12 MG1655 in the untreated control cells and those exposed to sublethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO were C12:0, C12 cyc, C14:0, C15 iso, C15 ante, C15:0, C16 iso, C16:1 *trans* 9, C16:1 *cis 9*, C16:0, C17 iso, C17 ante, C17 cyc, C17:0, C18:2 (*cis*,*cis*) 9-12, C18:1 9*cis*, C18:1 9*trans*, C18:1 *cis*11, C18:0 and C19 cyc. However, their levels varied according to exposure conditions (the presence/absence of ethanol or natural antimicrobials).The fatty acids subjected to significant changes in relation to the antimicrobial used are reported in Table 5. The main effect of the exposure to sublethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme EO on the total cell membrane fatty acid composition of *E. coli* consisted into a reduction of the unsaturation level. This reduction was due to the decrease of specific unsaturated FAs in relation to the antimicrobial considered. In fact, the one hour exposure to 500 mg/L of citral caused the highest reduction of the UL value compared to the untreated control cells, and the UL reductions was due to a marked diminutions of the relative amount of the C18:1 9*cis* and C18:1 *cis*11 UFAs. The decrease of these UFAs was accompanied by the increase of levels of C16:0, C17 iso and C17 ante, C17 cyc and C19 cyc saturated, branched and cyclopropanic fatty acids.

The UL reduction in cells exposed to carvacrol, (E)-2-hexenal and thyme EO was mainly due to reductions of C18:1 *cis*11 compared to the control cells. The exposure to carvacrol induced also a slight increase of unsaturated FAs such as C18:2 (*cis*,*cis*) 9-12 and C18:1 *trans* 9. The exposure to (E)-2-hexenal and thyme EO induced the increase of C18:1 *cis* and *trans* 9 isomers. However, these increases of UFAs were unable to counteract the dramatic decrease with respect to the control cells of C18:1 *cis* 11.

The exposure to the natural antimicrobial considered significantly modified the mean chain length value (CL). The citral and carvacrol exposure induced, a slight reduction of the mean chain length, mainly due the increase of the C12:0 and C14:0 FA percentages.

Carvacrol exposure provoked the highest relative augmentation of the short chain fatty acids, *i.e.* C12:0 and C14:0 (2.81% and 9.61% respectively). The exposure to (E)-2-hexenal and thyme EO induced a little increase of the CL value. As showed in Table 5, after the one hour exposure, the relative percentage of the C18:0 FA was dramatically increased in such conditions compared to the untreated control and to the other treatments. In addition, in all the condition tested, a significant increase of the cyclopropanic FA C17 cyc and C19 cyc cyclopropanic were observed (Table 5).

The free fatty acid released by *E. coli* K12 MG1655, after the exposure to natural antimicrobials is reported in Table 6. The main membrane FFAs detected in the control cells were C14:0, C16:0, C18:0, C17cyc, C19cyc, C18:2 *cis,cis* 9-12, C18:1 *cis* 9 and C18:1 *trans* 9. The one hour exposure to the natural antimicrobials tested induced a reduction of the unsaturation level and mean chain length values. The severity of the modification observed depended on the chemical characteristics of the natural antimicrobial applied. In fact, the highest effect on the *Escherichia coli* unsaturation level was observed after the one hour exposure to 500 mg/L of citral (Table 6). The exposure to such natural antimicrobial caused a consistent reduction of the free UFAs C18:2 *cis,cis 9*-12 and C18:1 *cis 9*, levels as well as, a significant increase of the C18:1 *trans* 9. Moreover, citral caused a the highest reduction of the mean chain length value, due the raise of the relative percentages of the C:12:0, C14:0, C16:0, C17 cyc , C19 cyc FAs.

A similar trend was observed after the exposure to carvacrol, (E)-2-hexenal and thyme EO sublethal concentrations. In fact, these natural antimicrobials increased the level of C:14, C:16, C17 cyc, C19 cyc and C18:1 *trans* 9 fatty acids as well as, a reduction of the C:18 long chain fatty acid. As described for citral, also a reduction of the UL values was recorded after the exposure to carvacrol, (E)-2-hexenal and thyme EO. The exposure to these natural antimicrobials reduced the C18:2 *cis,cis 9*-12, C18:1 *cis 9* percentages, while the C18:1 *trans* 9 concentration raised (Table 6) .

Table 5: Total membrane fatty acid composition of Escherichia coli k12 MG1655 in relation to the stress condition applied.

Total fatty acids (%)	C12:0	C14:0	C12 cyc	C15 iso	C15 ante	C15:0	C16 iso	C16:1 *trans* 9	C16:1 cis9	C16:0	C17 iso	C17 ante	C17 cyc	C17:0	C18:2 (*cis,cis*) 9-12	C18:1 *cis* 9	C18:1 *trans* 9	C18:1 *cis* 11	C18:0	C19 cyc	UL[a]	CL[b]
untreated control	0.33	4.02	2.59	0.15	0.21	0.47	0.08	0.30	0.00	37.39	2.11	2.32	3.60	2.85	1.27	4.63	0.30	37.38	0.00	1.43	0.45	1704.65
EtOH 1%	1.80	4.36	3.80	0.00	0.00	0.00	0.00	0.00	0.00	49.34	2.34	2.32	0.00	2.89	0.00	3.48	0.00	0.00	29.66	0.00	0.03	1642.64
Citral 500 mg/L	0.50	8.80	0.29	0.10	0.12	0.12	0.00	0.29	0.76	55.52	9.36	8.75	5.87	2.34	1.45	0.99	0.81	0.35	3.59	5.64	0.06	1726.82
Carvacrol 60 mg/L	2.81	9.61	0.39	0.26	0.43	0.96	0.19	0.14	0.82	49.09	2.22	2.28	4.23	2.73	3.78	4.20	0.63	0.44	14.78	1.95	0.14	1662.44
(E)-2-hexenal 200 mg/L	0.20	2.71	1.60	0.59	0.64	0.36	0.18	0.20	0.23	37.15	1.57	2.47	5.69	2.66	1.79	5.74	0.57	0.51	35.15	2.18	0.11	1727.17
Thyme EO 125 mg/L	0.12	2.80	0.60	0.15	0.15	0.28	0.00	0.25	0.24	35.83	8.51	2.58	4.46	2.77	1.54	5.45	0.54	0.16	33.56	1.83	0.10	1726.38

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%.

[a] *Unsaturation level calculated as [percentage monoenes + 2(percentage dienes) + 3(percentage trienes)]/100.0*

[b] *Mean chain length calculated as (FAP * C) (where FAP is the percentage of fatty acid and C the number of carbon atom*

Table 6: Free membrane free fatty acid composition of Escherichia coli k12 MG1655 in relation to the stress condition applied.

Free Fatty Acids (%)																	
	C12:0	**C14:0**	**C12 cyc**	**C15 iso**	**C15 ante**	**C16:1 *trans* 9**	**C16:1 *cis 9***	**C16:0**	**C17 cyc**	**C18:2 (*cis*,*cis*) 9-12**	**C18:1 *cis 9***	**C18:1 *trans* 9**	**C18:0**	**C19 cyc**	**C20:0**	**UL[a]**	**CL[b]**
untreated control	0.55	4.49	0.40	0.15	0.13	0.04	0.66	31.96	12.55	4.56	9.57	1.61	28.62	4.15	0.58	0.21	1702.91
EtOH 1%	0.41	4.01	0.26	0.11	0.14	0.05	0.53	25.19	10.76	3.08	8.28	1.33	42.15	3.71	0.00	0.16	1720.68
Citral 500 mg/L	2.74	7.85	0.11	0.08	0.06	0.10	1.17	41.58	16.64	2.04	3.56	4.46	12.63	6.64	0.34	0.13	1655.99
Carvacrol 60 mg/L	0.56	6.31	0.26	0.11	0.10	0.10	0.89	29.91	18.17	3.42	7.88	2.61	22.08	7.08	0.51	0.18	1697.40
(E)-2-hexenal 200 mg/L	0.76	6.72	0.18	0.09	0.09	0.10	0.84	32.61	19.61	3.03	6.76	3.46	17.84	7.59	0.35	0.17	1688.63
Thyme EO 125 mg/L	3.02	8.64	0.14	0.08	0.07	0.12	1.05	34.28	17.67	2.39	5.46	3.49	15.43	7.30	0.87	0.15	1666.50

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%.

[a] Unsaturation level calculated as [percentage monoenes + 2(percentage dienes) + 3(percentage trienes)]/100.0

*[b] Mean chain length calculated as (FAP * C) (where FAP is the percentage of fatty acid and C the number of carbon atom*

Principal component analysis of the fatty acid data after one hour exposure of *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655 to natural antimicrobials.

To better show the relationships between membrane fatty acid composition and chemical stress applied, a principal component analysis (PCA) of the total membrane fatty acid percentages detected was performed.

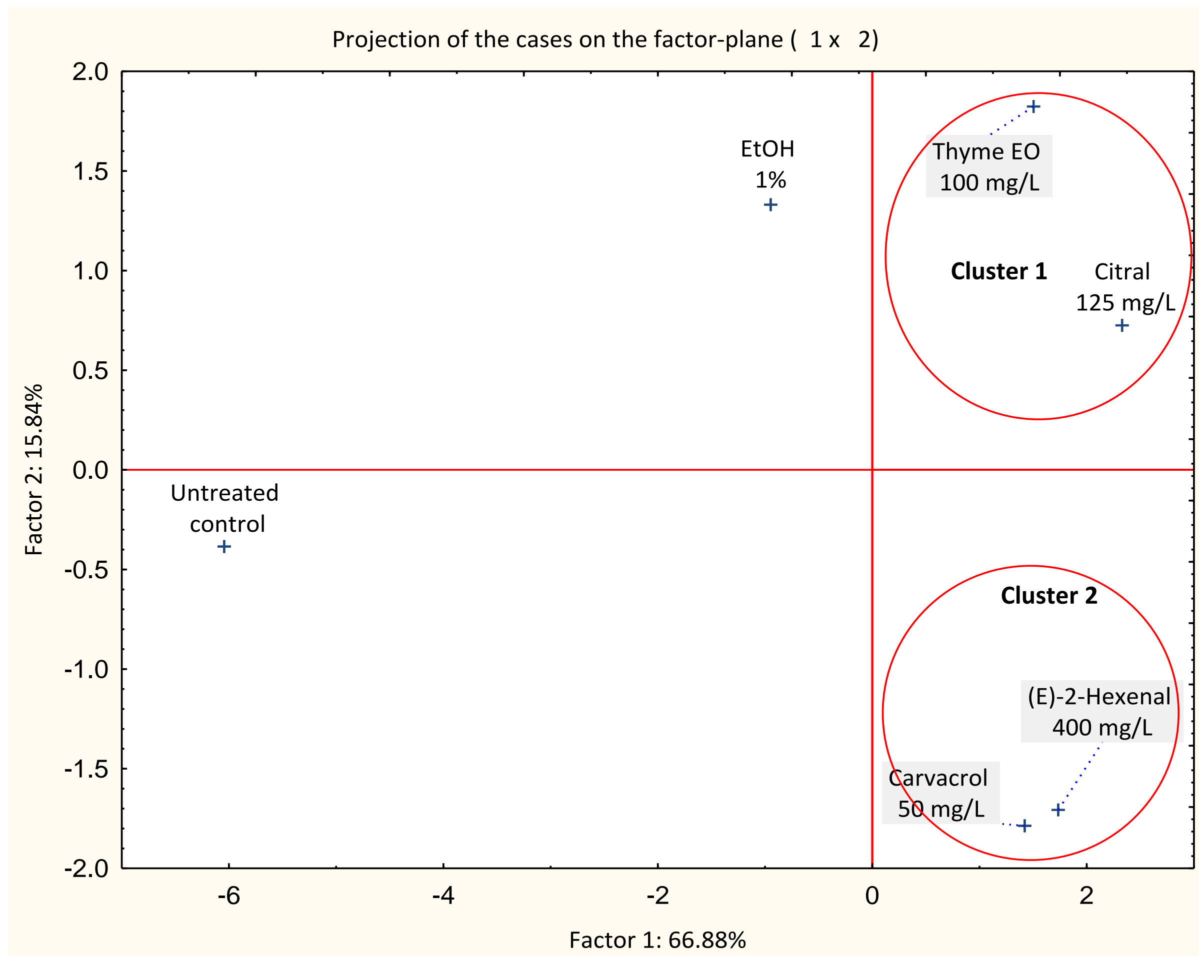

Figure 1: Principal component analysis loading plot of the total membrane fatty acids composition of Listeria monocytogenes Scott A in relation to the stress condition applied.

In Figure 1 the Principal Component Analysis (PCA) loading plots of *Listeria monocytogenes* Scott A cell membrane fatty acid profiles in relation to the natural antimicrobial tested are reported. All the samples of *L. monocytogenes* were mapped in the space spanned by the first two principal components PC1 *versus* PC2. PC1 accounted for 66.88% of the total variability, and PC2 for 15.84%, respectively. As highlighted by the PCA the responses of *L. monocytogenes* Scott A to the different natural antimicrobials tested were related to the chemical compound tested and they

were significantly different compared to the untreated controls and samples only exposed to 1% ethanol. The analysis allowed to group the other cells of *L. monocytogenes* into two different and defined clusters: cluster 1 represented by samples treated with sublethal concentrations of citral and thyme EO and cluster 2, including samples exposed to sublethal concentrations of carvacrol and (E)-2-hexenal (Figure 1). These two clusters were separated along the PCA2, accounting for the 15.84% of the total, variability. However, cluster 1 was very near along the PC1 to cells exposed exclusively to ethanol. By contrast, it was well separated by the untreated controls both along PC1 and PC2. The main positive effects on factor 1 were determined by C12:0, C14:0, C17 iso, C18:2 (*cis*,*cis*) 9-12, and C19:0 while the negative effects observed were related to the unsaturated and branched fatty acids, such asC14:1 *cis*11, C15 ante, C16 *trans* 9, C16:0, C17 iso, C17 ante, C18:1 *cis 9* and C18:1 *trans* 9. In addition, factor 2 was highly positively related to the short chain and branched FAs C12:0, C14:0 *cis*11, C14:0, C15 ante and to the UFAs C16:1 *trans* 9, C18:2 (*cis*,*cis*) 9-12 and negatively associated to the FAs C17 iso, C17 ante and C19:0 as well as, to the C18:1 *cis* and *trans* UFAs (Figure 2).

