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Transcriptomic approach and membrane fatty acid analysis to study the response mechanisms of *Escherichia coli* to thyme essential oil, carvacrol, 2-(E)-hexanal and citral exposure

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Abbreviated Running Headline:

***E. coli* stress response to natural antimicrobials**

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Abstract

Aims: The application of essential oils and their components as food preservatives is promising but requires a deeper understanding of their mechanisms of action. This study aims to evaluate the effects of thyme essential oil, carvacrol, citral and 2-(E)-hexenal, on whole-genome gene expression (the transcriptome), as well as the fatty acids composition of the cell membranes of *Escherichia coli* K12.

Methods and Results: Therefore, we studied the response against 1 hour of exposure to sublethal concentrations of natural antimicrobials, of exponentially growing *Escherichia coli* K12, using DNA microarray technology and a gas chromatographic method. The results show that treatment with a sublethal concentration of the antimicrobials strongly affects global gene expression in *E. coli* for all antimicrobials used. Major changes in the expression of genes involved in metabolic pathways as well as in fatty acid biosynthesis and protection against oxidative stress were evidenced. Moreover, the sub-lethal treatments resulted in increased levels of unsaturated and cyclic fatty acids as well as an increase of the chain length compare to the controls.

Conclusions: The down-regulation of genes involved in aerobic metabolism indicate a shift from respiration to fermentative growth. Moreover, the results obtained suggest that the cytoplasmic membrane of *E. coli* is the major cellular target of essential oils and their components. In addition, the key role of membrane unsaturated fatty acids in the response mechanisms of *E. coli* to natural antimicrobials has been confirmed in this study.

Significance and Impact of the Study: The transcriptomic data obtained signify a further step to understand the mechanisms of action of natural antimicrobials also when sub-lethal concentrations and short term exposure. In addition, this research goes in deep correlating the transcriptomic modification with the changes of *E. coli* fatty acid composition of cell membrane identified as the main target of the natural antimicrobials.

Keywords: *Escherichia coli*; Stress response; Gene expression; Mechanism of action; Microarray; Natural antimicrobials; Essential oils;

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 (Hylgaard *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; Abdollahzadeh *et al.*, 2014). 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 2-(*E*)-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 (Leite *et*

al., 2014). EOs are used in the food industry as flavoring agents since many years and are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) (EFSA, 2010; FDA, 2017) and the. 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, 2015a), 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. Many hypotheses on the mechanisms of actions of natural antimicrobials have been reported in literature (Patrignani *et al.*, 2008; Hyldgaard *et al.*, 2012; Picone *et al.*, 2013; Siroli *et al.*, 2015b; Nazzarro *et al.*, 2017). 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*.

However, the literature data on action mechanisms of natural antimicrobials are still fragmentary and far to be conclusive. In any case the modification of cell membrane is reported as fundamental for microbial cells, including those of *E. coli*, to face and survive to

environmental stresses also when antimicrobials were included in the growth media (Di Pasqua *et al.*, 2006; Gianotti *et al.*, 2009; Patrignani *et al.*, 2008; Siroli *et al.*, 2015b).

However, no data on the effect of a short term exposure on the membrane fatty acid composition of *E. coli* are reported in literature.

In order to promote 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. However, the understanding of microbial adaptive strategies cannot be completely drowning from phenotypic responses, which are the final expression of genomic information (Filannino *et al.*, 2018). In this framework, the comprehension of the relationship between the fatty acid modification of *E. coli* cell membrane, identified as main target of natural antimicrobials, and the overall transcriptome data analysis is fundamental for fully understanding its adaptive and survival strategies. This is particularly important in food system in which several hurdles act simultaneously triggering, sometimes, microbial responses resulting in increased pathogen resistance and virulence (Chung *et al.*, 2006; Newell *et al.*, 2010).