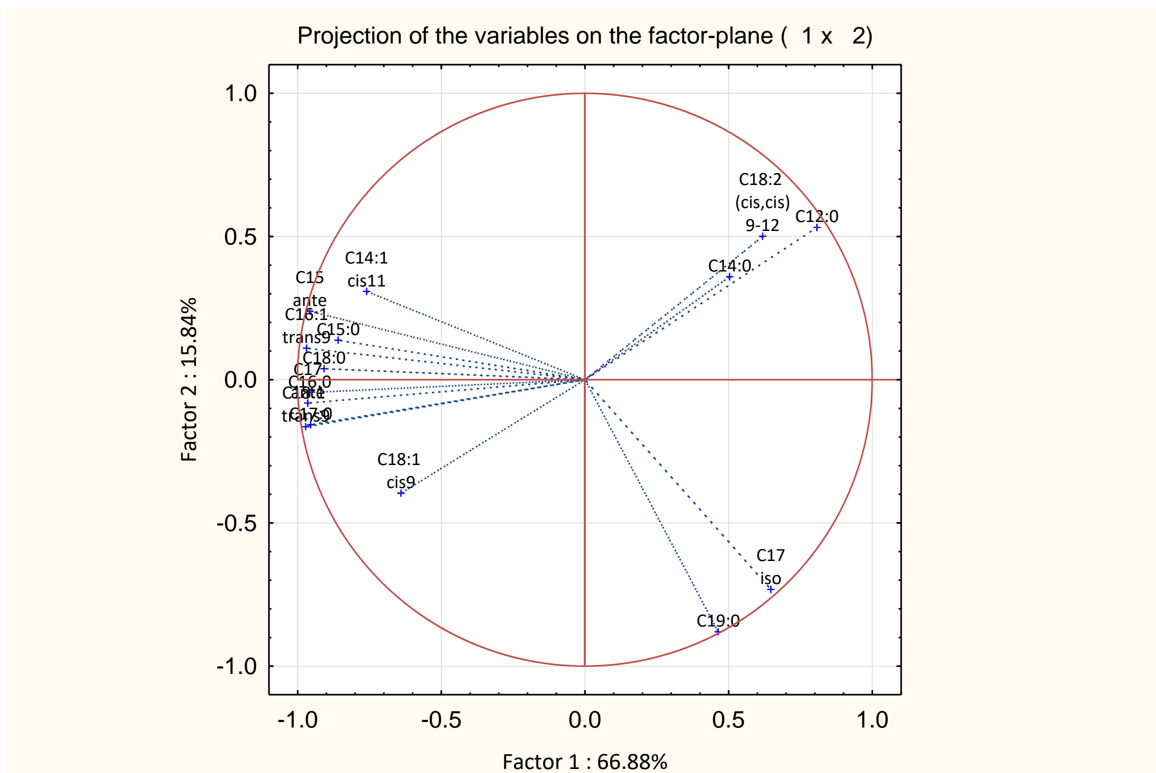

Figure 2: Principal component analysis factor coordinates for the two-first factors of the total membrane fatty acids composition of Listeria monocytogenes Scott A in relation to the stress condition applied.

Also for *Escherichia coli* K12 MG1655 the Principal Component Analysis (PCA) showed that samples were grouped in relation to the treatment applied (Figure 3). As reported in in Figure 3 the PCA

loading plots of the total membrane fatty acids composition of *E. coli* samples exposed for one hour to natural antimicrobials, were mapped in the space described by the first two principal components PC1 *versus* PC2. PC1 accounted for 35.22% of the total variability, and PC2 for 28.58%, respectively. Three different clusters were evident: cluster 1 formed by samples exposed to sublethal concentrations of carvacrol and (E)-2-hexenal; cluster 2 formed by samples exposed to sublethal concentrations of thyme EO and citral; cluster 3 grouped the control samples with or without the supplementation of ethanol (Figure 3). The three clusters were well separated both along the PC1 and PC2. The cluster 3, formed by the control samples, was clearly separated along the PC2 from the cluster 1 as well as separated along the PC1 from the cluster 2 (Figure 3). However, within the cluster 2, the samples treated exposed to citral were clearly separated along PC1 (explaining 35.22% of the total variability) from those exposed to thyme EO (Figure 3). In this case the projection of the variables on the factor plane for the PC1 and PC2 showed that C16:1 *cis 9*, C16: *trans* 9 and C18:1 *trans* 9 unsaturated fatty acids and to the C17 iso, C17 ante, C17 cyc and C19 cyc (branched and cyclopropanic FAs) accounted for the main positive effects on the factor 1. By the contrast the main negative effect on the PC1 were related to the C12 cyc cyclopropanic FAs, C17:0 and to C18:0 saturated FAs, and to C18:1 *cis 9*. Moreover, factor 2 was positively related to the C15 iso, C15 ante and C16 iso branched FAs and to the C16 and C18 UFAs while, the negative effects were related to C12 cyc, C19 cyc cyclopropanic fatty acids and to the C17 iso and anteiso branched fatty acids (data not showed).

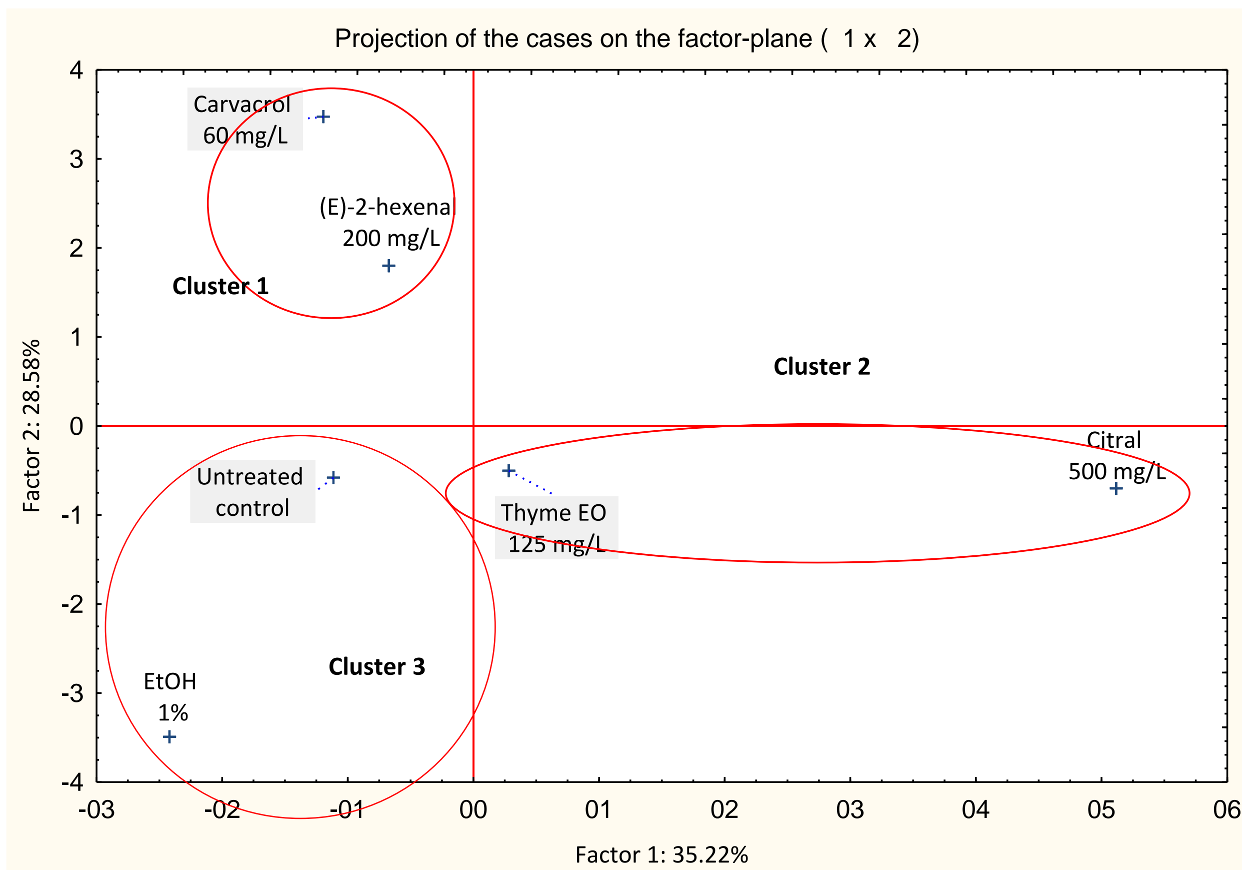

Figure 3: : Principal component analysis loading plot of Escherichia coli K12 MG1655 cell membrane fatty acid profiles in relation to the stress condition applied.

Discussion

Microbial cells have adopted proficient defense systems in order to survive to a huge variety of physicochemical adverse conditions and to adapt to environmental stresses. One of these strategies is represented by the home-viscous adaptation, a mechanism that enable microbial cell to maintain the integrity, viscosity and functionality of the membrane. 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 (Lanciotti et al., 2003;Patrignani et al., 2008;Siroli et al., 2015a). Also a wide literature on the membrane fatty acid modulation of *L. monocytogenes* and *E. coli* in response to several physic-chemical stresses is available (Brown et al., 1997;Denich et al., 2003;Ku et al.,

2007;Gianotti et al., 2008;Gianotti et al., 2009). However, the data on the membrane adaptation mechanisms of these two pathogens to natural antimicrobial are scarce and they are generally recorded during the growth in their presence (Di Pasqua et al., 2006;Patrignani et al., 2008;Siroli et al., 2015a). However, it is well known that the adaptation mechanisms are the results of a sequence of microbial short term responses, with time dependent gene over and down regulations (Serrazanetti et al., 2015). However, the comprehension of the short term response of pathogenic species is fundamental to set up food preservation strategies based on the use of natural antimicrobials alternative to traditional ones, particularly when they are used at sub-lethal concentrations. The analyses of membrane associated and released FAs showed different short term adaptation mechanisms for *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655, in relation to the natural antimicrobial used.

In our experimental conditions, the one hour exposure to sublethal concentrations (equal to ½ of the MIC value) of citral, carvacrol, (E)-2-hexenal and thyme EO reduced significantly the unsaturation level (UL) in both in *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655. The reduction of the UL was mainly related in *E.coli* to a severe reduction of the C18:1 *cis*11 UFA, while in *L. monocytogenes* the UL decrease was related to the C14:1 *cis*11, C16:1 *trans*9, C18:1 *cis*9, C18:1 *trans*9 UFAs levels. These data are apparently in disagreement with literature ones. In fact, a wide literature has shown a crucial role of unsaturated FAs in response to several different stresses, including low or high growth temperatures, high pressure homogenization as well as oxidative , acid , ethanol and salt addition stresses(Patrignani et al., 2008;Montanari et al., 2010;Wu et al., 2012;Siroli et al., 2014;Tabanelli et al., 2014). An increase of UFA was observed also in many microbial species, including *L. monocytogenes* and *E. coli* when grown in the presence of EOs or their components (Di Pasqua et al., 2006;Patrignani et al., 2008;Siroli et al., 2015a). In fact, in a previous study of Di Pasqua et al., (2006) detected an increase of some UFAs and of the membrane fluidity in *E. coli* and *Brochothrix thermosphacta* grown in the presence of sub-lethal concentrations of thymol, limonene, carvacrol, eugenol and cinnamaldehyde. Also Siroli et al., (2015a) highlighted an increase of the unsaturation level of *L. monocytogenes* and *E. coli* during the growth in the presence of sublethal concentrations of citral, carvacrol, thymol, oregano and thyme EOs. In addition, 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 (E)-2-hexenal when supplemented in the growth media. The crucial role of unsaturated FAs in the microbial stress adaptation is attributed to their role in the reduction of oxidative stress, in its turn resulting from the unbalance

unbalance between anabolic and catabolic pathways under stress conditions, since the desaturase of many microorganisms are oxygen dependent and, consequently, reduce the O_2 vapour pressure and its reactivity within cell membrane (Dodd et al., 1997;Chatterjee et al., 2000;Guerzoni et al., 2001). The apparent disagreement of our data with the literature data can be explained on the basis of the general FA biosynthetic pathways of *L. monocytogenes* and *E. coli* (Figure 4). The synthesis of fatty acids is one of the most ubiquitous pathways in organisms and, independently on the specificities related to the species, in both species the biosynthesis of UFA relays on a previous production of saturated FAs.

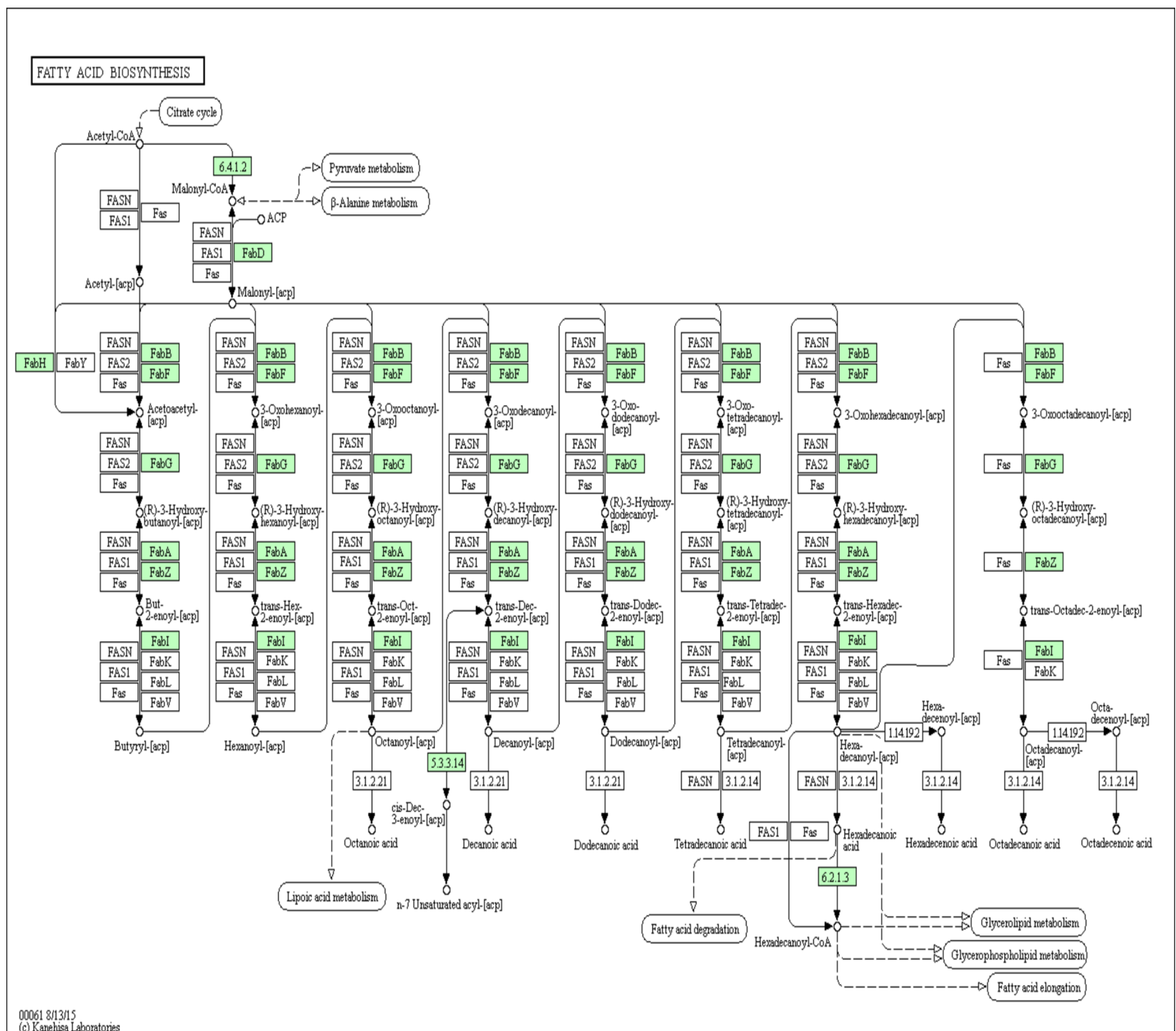

Figure 41: Fatty acids biosynthesis pathways in Listeria monocytogenes Scott A and Escherichia coli K12 MG1655 (Kanehisa et al., 2017).

In fact, the highly conserved *fabA, fabB, fabF, fabG, fabZ and fabI* genes, are involved in the unsaturated fatty acid synthesis of both the microorganisms considered (Feng and Cronan, 2009).

The short term exposure to the natural antimicrobials induced in both microorganisms a significant increase of short chain saturated FA, that are precursors of longer saturated or unsaturated FAs. The removal of the stress conditions surely prevented the further and significant biosynthesis of UFAs. In fact, a slight increase of the relative percentage of the C18:2 (*cis*,*cis*) 9-12 was observes in both microorganisms and in all the short time stress exposures adopted. In addition the exposure of *E. coli* to (E)-2-hexenal and thyme EO slight increased the relative abundances also of C18:1 *cis* 9 and C18:1 *trans* 9. On the other hand Serrazanetti et al. (2015) clearly showed a significant time dependence of the regulation of the gene involved in UFA biosynthesis in *Saccharomyces bayanus* L951 after a very short stress exposure (cells exposed to high pressure homogenization at 80 MPa and for few milliseconds). This hypothesis is confirmed also by the data relative to UL values. In fact, *L. monocytogenes* showed a reduction, compared to the untreated cells, of the chain length values, in all the stress conditions tested, due the dramatically increase of the C12:0 and C14:0 fatty acid levels (precursors of longer saturated and unsaturated FA). The reduction of CL, with respect to the untreated controls, was observed also *E. coli* in all conditions, except those in which the biosynthesis of UFA was extremely precocious, *i.e.* during the exposure to (E)-2-hexenal and thyme EO. More specifically, in *E. coli* exposure to citral and carvacrol sublethal concentrations, caused a reduction a of the CL value due reduction of the long chains C18 unsaturated and acylic FAs as well as, the augment of short and medium chains acylic fatty acids, C14 and C16. Differently *E. coli* exposure to (E)-2-hexenal and thyme EO, increased the mean chain length values increased due higher concentrations of FAs having 18 carbon atoms, both saturated that unsaturated ones.

The other modifications observed in membrane fatty acid profiles in relation to antimicrobial used were specific for each strain considered and they are mainly congruent with the maintenance of membrane proper fluidity and in agreement with literature (Patrignani et al., 2008;Gianotti et al., 2009;Montanari et al., 2010;Siroli et al., 2015a). In particular, in *L. monocytogenes* a marked increase of C19:0 and C17: iso percentages, associated to marked reductions of C15ante and C17 ante were observed after the exposure to antimicrobials used. Otherwise, in comparison to iso branched FA, anteiso FAs confer greater fluidizing properties as their structure disturb packing order to a greater extent (Heipieper et al., 1996;Gianotti et al., 2009;Montanari et al., 2010).The increase observed of C17 iso, together with the reduction of UL, contributed to counteract the fluidizing effect of the reduction of CL, observed in the cells exposed to the natural antimicrobials used. In *E. coli* to counteract the fluidity changes induced by the CL and UL modifications, the

relative percentages of cyclopropanic fatty acids (CFAs) were modulated. In particular, in the experimental conditions tested induced in *E. coli* a significative augment of the C17 cyc and C19 cyc cyclopropanic FAs. As previously described by Siroli et al., (2015a), a slight increase of the C17 cyc and C19 cyc relative percentages were observed in *Escherichia coli* 555 after the grown in presence of thyme EO. 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 and Marshall, 2004). Fatty acid cyclisation, was shown to be the one of the main response mechanism of *E. coli* to stress conditions such as the growth in acids environments (Gianotti et al., 2009). The literature data concerning the role of cyclic acid in the 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 (Grogan and Cronan, 1997). On the contrary, other authors reported that cyclopropane fatty acids confer fluidity upon the cell membrane and assist in tolerance towards disturbance factors (Denich et al., 2003). In particular, Denich et al. (2003) showed as cyclic FA, analogously to branched FA, increase the fluidity of cytoplasmic membrane as they retain the ability to slide past each other as they cannot form crystalline structure. Our experimental data seem to confirm the fluidizing effect of cyclic FA. In fact, they counteracted the reduction of membrane fluidity due to the increase of CL and the decrease of UL in the cells exposed to hexenal and thyme EO. Moreover, they counteracted also the reduction of membrane fluidity due to the decrease of UL and increase of *trans* isomers in cells exposed to citral and carvacrol. In fact, unsaturated FA in the *cis* conformation do not pack as efficiently, due to their bent steric structure. *Trans* UFA with their long linear structure behave more like saturated FA that lie in a linear manner, taking up less volume and creating a more ordered membranes (Diefenbach et al., 1992). Moreover the increase of *trans* isomers in these conditions can contribute to the resistance to these specific chemical stresses (citral and carvacrol exposure), since *cis*/*trans* isomerization of double bonds analogously to cyclopropanation are both able to confer membrane chemical stability and protection against toxic molecules (including H+) (Härtig et al., 2005). Moreover, they share the immediacy of response (Grogan and Cronan, 1997;Cronan, 2002;Ku et al., 2007).

The free fatty acids profiles after the exposure to natural antimicrobials of *Escherichia coli* K12 MG1655 confirmed the effects observed on the membrane fatty acids profiles. In fact, allthe stress exposures induced in the released FA profiles of *E. coli* K12 an increase of relative percentages of

the cyclopropanic FAs (C17 cyc and C19 cyc), the short and medium chains FAs (C12:0, C14:0 and C16:0) and a reduction of the free C18 UFAs with consequent reduction of both the UL and CL values. As described for the total membrane fatty acids the concentrations of the free cyclopropanic FAs C17 cyc and C19 cyc raised in all condition tested.

Similar response patterns were observed for *L. monocytogenes* Scott A cells after the exposure to natural antimicrobials tested. The free fatty acids profiles of *L. monocytogenes* highlighted a reduction of the unsaturation levels mainly due to a significative reduction of both *cis*/*trans* of C18:1. Also the linoleic acid (C18:2 *cis*,*cis* 9-12) was significantly reduced except after the exposure to citral. The UFA level reduction of *L. monocytogenes* cells was also in this case associated with a significant increase of the C12:0, C14:0 and C16:0. The increase of these free FAs, associated with the reduction both membrane and free UFAs, is in agreement with a wide literature (Guerzoni et al., 2001;Patrignani et al., 2008;Gianotti et al., 2009;Montanari et al., 2010;Tabanelli et al., 2014;Siroli et al., 2015a). In fact, their increase is attributed to the oxylipin synthesis during stress. Exposure in many microorganisms. In fact, short and medium chain FAs, within their esters and some saturated and unsaturated aldehydes, lactones, alcohols and furanones, belong to the oxylipin family and are considered as UFA oxidation products (Vannini et al., 2007;Montanari et al., 2013;Siroli et al., 2015a). The UFA oxidation and the production of oxylipin are ubiquitous defense mechanisms against the radical species of the oxygen (ROS). However, as already underlined, all stress conditions 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 (ROS) in the cells (Dodd et al., 1997). Free radicals can react with UFAs and initiate a lipid peroxidation producing peroxy fatty acids and hydroxy aldehydes. These molecules decrease the membrane fluidity causing a significant disruption of the membrane bounded proteins (Cabiscol Català et al., 2000;Guerzoni et al., 2001). The enzymatic cleavage of peroxy fatty acids and their conversion into oxylipin counteract their negative effect of the cell membrane reducing also reactivity of ROS (Montanari et al., 2013).

Conclusions

In conclusion the findings of this work contribute to the comprehension of the membrane FAs modulation mechanisms of *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655 in relation to their exposure to sublethal concentrations of citral, carvacrol (E)-2-hexenal and thyme EO. However, a deeper investigation to clarify if the changes in released or associated membrane FAs are the consequences of or the trigger for stress-related gene expression is necessary. These information are necessary in order to avoid any kind of microbial resistance phenomena even if the natural antimicrobials are generally used at sublethal concentrations and in combination with other non-thermal preservative strategies. Consequently, the detailed knowledge of the action mechanisms of natural antimicrobials considered in relation to the others hurdles applied is mandatory for their implementation at industrial level as innovative preservation strategies. The implementation processes should be also related to the food matrices and production processes.

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Effects of (E)-2-Hexenal combined with High pressure Homogenization on apple juice safety and shelf-life

Introduction

Fruit juices are perceived by consumers as health-promoting foods due to their low sodium and lipid content as well as their high vitamin C, polyphenol and flavonoid concentration that contribute to their antioxidant properties (Patrignani et al., 2009). Unfortunately, these products are susceptible to alteration and have a limited shelf-life. Their spoilage is mainly determined by yeasts, responsible for fermented taste and carbon dioxide production, lactic acid bacteria, producing buttermilk off-flavours (diacetyl), and moulds that can contribute to the spoilage with their superficial growth (Tournas et al., 2006). Although fruit juices, due to their low pH, are generally considered as safe, the literature data have reported that unpasteurized ones, contaminated with food-borne pathogenic microorganisms like *Listeria monocytogenes* and *Escherichia coli* O157: H7, can be a vehicle of illness (Brinez et al., 2006; Briñez et al., 2006; Diels and Michiels, 2006; Patrignani et al., 2013).

In order to achieve the safety and the desired shelf life of fruit and vegetable juices, heat treatment is the most used method due to its efficacy on the microbial inactivation (Tribst et al., 2008). However, causing a depletion of the vitamin continent and the production of off-flavors, it has negative effects on the nutritional and organoleptic food properties (McDonald et al., 2000, Vachon et al., 2002; Pathanibul et al., 2009; Calligaris et al., 2012).

The increased demand for healthy fresh juices without synthetic antimicrobials has stimulated the researchers to find alternative strategies to heat treatment, able to ensure safety and adequate shelf-life without detrimental effects on the product nutritional, chemico-physical and organoleptic characteristics.

In this contest, essential oils (EOs) and their bioactive compounds represent an interesting option to synthetic antimicrobials. Most of them are generally recognized as safe (GRAS) (Newberne et al., 2000) and their antimicrobial activity is well known and documented in model and real systems (Burt, 2004; Bakkali et al., 2008; Hyldgaard et al., 2012; Patel, 2015; Hassoun and Çoban, 2017). The role of EOs and their constituents to improve the stability of drinks, fruit beverages, juices and ready-to-eat foods were previously reported in literature (Belletti et al., 2004; Ndagijimana et al.,

2004; Belletti et al., 2007; Belletti et al., 2008; Belletti et al., 2010; Patrignani et al., 2013; Patrignani et al., 2015; Siroli et al., 2015a; Siroli et al., 2015b).

Among natural antimicrobials, hexanal and (E)-2-hexenal are promising alternatives to synthetic antimicrobials for the fruit beverage safety. These six carbon atom unsaturated aldehydes characterized by fresh vegetable and fruit aroma have proved their antimicrobial activity both in model (Caccioni et al., 1997; Gardini et al., 1997) and real food systems such as salads (Lanciotti et al., 2003) Corbo et al., 2000 and fruit beverages (Belletti et al., 2008).

Although antimicrobials properties of essential oils and their bioactive compounds are well documented their application in food industries is limited due their high volatility and low sensory threshold that could cause alterations to the organoleptic properties of food matrixes. For these reasons several authors have suggested their application in combination with other non-thermal strategies to promote the microbial safety of foods.

The non-thermal alternative technologies proposed for preservation of fruit juices include pulsed electric fields (Evrendilek et al., 1999; Somolinos et al., 2010), high hydrostatic pressure (Houška et al., 2006; Somolinos et al., 2008) and high pressure homogenization (HPH) (Lacroix et al., 2005; Brinez et al., 2006; Briñez et al., 2006; Diels and Michiels, 2006; Kumar et al., 2009; Pathanibul et al., 2009; Patrignani et al., 2009; 2010; Patrignani and Lanciotti, 2016).

High Pressure Homogenization represents one of the most promising alternative to thermal treatments for food preservation. Different authors showed fruit juices can be considered as an interesting field of application of HPH in order to reduce the microbial cell load without loss of quality and freshness attributes (Patrignani et al., 2009; 2010; Donsì et al., 2011). In fact, different authors studied the ability of HPH to increase the safety and shelf-life of orange, carrot and apple juices (Lanciotti et al., 1994; Guerzoni et al., 1999; Kheadr et al., 2002; Wuytack et al., 2002; Vannini et al., 2004; Diels and Michiels, 2006; Bevilacqua et al., 2009; Patrignani et al., 2009; 2010; Patrignani et al., 2013; Ferragut et al., 2015). According to the literature data, HPH treatments also caused the inactivation of *E.coli* O157:H7 ATCC 35150, *E. coli* O58:H21 ATCC 10536, *E. coli* O157:H7 CCUG 44857, *Lactobacillus plantarum* ATCC 14917, *Leuconostoc mesenteroides* ATCC 23386 in deliberately inoculated in orange juices as well as of wild strains of *Saccharomyces cerevisiae* and *Penicillium* ssp. (Brinez et al., 2006; Briñez et al., 2006; Tahiri et al., 2006). As showed by Donsì et al., (2011) high pressure homogenization processes cause changes on different physical characteristics of the juices such as the average particle size of suspended solids and viscosity of the products. Moreover, functional compounds naturally present in fruits such as

flavonoids, as well as the attributes of freshness and texture are more preserved by homogenizing high pressure treatment than thermal treatment (Lacroix et al., 2005; Betoret et al., 2009). The literature data have also underlined that the inactivation potential of HPH can be increased when this technology is used in combination with other mild hurdles. For example, the HPH treatment, used in combination with a mild pre-warming treatment of 50 C for 10 min showed good potential to increase the shelf-life of orange juices and to reduce its opalescence (Lacroix et al., 2005). However, as previously reported, the consumers expectation are more focused on natural treatments alternative to thermal ones and able to maintain high juice functionalities. About this, some Authors have pointed out the combined use of HPH and natural antimicrobials, such as essential oils or their bioactive compounds, to increase safety and shelf life of fruit and veg juices (Bevilacqua et al., 2012; Patrignani et al., 2013).

In this perspective, this part of my research evaluated the effects hexanal (70 mg/L) and 2-(E)-hexenal (35 mg/L) in combination with repeated HPH treatments at 100 MPa and 200 MPa on different fruit juice spoilage agents and food-borne pathogens inoculated on apple centrifuged juices. The considered microorganisms were *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae*. The data obtained were compared with those obtained using mild thermal treatments (55 °C from 3.3 to 25 minutes), for juice stabilization, in presence of hexanal and 2-(E)-hexenal, at the same concentrations used for HPH and using the same food matrix and microorganisms. Moreover, the microbial recovery ability in the apple juices, in relation to the applied treatments, was evaluated during the juice storage in thermal abuse conditions at 10°C. Also, the effects of the adopted treatments on the apple juice organoleptic properties were investigated. In particular, pH and color changes and volatile molecular and organic acid profiles were evaluated over storage time.

Material and methods

Natural antimicrobials

For all the assessed conditions, the apple juices were prepared by the centrifugation of *Golden delicious* apples supplemented with 2 g/L of citric acid (antioxidant factor) and added of hexanal and (E)-2-hexenal at concentrations of 70 mg/L and 35mg/L, respectively. The obtained apple juice

was inoculated with the target microbial species with a final cell loads of about 4 log CFU/mL. Samples after the inoculation were immediately treated with HPH or heat treatments.

High pressure homogenization (HPH) treatments

The high pressure homogenization (HPH) treatments were achieved, using a continuous high pressure homogenizer PANDA (Gea, Parma, Italy), previously sanitized according to manufacturer suggestions. The inoculated batches were subjected to HPH treatments, performed at 100 MPa for 2 and 3 cycles and at 200 MPa for 3 cycles. The machine was supplied with a homogenizing PS type valve with a flow rate of 10 l/ h; the valve assembly includes a ball-type impact head made of ceramics, a stainless steel large inner diameter impact ring and a tungsten-carbide passage head. The inlet temperature of the treated apple juices was 4 °C and it increased of about 1.5 °C/ 10 MPa. After each pass at 100 MPa and 200 MPa, apple juices were cooled by using a thermal exchanger (Niro Soavi, Parma, Italy). The maximum temperature reached by the samples did not exceed 40 °C. As control samples, inoculated centrifuged apple juice were used subjected or not to the natural antimicrobials supplementation and HPH treatments.

Mild thermal treatments

Inoculated apple juice, supplemented or not with hexanal and (E)-2-hexenal, were also thermally treated in water bath at 55 °C from 3.3 to 25 minutes. In order to obtain a constant temperature of inactivation, 4 ml of inoculated apple juices were introduced in 5 mL sterile glass vials and its temperature was monitored using a thermocouple.

Total Viable Cell Count

The viable cell loads of the microorganisms used in these experiments, immediately after the application of the proposed treatments and during the product storage, were determined in duplicate by plate count sampling. Samples were serially diluted in glass tubes using, as dilution solution, physiological saline buffer (9 g/L NaCl). One hundred microliter of each dilution were spread or included in different selective culture media such as De Man, Rogosa and Sharpe agar (MRS) supplemented with cycloheximide (0.2% p/v) (Oxoid, Milano, italy) for *Lb. plantarum*; Violet

Reb Bile Agar supplemented with MUG (VRBA) (Oxoid, Milano, italy) for *E. coli;* Listeria Selective Agar (LSO) (Oxoid, Milano, italy) for *L. monocytogenes*; Braid Parker Agrar added with Egg yolk tellurite (50mL/L) (Oxoid, Milano, italy) for *St. aureus* and Yeast extract, Peptone and Dextrose Agar (YPD) (Oxoid, Milano, italy) for *S. cerevisiae.* The plates were then incubated at 37°C for 24-48 h for bacteria and at 30°C for 48 h for *S. cerevisiae.* Cell loads were monitored up to the product spoilage threshold was reached.

Physico-chemical analyses

The sample pH was measured in triplicate immediately after treatments and over the product storage by using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain).

GC/MS/SPME volatile molecule profiles

For each experimental condition, 5 mL of centrifuged apple juices were sterilely taken and placed in a 10-mL vial sealed with a PTFE/silicon septum. For 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., (2013) was used.

Organic acid analysis

The qualitative and quantitative amount of organic acids in the samples were evaluated by HPLC chromatography. Organic acids were extracted both from HPH and mild thermally treated samples, supplemented or not with antimicrobial compounds. From each condition tested, the extraction were performed on 5 mL of samples by adding 0.1N H_2SO_4 .Samples were subsequently vortexed for 1 minute, incubated at room temperature for 9 minutes, centrifuged at 4000 rpm for 10 minutes and filtered using 0.2 µm filter membranes. For the analysis and the HPLC-chromatographic conditions, the method reported by Tabanelli et al. (2016) was used.

Colour analysis

Colour was measured using a colour spectrophotometer mod. Colorflex (Hunterlab, USA). Colour was measured using the CIELab scale and Illuminant D65. The instrument was calibrated with a

white tile (L* 98.03, a* _0.23, b* 2.05) before the measurements. Results were expressed as L* (ranging from 0 to 100),a* and b*.

Results and discussion

In this work the effects of different high pressure homogenization (HPH) treatments at 100 MPa and 200 MPa and mild heat treatments were evaluated on the death kinetics and microbial growth ability of different food-borne pathogen and spoilage agents, deliberately inoculated in centrifuged apple juices as real food system. In my experiments, apple juice was considered a food model system due its high instability which lead to rapid a sediment formation and color changes.
In addition, the effects of the hyperbaric and heat treatments were also evaluated in presence of natural antimicrobials conveyed in ethanol. As described by different authors (Corbo et al., 2009; Patrignani et al., 2013), the combined application of HPH and natural antimicrobials represent a reliable alternative to traditional thermal treatments for promote the foods safety and shelf-life. In this framework, the effect HPH treatments in combination with sublethal concentration of hexanal and (E)-2-hexenal were evaluated on centrifuged apple juices inoculated with food-borne pathogens such as *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* and with the spoilage agents *Lactobacillus plantarum* and *Saccharomyces cerevisiae*. In order to understand the effects of hyperbaric treatment, antimicrobial substances and ethanol used to convey the substances into the system, three different apple juice batches were considered:

- centrifuged apple juice, inoculated with targeted microorganisms and treated at 0.1MPa, 100 MPa, 100 MPa x 2 cycles, 100 MPa x 3 cycles and 200 MPa x 2 cycles;
- centrifuged apple juice, inoculated with target microorganisms, added with ethanol (1% v/v) and treated at 0.1MPa, 100 MPa, 100 MPa x 2 cycles, 100 MPa x 3 cycles and 200 MPa x 2 cycles;
- centrifuged apple, inoculated with target microorganisms, supplemented with hexanal (70 ppm) and 2-(E)-hexenal (35 ppm) and treated at 0.1MPa, 100 MPa, 100 MPa x 2 cycles, 100 MPa x3 cycles and 200 MPa x 2 cycles;

Moreover, these data were compared with those obtained from inoculated juices treated by mild thermal treatments.

Evaluation of colour (brightness) of apple juice samples treated with high homogenization pressures (HPH) with or without natural antimicrobials supplementation

In Figure 1, the brightness values, determined during storage at 10 ° C of high-pressure homogenized apple juices, are reported. It is evident that, as the pressure increased, the brightness of the samples was enhanced. The highest effect was observed after the homogenization at 200 MPa x 2 cycles in presence of hexanal. On the other hands, the positive effect of the high homogenization pressures and natural antimicrobials on the maintenance of the colour of fruit-based products is well known (Lanciotti et al., 1999; Corbo et al., 2000; Suárez-Jacobo et al., 2012; Fernández-Sestelo et al., 2013). The increase of L* parameter can be attributed to the different properties of the reduced particles to reflect the light. Moreover, the reduction of the mean macromolecules of the system induced, in the treated samples, a delay in separation and sedimentation. Moreover, also the detoxifying molecules such as hexanol, deriving from the enzymatic reduction of the hexanal, is proved to have an inhibiting effect on the polyphenoloxidase of the considered matrix.

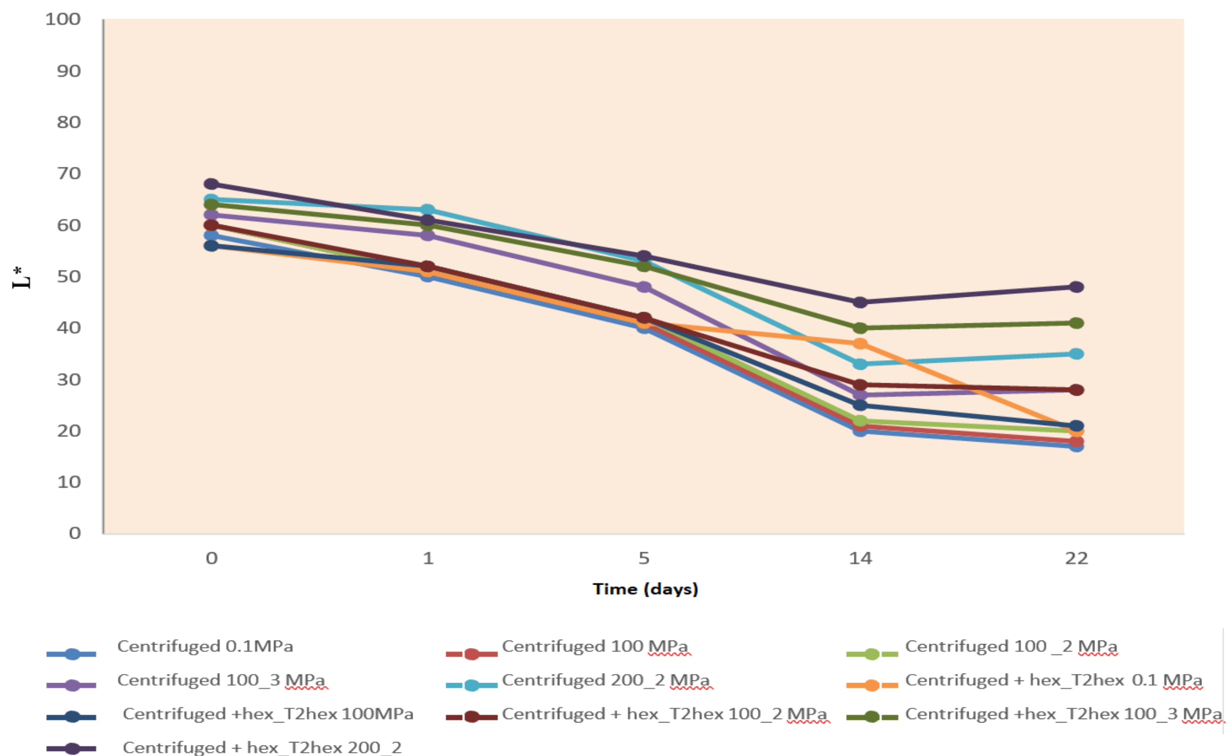


*Figure 1: Evolution of brightness (L *) in apple centrifuged samples in relation to hyperbaric treatment and presence of hexanal and (E)-2-hexenal.*

Effects of HPH and thermal treatments on *Listeria monocytogenes*, *Escherichia coli, Staphylococcus aureus, Saccharomyces cerevisiae* and *Lactobacillus plantarum* inoculated in apple juices with or without supplementation of natural antimicrobials.

Concerning the microbial inactivation obtained by high pressure homogenization treatments, the data showed that for all the microorganisms considered, with the exception of *Escherichia coli*, the effect of pressure was maximum when the juice were treated at 200 MPa for 2 repeated cycles. On the other hand, *E. coli* death kinetic was strongly influenced by the pH of the system (Table 1). The combined effect of pressure and natural antimicrobials appears to be very effective on *Listeria monocytogenes* (Figure 2*).* The *L. monocytogenes* cell load after the homogenization treatment at 100 MPa repeated 3 times, were below the detection limit (Table 2). Also on *Saccharomyces cerevisiae*, the combined effect of the two hurdles is evident (Figure 3 and Table 3). Although the yeasts resistance to homogenization treatments is well known, data from the literature indicate a good inactivation after a combined treatment with natural antimicrobials and high pressures (Patrignani et al., 2013). The highest efficacy of the combined treatment can be can be attributed to the increase in the vapor pressure of antimicrobial molecules during the hyperbaric treatment. As reported by Lerici et al. (1996), the rise of the vapor pressure increases the affinity of hexanal and (E)-2-hexenal with the lipidic cell membrane layers resulting in an augmented antimicrobial activity. In addition, the hyperbaric treatments provoked severe damage to the extracellular cell structure*. Lactobacillus plantarum* and *Staphylococcus aureus* (Figure 4-5) showed a higher resistance to the HPH treatments and natural antimicrobials. Gram-positive bacteria, due to their cellular structure and the presence of the thickest cell wall, are more resistant compared to the Gram-negative bacteria to high pressure homogenization. (Lanciotti et al., 2004). By contrast, the presence of lipopolysaccharides in the outer cell membrane increases their resistance to antimicrobials used in food industry (Helander et al., 1997). On the other hand, some natural antimicrobials, including diacetyl, hexanal and 2-(E)-hexanal, have shown an interesting activity also against Gram negative bacteria (Lanciotti et al., 2003; Patrignani et al., 2008). The effect of natural antimicrobials on target microorganisms is more evident during the storage of centrifuged apple samples in conditions of thermal abuse at 10 °C. For all bacteria tested, after 2 days of storage, the death kinetics, also influenced by the low pH of the system, were accelerated in the presence of hexanal and 2- (E) -hexenal (Table 2,4,5). *Saccharomyces cerevisiae*, characterized by a higher acid residence and osmo-tolerance, showed a reduced growth in samples homogenized at 100 MPa and 100 MPa repeated 2 times in presence of the

tested natural antimicrobials (Table 3). In samples treated with 100 MPa repeated 3 times and 200 MPa repeated 2 times, the *S. cerevisiae* cell loads were below the detection limit after 22 storage days at 10 °C (Table 3). The use of pressure, alone or in combination with ethanol, prevented the proliferation of Lactobacillus plantarum during storage at 10 °C over the period considered. The effects were more severe in presence of the natural antimicrobials. In presence of hexanal and 2- (E) -hexenal, after two day of storage, the *L. plantarum* cell loads were below the detention limits in the samples homogenized at 100 MPa for 3 times and 200 MPa repeated 2 times (Table 4).

Table 1: Death kinetics of Escherichia coli in relation to the pressure applied with or without the supplementation of natural antimicrobials.

Escherichia coli	
Treatments	**Cell loads after treatments (log CFU/mL)**
Centrifuged+m.o	
0.1 MPa	3.5 ±0.15
100 MPa	-*
100_(2) MPa	-
100_(3) MPa	-
200_(2) MPa	-
Centrifuged+m.o+ethanol	
0.1 MPa	3.5 ±0.25
100 MPa	-
100_(2) MPa	-
100_(3) MPa	-
200_(2) MPa	-
Centrifuged+m.o+ethanol+hex+T2_hex	
0.1 MPa	3.5 ±0.17
100 MPa	-
100_(2) MPa	-
100_(3) MPa	-
200_(2) MPa	-

- below the detection limit*

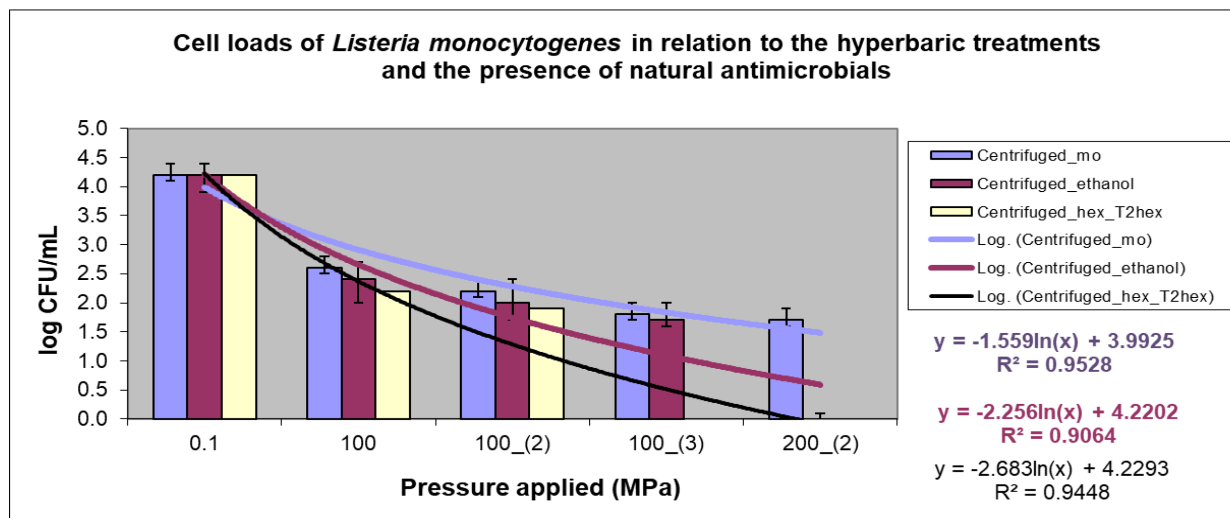


Figure 2: Death kinetics of Listeria monocytogenes in relation to the pressure applied with or without the supplementation of natural antimicrobials.

Centrifuged_mo: centrifuged apple juice inoculated with L. monocytogenes and treated with HPH.

Centrifuged_ethanol: centrifuged apple juice inoculated with L.monocytogens supplemented with ethanol (1% v/v) and treated with HPH.

Centrifuged_hex_T2hex: centrifuged apple juice inoculated with L.monocytogens supplemented with hexenal and (E)-2-hexenal (70-35 mg/L) and treated with HPH.

Table 2: Evolution of the total viable cell counts (log CFU/ mL) of Listeria monocytogenes inoculated (4 log CFU/mL) in apple centrifugated juices in relation to HPH treatments and the supplementation of natural antimicrobials.

Listeria monocytogenes	*Cell loads after HPH treatments (log CFU/mL)*						
		Days of storage at 10 °C after the HPH treatments					
Treatments	**Cell loads after treatments**	**1**	**2**	**5**	**8**	**12**	**14-22**
Centrifuged+m.o							
0.1 MPa	4.2 ±0.11	2.8 ±0.10	2.8 ±0.13	2.3 ±0.19	1.2 ±0.27	1.0 ±0.11	-*
100 MPa	2.6 ±0.18	1.0 ±0.15	1.0 ±0.09	1.0 ±0.14	1.0 ±0.14	1.0 ±0.16	-
100_(2) Mpa	2.2 ±0.14	1.0 ±0.25	1.0 ±0.17	1.0 ±0.19	1.0 ±0.15	-	-
100_(3) Mpa	1.8 ±0.22	1.2 ±0.10	1.1 ±0.08	1.0 ±0.20	1.0 ±0.15	-	-
200_(2) Mpa	1.7 ±0.21	1.3 ±0.12	1.3 ±0.14	1.0 ±0.25	1.0 ±0.10	-	-
Centrifuged+m.o+ethanol							
0.1 Mpa	4.2 ±0.24	2.7 ±0.07	2.7 ±0.22	2.0 ±0.23	1.0 ±0.15	0.9 ±0.13	-
100 MPa	2.4 ±0.18	2.0 ±0.09	2.0 ±0.15	2.0 ±0.18	0.9 ±0.06	0.9 ±0.16	-
100_(2) MPa	2.0 ±0.11	2.0 ±0.22	2.0 ±0.10	2.0 ±0.15	1.0 ±0.14	-	-
100_(3) MPa	1.9 ±0.07	1.7 ±0.12	1.7 ±0.12	1.1 ±0.15	-	-	-
200_(2) MPa	-	-	-	-	-	-	-
Centrifuged+m.o+ethanol+hex+T2_hex							
0.1 MPa	4.2 ±0.10	1.9 ±0.17	1.9 ±0.29	-	-	-	-
100 MPa	2.2 ±0.12	-	-	-	-	-	-
100_(2) MPa	1.9 ±0.16	-	-	-	-	-	-
100_(3) MPa	-	-	-	-	-	-	-
200_(2) MPa	-	-	-	-	-	-	-

- below the detection limit*

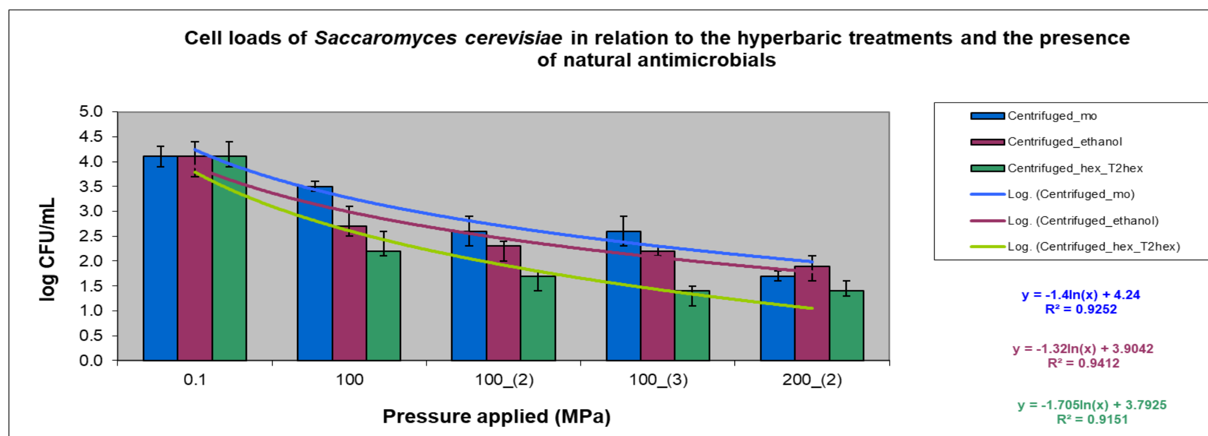


Figure 3: Death kinetics of Saccharomyces cerevisiae in relation to the pressure applied with or without the supplementation of natural antimicrobials.

Centrifuged_mo: centrifuged apple juice inoculated with S. cerevisiae and treated with HPH.

Centrifuged_ethanol: centrifuged apple juice inoculated with S. cerevisiae supplemented with ethanol (1% v/v) and treated with HPH.

Centrifuged_hex_T2hex: centrifuged apple juice inoculated with S. cerevisiae supplemented with hexenal and (E)-2-hexenal (70-35 mg/L) and treated with HPH.

Table 3: Evolution of the total viable cell counts (log CFU/ mL) of Saccharomyces cerevisiae inoculated (4 log CFU/mL) in apple centrifugated juices in relation to HPH treatments and the supplementation of natural antimicrobials.

Saccharomyces cerevisiae	*Cell loads after HPH treatments (log CFU/mL)*					
		Days of storage at 10 °C after the HPH treatments				
Treatments	**Cell loads after treatments**	**1**	**6**	**12**	**14**	**22**
Centrifuged+m.o						
0.1 MPa	4.1 ±0.11	3.6 ±0.21	5.2 ±0.17	5.2 ±0.09	6.6 ±0.14	-[a]
100 MPa	3.5 ±0.21	3.7 ±0.16	4.8 ±0.13	4.8 ±0.12	6.8 ±0.11	-[a]
100_(2) MPa	2.6 ±0.12	3.0 ±0.11	±0.14	4.4 ±0.16	6.7 ±0.19	-[a]
100_(3) MPa	2.6 ±0.22	2.9 ±0.26	4.3 ±0.15	4.3 ±0.17	6.7 ±0.10	-[a]
200_(2) MPa	1.7 ±0.09	2.0 ±0.25	4.5 ±0.22	0 ±0.21	6.7 ±0.22	-[a]
Centrifuged+m.o+ethanol						
0.1 MPa	4.1 ±0.09	4.2 ±0.11	5.0 ±0.14	5.3 ±0.16	6.7 0.08	-[a]
100 MPa	2.7 ± 0.12	4.1 ±0.23	4.0 ±0.12	4.0±0.27	5.9 ±0.11	6.5±0.09
100_(2) MPa	2.3 ± 0.15	3.3 ± 0.16	4.0 ± 0.18	4.0 ± 0.12	5.9 ± 0.10	6.8 ± 0.11
100_(3) MPa	2.2 ± 0.13	2.0 ± 0.17	3.9 ± 0.15	3.9±0.09	4.3 ± 0.19	6.1 ± 0.12
200_(2) MPa	1.9 ± 0.20	2.0 ± 0.25	3.0 ± 0.22	3.0 ± 0.17	4.1 ± 0.21	6.2 ± 0.23
Centrifuged+m.o+ethanol+hex+T2_hex						
0.1 MPa	4.1 ± 0.15	3.9 ± 0.10	2.0 ± 0.17	2.0± 0.15	1.5 ± 0.18	1.4 ± 0.17
100 MPa	2.2 ± 0.11	3.6± 0.15	-[b]	-[b]	-[b]	-[b]
100_(2) MPa	1.7± 0.15	3.4 ± 0.12	-[b]	-[b]	-[b]	-[b]
100_(3) MPa	1.4 ± 0.10	3.4± 0.15	-[b]	-[b]	-[b]	-[b]
200_(2) MPa	1.4 ± 0.09	2.5 ± 0.22	-[b]	-[b]	-[b]	-[b]

-[a]Spoiling threshold reached

-[b]Below the detenction limit

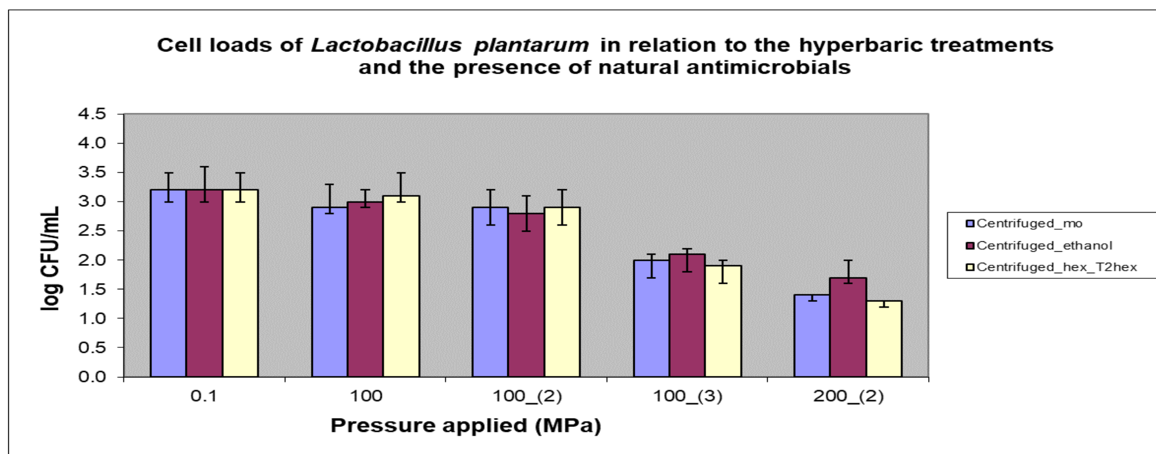


Figure 4: Death kinetics of Lactobacillus plantarum in relation to the pressure applied with or without the supplementation of natural antimicrobials.

Centrifuged_mo: centrifuged apple juice inoculated with Lb. plantarum and treated with HPH.

Centrifuged_ethanol: centrifuged apple juice inoculated with Lb. plantarum supplemented with ethanol (1% v/v) and treated with HPH.

Centrifuged_hex_T2hex: centrifuged apple juice inoculated with Lb. plantarum supplemented with hexenal and (E)-2-hexenal (70-35 mg/L) and treated with HPH.

Table 4: Evolution of the total viable cell counts (log CFU/ mL) of Lactobacillus plantarum inoculated (4 log CFU/mL) in apple centrifugated juices in relation to HPH treatments and the supplementation of natural antimicrobials.

Lactobacillus plantarum	*Cell loads after HPH treatments (log CFU/mL)*					
		Days of storage at 10 °C after the HPH treatments				
Treatments	**Cell loads after treatments**	**2**	**5**	**8**	**14**	**19**
Centrifuged+m.o						
0.1 MPa	3.2 ±0.18	2.0 ±0.29	2.0 ±0.18	2.1 ±0.21	2.3 ±0.24	-*
100 MPa	2.9 ±0.10	1.3 ±0.09	1.7 ±0.12	2.0 ±0.14	1.9 ±0.15	-
100_(2) MPa	2.9 ±0.21	2.0 ±0.22	1.3 ±0.15	1.8 ±0.19	1.9 ±0.18	-
100_(3) MPa	2.0 ±0.13	1.5 ±0.15	1.3 ±0.17	1.8 ±0.11	1.9 ± 0.15	-
200_(2) MPa	1.4 ±0.18	-	-	0.7 ±0.12	1.3 ±0.13	-
Centrifuged+m.o+ethanol						
0.1 MPa	3.2 ±0.24	1.9 ±0.19	1.9 ±0.21	2.3 ±0.22	2.6 ±18	-
100 MPa	3.0 ±0.20	1.9 ±0.17	1.4 ±0.28	2.0 ±0.29	1.9 ± 0.15	1.1 ±0.13
100_(2) MPa	2.8 ±0.14	1.2 ±0.11	1.1 ±0.17	2.0 ±0.18	2.1 ±0.19	0.9 ±0.16
100_(3) MPa	2.1 ±0.15	1.1 ±0.16	1.0 ±0.15	2.0 ±0.13	1.9 ±0.12	-
200_(2) MPa	1.7 ±0.09	-	-	1.6 ±0.17	2.2 ±0.10	-
Centrifuged+m.o+ethanol+hex+T2_hex						
0.1 MPa	3.2 ±0.10	1.8 ±0.17	1.4 ±0.13	1.5 ±0.19	0.9 ±0.11	-
100 MPa	3.1 ±0.08	1.8 ±0.16	1.0 ±0.15	1.5 ±0.21	0.8 ±0.20	-
100_(2) MPa	2.9 ±0.21	0.9 ±0.14	1.0 ±0.19	1.4 ±0.17	0.9 ±0.13	-
100_(3) MPa	1.9 ±0.17	-	-	-	-	-
200_(2) MPa	1.3 ±0.16	-	-	-	-	-

- below the detection limit*

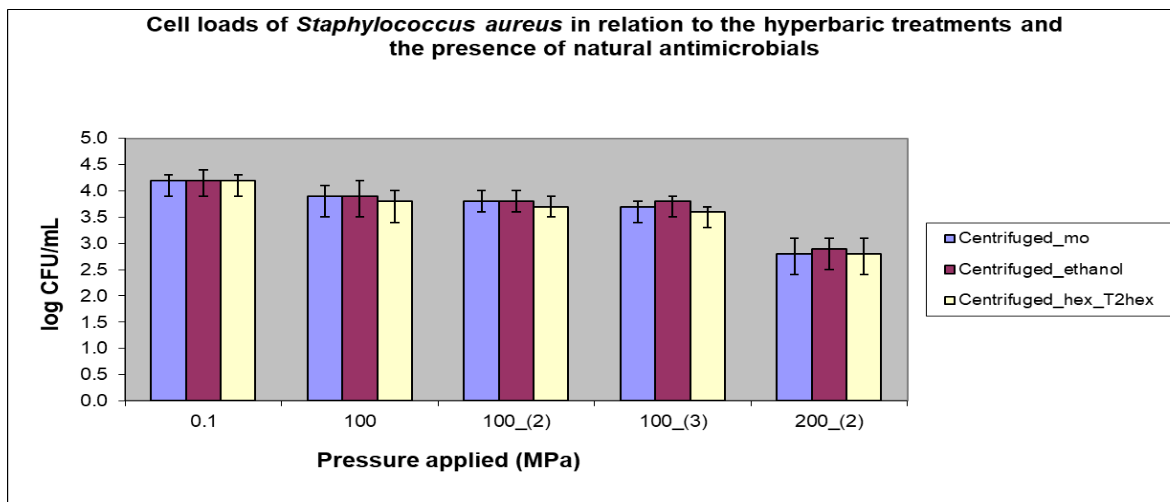


Figure 5: Death kinetics of Staphylococcus aureus in relation to the pressure applied with or without the supplementation of natural antimicrobials

Centrifuged_mo: centrifuged apple juice inoculated with S. aureus and treated with HPH.

Centrifuged_ethanol: centrifuged apple juice inoculated with S. aureus supplemented with ethanol (1% v/v) and treated with HPH.

Centrifuged_hex_T2hex: centrifuged apple juice inoculated with S. aureus supplemented with hexenal and (E)-2-hexenal (70-35 mg/L) and treated with HPH.

Table 5: Evolution of the total viable cell counts (log CFU/ mL) of Staphylococcus aureus inoculated (4 log CFU/mL) in apple centrifugated juices in relation to HPH treatments and the supplementation of natural antimicrobials.

Staphylococcus aureus	*Cell loads after HPH treatments (log CFU/mL)*					
		Days of storage at 10 °C after the HPH treatments				
Treatments	**Cell loads after treatments**	**1**	**2**	**5**	**8**	**12 22**
Centrifuged+m.o						
0.1 MPa	4.2 ±0.12	3.5 ±0.16	3.5 ±0.15	2.5 ±0.14	1.6 ±0.15	-*
100 MPa	3.9 ±0.15	3.3 ±0.16	3.4 ±0.17	2.4 ±0.18	1.7 ±0.12	-
100_(2) MPa	3.8 ±0.19	3.2 ±0.13	3.0 ±0.14	2.3 ±0.21	1.5 ±0.11	-
100_(3) MPa	3.7 ±0.14	3.0 ±0.16	2.9 ±0.12	2.4 ±0.21	1.5 ±0.13	-
200_(2) MPa	2.8 ±0.10	2.3 ±0.11	2.2 ±0.10	1.8 ±0.15	1.0 ±0.16	-
Centrifuged+m.o+ethanol						
0.1 MPa	4.2 ±0.09	3.6 ±0.18	3.6 ±0.12	2.7 ±0.13	1.9 ±0.17	-
100 MPa	3.9 ±0.22	3.5 ±0.17	3.5 ±0.25	2.7 ±0.23	1.7 ±0.19	-
100_(2) MPa	3.8 ±0.23	3.7 ±0.10	3.4 ±0.11	2.4 ±0.15	1.8 ±0.16	-
100_(3) MPa	3.8 ±0.13	3.4 ±0.12	3.4 ±0.18	2.5 ±0.19	1.7 ±0.26	-
200_(2) MPa	2.9 ±0.18	2.2 ±0.20	1.8 ±0.24	1.0 ±0.17	1.0 ±0.12	-
Centrifuged+m.o+ethanol+hex+T2_hex						
0.1 MPa	4.2 ±0.18	3.3 ±0.16	3.0 ±0.27	1.5 ±00.28	-	-
100 MPa	3.8 ±0.12	3.1 ±0.11	2.6 ±0.21	1.0 ±0.09	-	-
100_(2) MPa	3.7 ±0.08	3.1 ±0.15	2.6 ±0.16	1.0 ±0.12	-	-
100_(3) MPa	3.6 ±0.13	3.0 ±0.12	2.4 ±0.11	-	-	-
200_(2) MPa	2.8 ±0.16	2.3 ±0.15	1.1 ±0.29	-	-	-

- below the detection limit*

Concerning the conventional thermal treatments, all the tested bacterial pathogenic species were extremely sensitive to the thermal treatments adopted described as instantaneous inactivation (Table 6), with the exception of *S. aureus*. On the other hands, the thermal resistance of this microorganism is well known, especially when heat treatments occur at low temperatures and the temperature rises slowly, in 1-2 min. In these conditions, heat stress response may occur resulting in the expression of heat shock proteins. However, the resistance of *S. aureus* to higher thermal treatment was also highlighted by Montanari et al., (2015) who found similar behaviours for some *S. aureus* strains heat treated at 80°C for 20 min. In our work, after 48 hours from the treatments and during the storage at 10 °C, all the bacteria considered decreases below the detention limits (Table 6). *Saccharomyces cerevisiae* SPA showed the highest resistance to the thermal inactivation (Table 7). Its thermal resistance is already documented in the literature (Belletti et al., 2008). The instant deactivation after the thermal treatments at 55 ° C for 200 seconds, in presence of natural antimicrobials or ethanol, were comparable with those obtained by heat treatments at 55 °C for longer times without antimicrobials. (Table 7). On the other hand, the increase in temperature resulted in a significant increase of the hexanal and (E)-2-hexenal vapor pressure and consequently in an increase of their toxicity. Also the *S. cerevisiae* recovery during the storage was significantly influenced by the added natural antimicrobials. In fact, with the exception of the samples characterized by the presence of hexanal and (E)-2-hexenal and/or treated for 200 and 360 seconds (6 minutes) at 55 ° C respectively, between 5th and 12th days of storage, spoilage processes were observed (Table 7).

Table 6: total viable cell counts (log CFU/ mL) of inoculated pathogenic bacteria (4 log CFU/mL) in apple centrifugated juices in relation to thermal treatments and the supplementation of natural antimicrobials.

Samples	Thermal treatment	*Cell loads (CFU/mL)*			
		L. monocytogenes	*E. coli*	*Lb. plantarum*	*S. aureus*
Centrifuged+m.o	12 min - 55 °C	-*	-	-	-
Centrifuged+m.o+ethanol	200 sec - 55 °C	1.40 ±0.21	0.40 ±0.18	1.00 ±0.22	2.00 ±0.13
Centrifuged+m.o+ethanol+hex+T2_hex	200 sec - 55 °C	1.20 ±0.13		0.80 ±0.11	1.60 ±0.06
Centrifuged+m.o	25 min - 55 °C	-	-	-	-
Centrifuged+m.o+ethanol+hex+T2_hex	6 min - 55 °C	1.00 ±0.16	-	-	1.40 ±0.10
Centrifuged+m.o+ethanol	6 min - 55 °C	1.00 ±0.08	-	0.40 ±0.11	1.80 ±0.23

** below the detection limit*

Table 7: Evolution of total viable cell counts (log CFU/ mL) of Saccharomyces cerevisiae inoculated at 4 log CFU/mL in apple centrifuged juices in relation to thermal treatments and the presence of natural antimicrobials.

Samples	Thermal treatment	Cell loads log CFU/mL				
				Days of storage at 10 °C after the thermal treatments		
		Initial cell loads	Cell loads after treatment	5	12	15
Centrifuged+m.o	12 min - 55 °C	5.50 ±0.12	3.90 ±0.14	5.40 ±0.12	7.60 ±0.13	-*
Centrifuged+m.o+ethanol	200 sec - 55 °C	5.50 ±0.25	4.30 ±0.09	5.20 ±0.11	7.40 ±0.15	-
Centrifuged+m.o+ethanol+hex+T2_hex	200 sec - 55 °C	5.40 ±0.14	4.20 ±0.19	3.50 ±0.12	3.50 ±0.23	3.9 ±0.16
Centrifuged+m.o	25 min - 55 °C	5.40 ±0.21	3.50 ±0.06	4.70 ±0.16	7.40 ±0.13	-
Centrifuged+m.o+ethanol+hex+T2_hex	6 min - 55 °C	5.50 ±0.12	4.20 ±0.13	3.10 ±0.10	3.70 ±0.14	4.2 ±0.18
Centrifuged+m.o+ethanol	6 min - 55 °C	5.50 ±0.17	4.50 ±0.11	5.00 ±0.15	7.50 ±0.10	-

- Spoiling threshold reached*

Volatile molecule profile changes on centrifuged apple juices in relation to the inoculated microbial strain, the adopted inactivation treatment and the supplementation of natural antimicrobials

Reached the *Saccharomyces cerevisiae* SPA spoilage threshold (6 log CFU/mL), the samples were analyzed by GC/MS-SPME, in order to underline potential spoilage markers in relation to the adopted treatment. The analysis allowed to detect 70 different molecules belonging to different chemical classes: alcohols, aldehydes, ketons, acids and esters. Regarding the HPH treatments, the principal component analysis (PCA) of the volatilome underlined a clear clusterization of the samples on the basis of the performed HPH treatments and the added natural antimicrobials (Figure 6). The projection of the different samples on the factorial plan, defined by the Principal Component 1 and 2 (PC1 and PC2), allowed to explain 50% of the variance observed and highlighted a distribution of the apple centrifuged samples in relation to the initial addition of hexanal and (E)-2-hexenal. Except for the untreated control samples inoculated with the target microorganisms, others samples clearly forms two defined clusters among the PC1 (Figure 6). The cluster 1included samples only homogenized with or without ethanol supplementation, while the cluster 2 represented samples treated with HPH in presence of hexanal or (E)-2-hexenal. These data are not surprising as the volatile profiles of the untreated control sample were due to the earlier development of spoilage inoculated microorganisms.

The projection of the variables on the factor plane for the first two factors showed how the highest production of acetic acid (Figure 7b) and ethyl acetate (Figure 11 a and b) were associated to the untreated samples. By contrast, the different applied treatments induced a significant variation on the samples volatile molecular profiles. The HPH treatments in combination with

natural antimicrobials, reducing the microbial growth, delayed the accumulation of the volatile molecules associated with the microbial metabolisms. In addition, the homogenization processes, modifying the microstructure of the system, also modified the retention of volatile molecules and consequently their release into the head space. Finally, the addition of hexanal and 2-(E)-hexenal changed the system further. Although these molecules are, from an organoleptic point of view, compatible with apple juice, they significant affected the sensorial properties of samples and also they induced a specific response from the spoilage microorganisms and vegetable tissue with a strong effects on the volatiloma. As described for the samples subjected to hyperbaric treatments, the volatile molecular profiles of mild thermal treated samples were mainly influenced by the type of the inoculated microbial strains and by the added natural antimicrobials. Indeed, the addition of hexanal and (E)-2-hexenal underlined a clear clusteraization, as described by the principal component analysis (PCA), of the volatilome profiles. Samples added with natural antimicrobials, and characterized by a significant inhibition of the microbial growth, formed a well-defined and separated cluster along the PC1 component that explains 38% of the variance. On the other hand, the other samples (only thermal treated) were distributed in two separate clusters along PC2 which explains 26% of the variance, on the base of their faster spoilage kinetics and the severity of the thermal treatment (Figure 12).

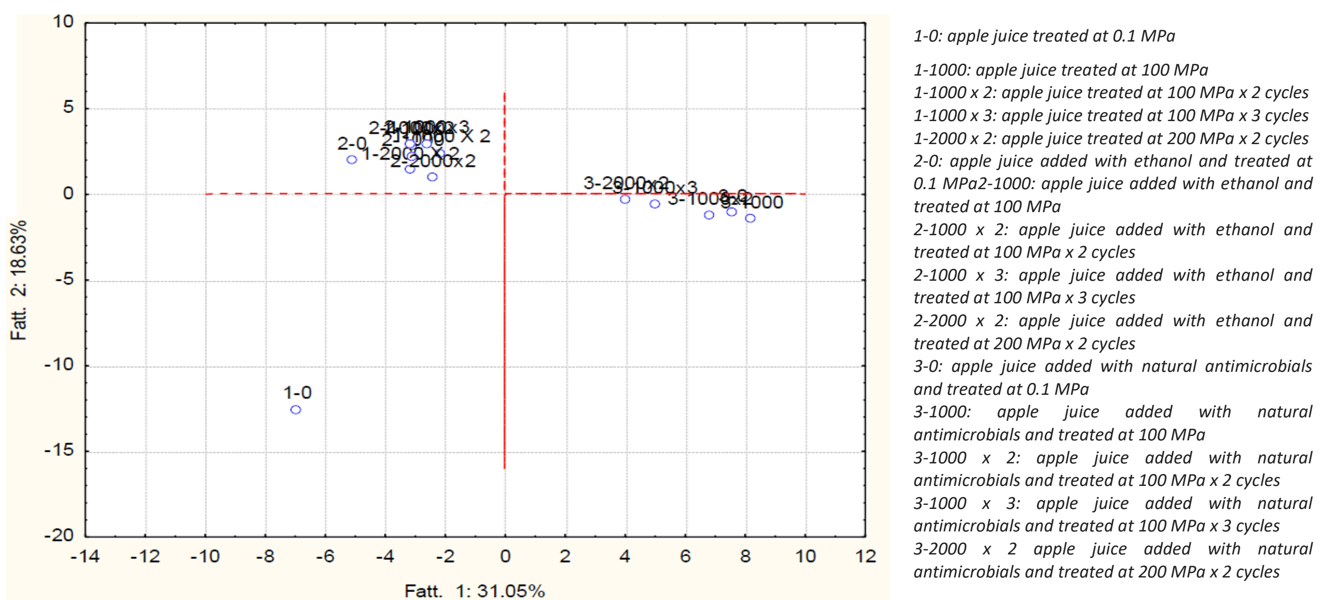
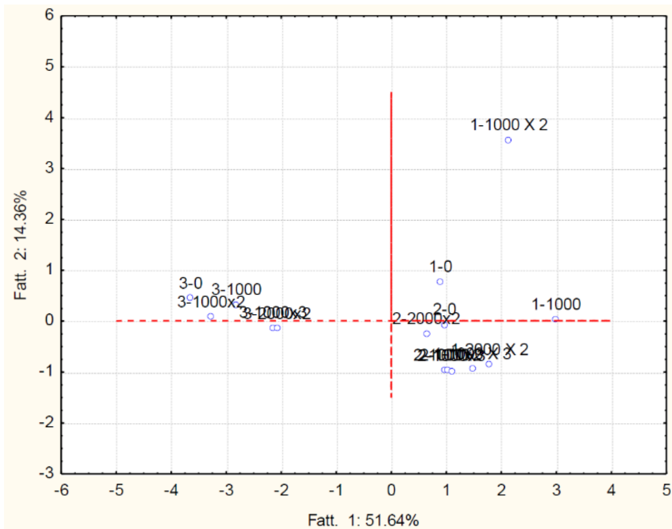
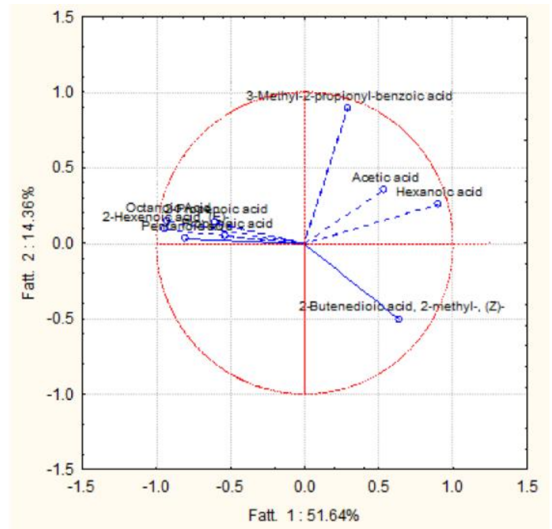


Figure 6: Principal component analysis loading plot of the volatile molecular profiles of centrifuged apple juices in relation to the microbial strain inoculated, HPH treatment with or without natural antimicrobials supplementation.

a **b**

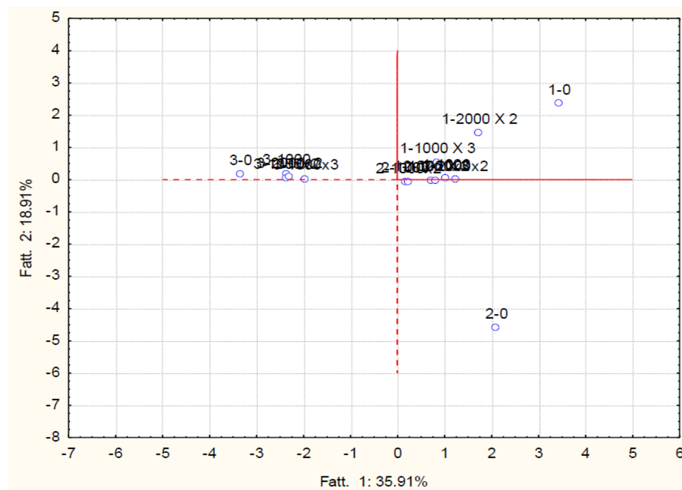


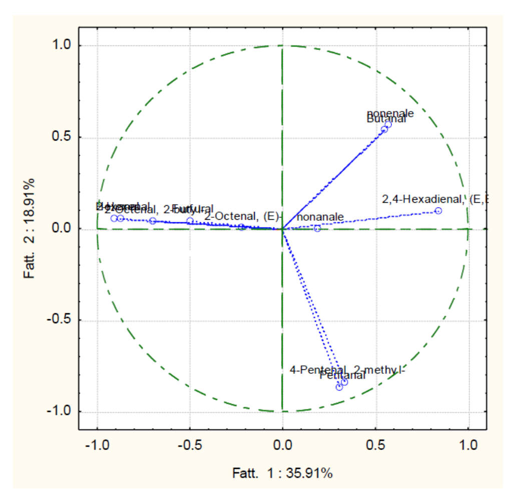


*see figure 6 for the caption

Figure 7 a and b: Principal component analysis loading plot of the organic acids profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated, HPH treatment with or without natural antimicrobials supplementation.

a **b**

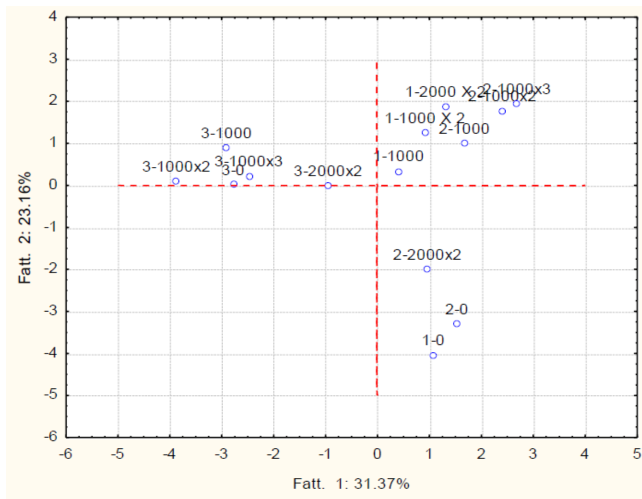


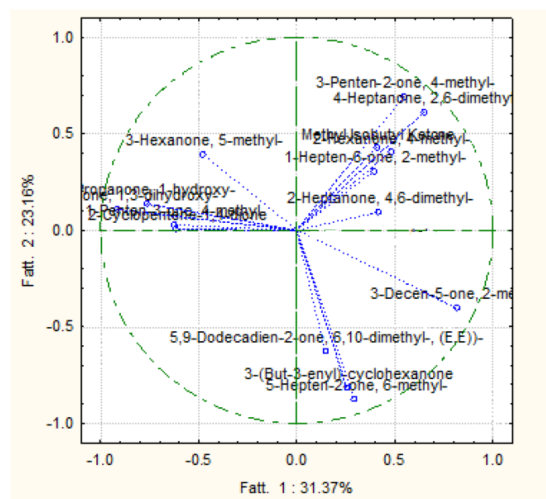


*see figure 6 for the caption

Figure 8 a and b: Principal component analysis loading plot of the aldehydes profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated, HPH treatment with or without natural antimicrobials supplementation.

a **b**

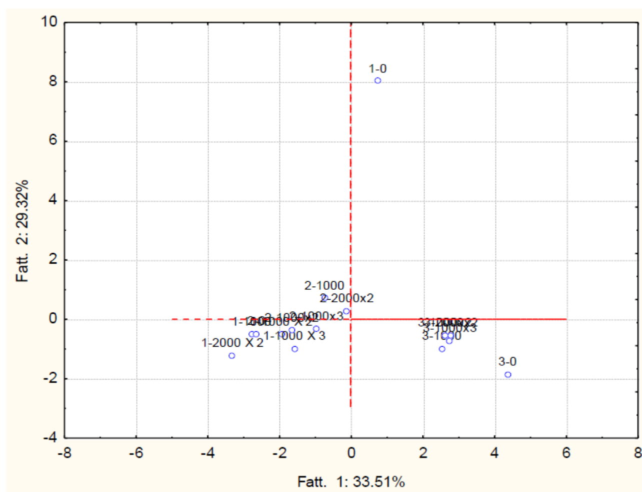


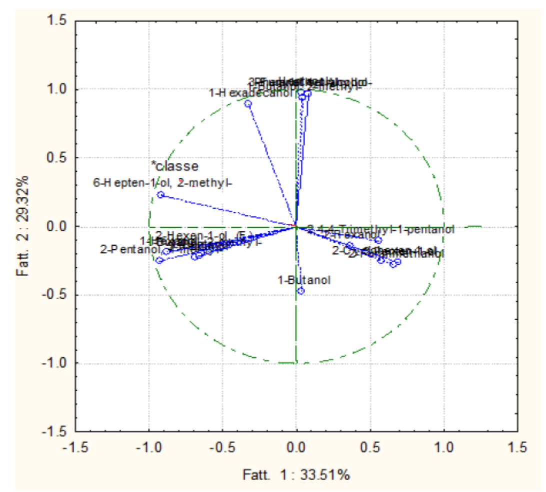


*see figure 6 for the caption

Figure 9 a and b: Principal component analysis loading plot of the ketons profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated, HPH treatment with or without natural antimicrobials supplementation.

a **b**

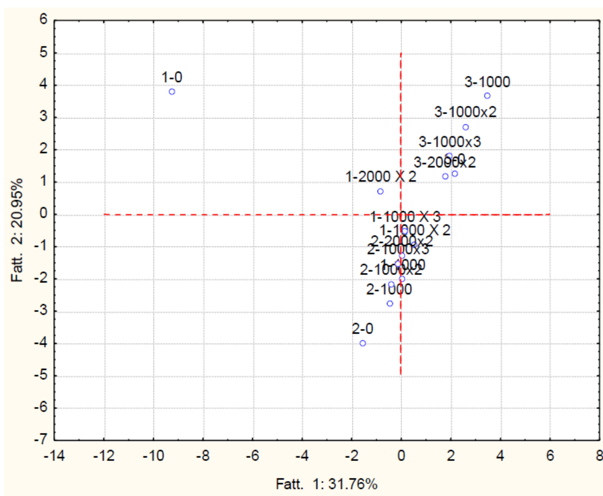




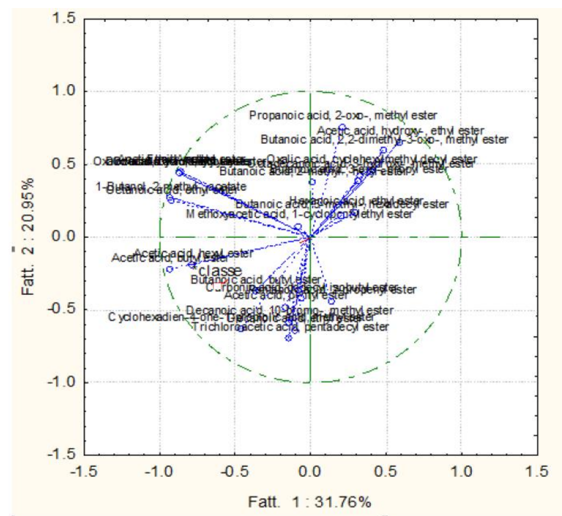
*see figure 6 for the caption

Figure 10 a and b: Principal component analysis loading plot of the alcohols profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated, HPH treatment with or without natural antimicrobials supplementation.

a

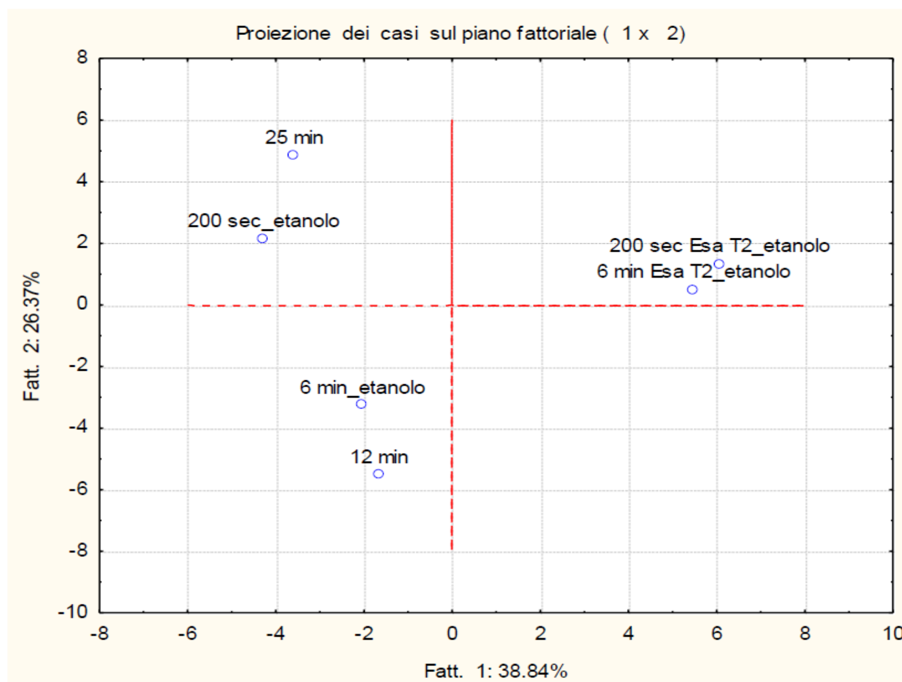


b



*see figure 6 for the caption

Figure 11 a and b: Principal component analysis loading plot of the esters profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated, HPH treatment with or without natural antimicrobials supplementation.



-12 min: centrifuged apple juice thermally treated at 55 ° C for 12 min.

- 25 min: centrifuged apple juice thermally treated at 55 ° C for 25 min.

- 6 min etanolo: apple centrifuged juice added with ethanol and thermally treated at 55 ° C for 6 min.

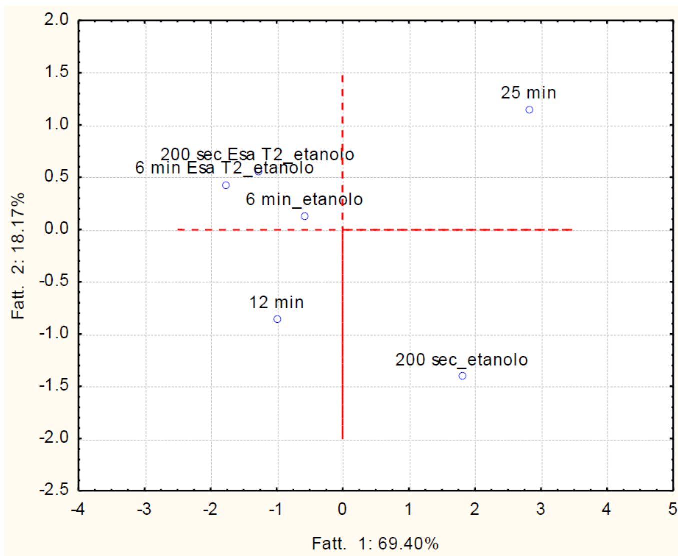
- 200 sec etanolo: apple centrifuged juice added with ethanol and thermally treated at 55 ° C for 200 sec.

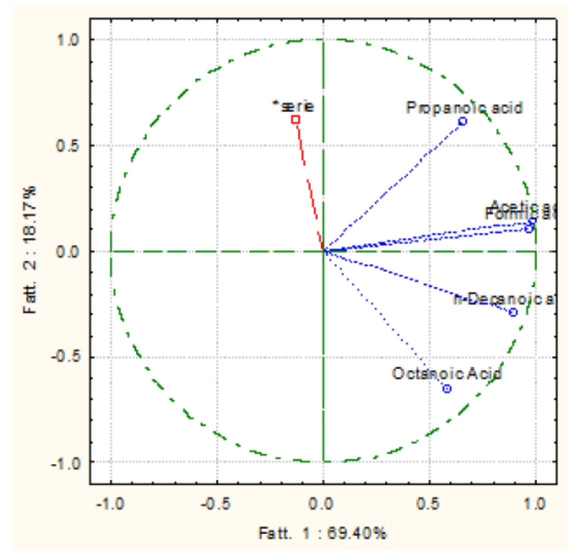
- 200 sec etanolo Esa _T2 Ese: apple centrifuged juice added with ethanol and natural antimicrobials and thermally treated at 55 ° C for 200 sec.

- 6 min etanolo _Esa _T2 Ese: apple centrifuged juice added with ethanol and natural antimicrobials and thermally treated at 55 ° C for 6 min.

Figure 12: Principal component analysis loading plot of the volatile molecular profiles of centrifuged apple juices in relation to the microbial strain inoculated and mild heat treatments with or without natural antimicrobials supplementation.

a **b**

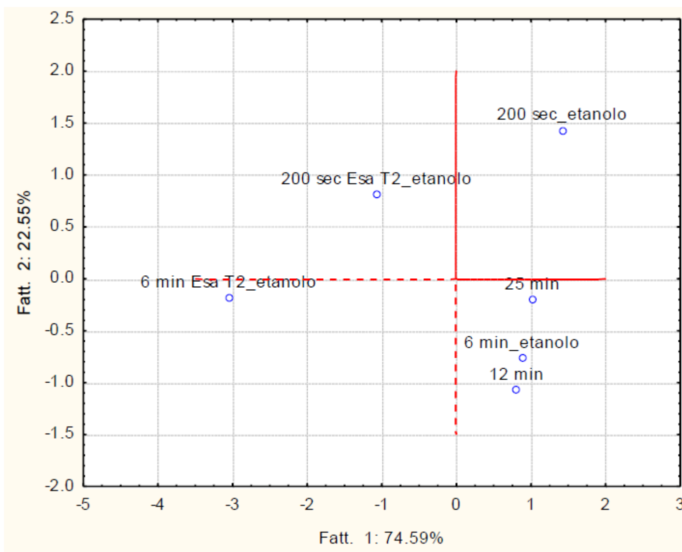


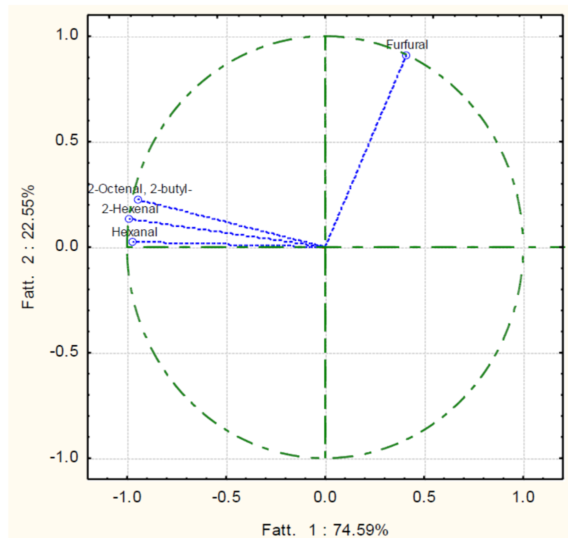


*see figure 12 for the caption

Figure 13 a and b: Principal component analysis loading plot of the organic acids profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated and mild heat treatments with or without natural antimicrobials supplementation.

a **b**

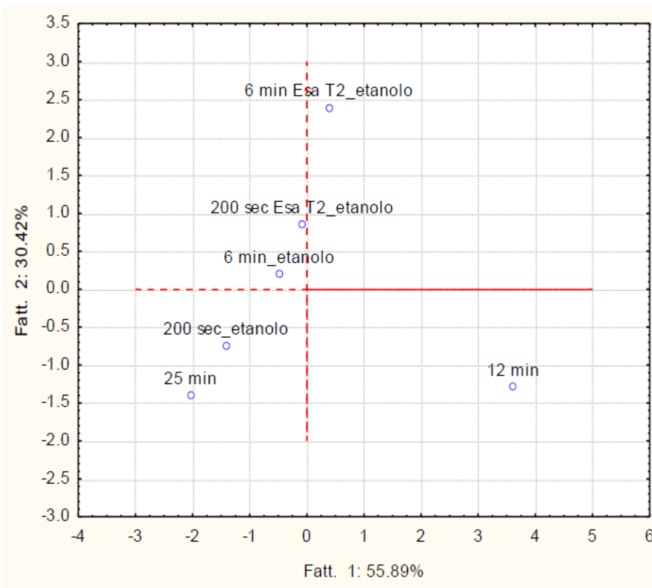


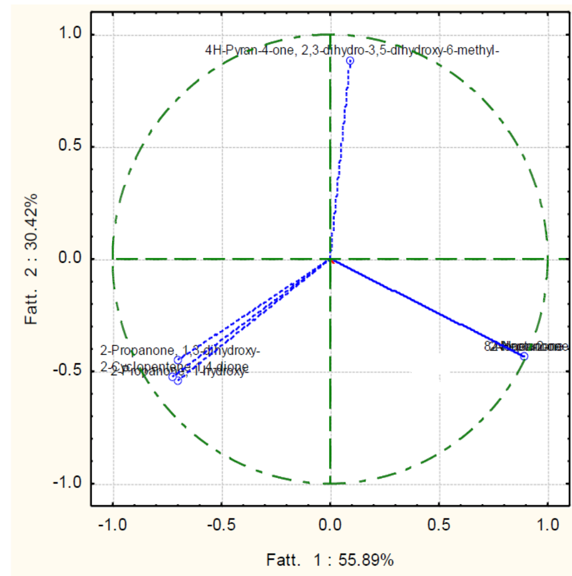


*see figure 12 for the caption

Figure 14 a and b: Principal component analysis loading plot of the aldehydes profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated and mild heat treatments with or without natural antimicrobials supplementation.

a **b**

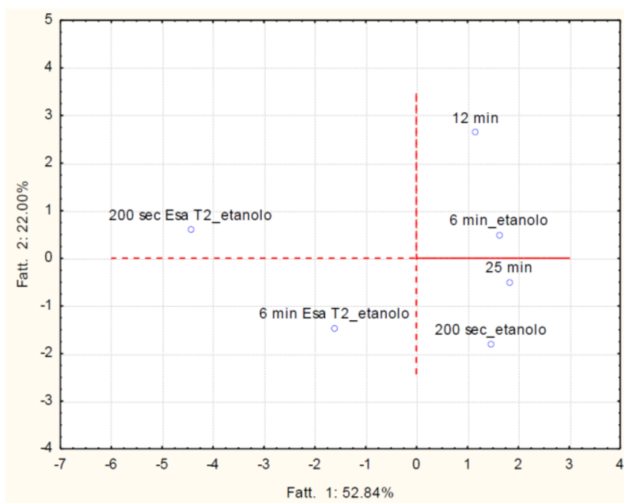


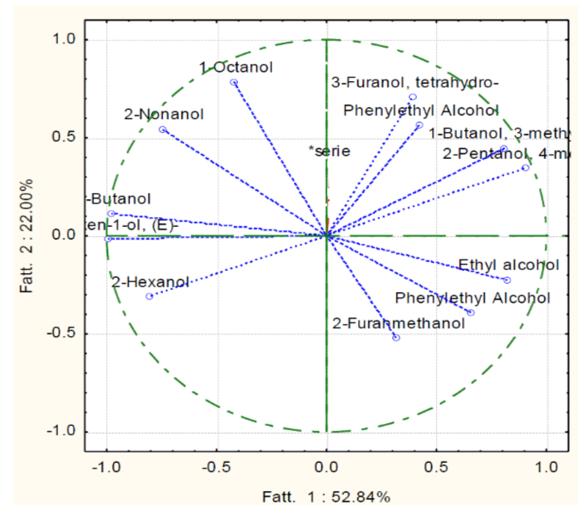


see figure 12 for the caption

Figure 15 a and b: Principal component analysis loading plot of the ketons profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated and mild heat treatments with or without natural antimicrobials supplementation.

a **b**





see figure 12 for the caption

Figure 16 a and b: Principal component analysis loading plot of the alcohols profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated and mild heat treatments with or without natural antimicrobials supplementation.

a

b

*see figure 12 for the caption

Figure 17 a and b: Principal component analysis loading plot of the ester profiles (SPME) of centrifuged apple juices in relation to the microbial strain inoculated and mild heat treatments with or without natural antimicrobials supplementation.

Effect of HPH, thermal treatments and natural antimicrobials on the content of organic acids in centrifuged apple juices.

Table 8: Juice organic acid concentration (mg/L) in relation to the applied treatment (hyperbaric or thermal) and the presence of natural antimicrobials.

Treatments	Citric acid	Malic acid	Ossalic acid	Ascorbic acid
	mg/L	mg/L	mg/L	mg/L
Centrifuged 0.1 MPa+m.o	1090.50	5189.70	208.89	8.00
Centrifuged 100 MPa+m.o	822.13	4588.28	204.80	15.04
Centrifuged 200MPa_2+m.o	887.38	4578.65	203.27	14.34
Centrifuged 0.1 MPa+m.o + hex+T2hex	1073.34	5253.85	202.98	0.51
Centrifuged 100 MPa+m.o + hex+T2hex	1122.42	5161.81	195.31	0.44
Centrifuged 200MPa_2+m.o +hex+T2hex	1392.31	5526.58	190.96	0.50
Centrifuged+m.o 12 min - 55 °C	1370.91	6232.45	184.34	0.29
Centrifuged+m.o+ethanol 200 sec - 55 °C	1286.9	5986.82	194.97	-*
Centrifuged+m.o+ethanol+hex+T2_hex 200 sec - 55 °C	1186.96	6351.96	203.97	-
Centrifuged+m.o 25 min - 55 °C	1224.3	5782.35	258.05	-
Centrifuged+m.o+ethanol+hex+T2_hex 6 min - 55 °C	1398.9	6367.14	224.1	-
Centrifuged+m.o+ethanol 6 min - 55 °C	1258.34	6189.31	220.45	-

-* *below the detection limit*

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%.

As highlighted in Table 8, the citric and malic acid concentrations decreased only in the samples inoculated with both spoilage and pathogenic microorganisms and only treated with HPH. These samples were characterized by faster microbial development and subsequently they quickly reached the spoilage threshold. On the other hand, the citric and the malic acids are characterized by a low microbiological stability due the metabolic activity of many spoilage agents such as yeast and lactic acid bacteria. However, in high-pressure treated samples, compared to the untreated control, the ascorbic acid content significantly raised. The higher ascorbic acid content recorded could be related to a reduced injury of the apple juice matrix and to a higher extraction as a consequence of the hyperbaric treatments (Suárez-Jacobo et al., 2012). Moreover, samples treated with HPH in combination with natural antimicrobials, showed an ascorbic acid content similar to that detected in thermally treated samples. Under these conditions, the oxidation of ascorbic acid, presumably, contributes significantly to maintaining the color in the apple centrifuged samples.

Conclusions

The present research evidenced the potential of high pressure homogenization treatments to increase the safety and shelf-life of centrifuged apple juices. In particular, the natural antimicrobials significantly increased the effectiveness of HPH and heat treatments applied, significantly increasing the apple juice shelf-life and safety even under conditions of thermal abuse (10 °C). In fact, they significantly factened the death kinetics of all the pathogenic species considered including *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655. Moreover, samples treated with a combination of HPH and natural antimicrobials showed enhanced texture properties and better color preservation. Furthermore, hexanal and (E)-2-hexenal, due their low sensorial threshold, directly influenced the volatile molecular profile of centrifuged apple juices contributing to an higher products diversification without any negative effect on their microbial safety. In fact, both the hyperbaric and thermal treatments in combination with natural antimicrobials, dramatically d the death kinetics of the pathogens species even when inoculated at significantly higher levels compared to those detected under real food process conditions. Moreover, the data obtained showed how the supplementation of natural antimicrobials represent a reliable alternative to reduce the severity of thermal treatments for the microbial

stabilization of the apple juices and, consequently, to reduce the damage to the different thermo-sensitive components of the apple juice matrixes.

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General conclusions

Consumer demand for minimally processed, and ready-to-eat foods with a reduced content of synthetic preservatives has stimulated the research of alternative preservation strategies. Essential oils (EOs) or their components represent one of the most promising natural feasible alternatives to improve food safety, shelf-life and quality. Although their antimicrobial properties are well documented few and fragmented are the information about their mechanisms of action, cellular targets and on the stress response strategies of microorganisms after the exposure to such compounds. In this framework, the main aim of the PhD project was to investigate on the effects of one hour exposure to sublethal concentrations of selected natural antimicrobials, such as citral, carvacrol, (E)-2-hexenal and thyme EO, on the food borne pathogens *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655. The action mechanisms of the natural antimicrobials and the cellular targets were studied through multiple approaches able to give information on cell morphological, physiological, tracriptome and proteome changes. In particular, the transcriptome of *L. monocytogenes* Scott A was studied by RT-qPCR on a pool of gene representative of different metabolisms: energetic, ferric uptake, stress response, gene transcription, cell division, virulence, motility, while the proteome effects were determined by bi-dimensional electrophoresis (2DE). By contrast, the transcriptome changes on *Escherichia coli* K12 MG1655 were evaluated using the microarray technology. In addition, for both the microbial strains the effects on the membrane fatty acid profiles were studied using GC/MS approach while single cell responses to the one hour exposure to natural antimicrobials of the whole populations were studied by flow cytometry. Finally the antimicrobial effect of (E)-2-hexenal, in combination with high pressure homogenization or traditional thermal treatments was verified in a real food system, i.e. apple juice, deliberately inoculated with spoilage and pathogenic microorganisms including pathogens *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655.

The results obtained allowed to define, for each strain and each antimicrobial used, the cell targets and the response mechanisms, respectively. The use of the different multi-parametric approaches provided useful information on citral, carvacrol, (E)-2-hexenal and thyme EO action mechanisms and their cell targets. Moreover, they allowed to elucidate the specific behavior and the response strategies used by *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655 to overcome the different stress conditions applied. In fact, the transcriptome analysis of *L. monocytogenes* Scott A

highlighted how citral and carvacrol induced an overexpression of genes involved in the stress response to adverse environmental conditions, i.e. *cspL* and *bsh* genes. (E)-2-hexenal and thyme EO created a clear unbalance in the energy metabolism determining a shift from respiration to fermentation, under-expressing *pgm* and *pdhD* involved in glycolysis. Moreover, these natural antimicrobials reduced the expression of the genes involved in the general stress response, in proteins synthesis and in DNA protection and repair after environmental shocks.

The transcriptome changes on *Escherichia coli* K12 MG1655 showed that the sub-lethal concentrations of citral, carvacrol, (E)-2-hexenal and thyme essential oil affected mainly the expression of genes involved in fatty acid biosynthesis, energy metabolism and protection against oxidative stress.

The proteomic data confirmed and deepened the effects evidenced by the transcriptome data. In fact, the sublethal concentrations of the natural antimicrobials used induced an over expression of the transporters, enzymes and cofactors involved in the less efficient energy generation mechanisms adopted by *L. monocytogenes* cells in response to the stress exposure and in the maintenance of the cell oxidoreductive potential.

In addition, natural antimicrobials tested caused the modulation of several proteins involved in the stress response, cell morphology, motility and protein synthesis of L. monocytogenes.

Both proteomic and trascritomic approaches showed the cell cytoplasmic membrane and the outer structures as the main targets of the selected natural antimicrobials. Consistent to these results, the evaluation of the membrane fatty acid profiles after the one hour exposure clarified the short term adaptation of *Listeria monocytogenes* Scott A and *Escherichia coli* K12 MG1655 to the selected antimicrobials. Mainly the one hour exposure to sublethal stresses caused a reduction of the unsaturation and chain length levels due a significant reduction of C18 UFAs as well as, an increase of the short chain saturated FAs. This common modulation mechanism was associated with homeviscous adaptation processes which differed in relation to the strain and antimicrobial used. In particular, *L. monocytogenes* increased C19:0 and C17: iso percentages reducing C15ante and C17 ante percentages after the exposure to all the antimicrobials used to counteract the fluidizing effect of the CL reduction. By contrast, *E. coli* counteracted the fluidity changes induced by the CL and UL modifications, modulating the relative percentages of cyclopropanic fatty acids such as C17 cyc and C19 cyc FAs. Moreover, *E. coli* when exposed to citral and carvacrol counteracted the reduction of membrane fluidity also by the increase of *trans* isomers.

Since the microbial resistance to stresses is reported to occur as a monomodal Gaussian with a narrow or broad distribution, or as a multimodal distribution comprising subpopulations of similar or vastly different numbers of individuals, a flow cytometric approach was used to analyze the whole population responses to citral, carvacrol, (E)-2-hexenal and thyme EO of *L. monocytogenes* Scott A and *E.coli* K12 MG1655.

The flow cytometry data reveled mainly permeabilization processes on the cytoplasmic cell membrane. These effects were more severe on *Escherichia coli*. By contrast no effect on the intracellular esterase activity were observed for both the strains considered. These evidences suggested a reversible injuring process of the cell membranes after the one hour exposure to the selected antimicrobials, used at different concentrations.

The validation in apple juice allowed to understand the real potential of one of the antimicrobials, chosen on the basis of its sensory compatibility with the food matrix, to improve food safety and shelf life. The effect of (E)-2-hexenal, in combination with high pressure homogenization or traditional thermal treatments, was evaluated on the safety, shelf-life and quality on apple juices inoculated with different food borne pathogens, including *L. monocytogenes* Scott A and *E.coli* K12 MG1655, and spoilage agents. In particular, the combination of between the proposed treatments and the natural antimicrobial, applied at sublethal concentration, resulted in a significant shelf life increase of the apple juices even under conditions of thermal abuse (10 °C).

Moreover, the natural antimicrobials dramatically increased the effectiveness of mild hyperbaric or thermal treatments accelerating the death kinetics of the pathogenic species considered even when inoculated at significantly higher levels compared to those detected under real food process conditions. Moreover, the data obtained showed how the supplementation of natural antimicrobials represent a reliable alternative to reduce the severity of thermal treatments for the microbial stabilization of the apple juices and, consequently, to reduce the damage to the different thermo-sensitive components of the apple juice matrixes and to improve their sensory properties. The data obtained, elucidating the effects of natural antimicrobials on the microbial cell physiology and responses also in real systems and in combination with other hurdles, can fasten their exploitation at industrial level to increase the food safety, shelf-life, functionality and sensory properties.