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 (unpasteurized and improperly pasteurized) 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, consequently, to increase 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), as well as the fatty acids composition of the cell membranes, of sub-lethal concentrations of thyme EO and some of the major components of EOs such as carvacrol, citral and 2-(E)-hexenal were studied in depth using DNA microarray technology.

Material and Methods

Natural antimicrobials

Citral, 2-(E)-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. Thyme EO used in this work was previously characterized through GC/MS-SPME analyses (Siroli *et al.*, 2015a).

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

For the determination of the MIC and MBC values of 2-(E)-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. The MBC values were defined as the lowest concentrations of the natural antimicrobials tested which lead to the death of the inoculated cells and, consequently, to the absence of growth after 24h of incubation at 37 °C onto BHI agar.

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

The concentrations employed were 175, 60 and 125 mg/l for of 2-(*E*)-hexenal, carvacrol and thyme EO, respectively. Each compound was used half of the determined MIC values after 18h at an inoculum level of 2 log CFU/ml. Citral was used at 500 mg/L since its MIC value resulted higher than 2000 mg/L, concentration not suitable to be used in food matrixes. These sub-lethal concentrations were chosen because exploitable in foods without changing their sensory profiles and to assess the response mechanisms of living cells.

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 in three different days, and for each experiment, three tubes, for

each condition, were used (one used for measuring OD and microbial plate counting while the remaining were used for transcriptomic analyses). 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 (Smyth, 2004) 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).

Fatty acids analyses

The analyses were performed at the same conditions adopted for microarray experiments after 1 h of exposure to the various compounds. Lipid extraction and membrane fatty acid analyses were performed according to Suutari *et al.* (1990) while gas-chromatography analyses were performed according to Siroli *et al.* (2015b). Fatty acids (FAs) were identified by comparing their retention times and mass fragmentation profiles with those of the standards mix, Bacterial Acid Methyl Esters (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.

Statistics 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.

The microbiological and membrane fatty acids data, after the treatment with the natural antimicrobials, were compared using ANOVA followed by LSD test at $p < 0.05$ level in order to monitor differences among treatments.

Results

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

The MICs and MBCs of citral, 2-(E)-hexenal, thyme EO or carvacrol against three density levels of the target microorganisms *E. coli* K12 are reported in Table 1. Differences in the

MICs and MBCs were observed in relation to the substances and, in some cases, the inoculum level used. Citral showed a low antimicrobial effectiveness against the target strain, independently on the inoculation level compared to the other natural antimicrobials. 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. By contrast, the influence of the initial inoculum on MICs and MBCs was evident for 2-(E)-hexenal and thyme EO. The latter 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 2-(E)-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 2-(E)-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 at 18h 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.

Fatty acids analyses of *E. coli* K12 treated with sub-lethal concentrations of natural antimicrobials

The main fatty acids detected in *Escherichia coli* k12 MG1655 independently to the antimicrobial tested 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 *9cis*, C18:1 *9trans*, C18:1 *cis*11, C18:0 and C19 cyc (data not shown). Table 2 report the relative percentages of saturated, unsaturated and branched fatty acids as well as their mean chain length (CL) in relation to the exposure to the antimicrobial used. The main effect of the treatments on *E. coli* cells consisted into an increase of the unsaturated fatty acids (UFAs)

that resulted significantly higher after the treatment with carvacrol, thyme EO and 2-(E)-hexenal compared to the control samples. The greatest modifications of UFAs level were observed after the exposure to carvacrol. On the contrary, samples treated with citral showed a significant ($p<0.05$) increase of branched fatty acids compared to all the other samples. All the treatments caused a significant increase ($p<0.05$) of cyclic fatty acids compared to the controls. The one-hour exposure to the tested molecules resulted as well in an increase of CL, in particular after the exposure to citral, (E)-2-hexenal and thyme EO.

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. 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 by OD measurement and plate counting (Table 3). The addition to *E. coli* of 175 mg/l of 2-(E)-hexenal or 60 mg/l of carvacrol did not affect the cell loads of the organism compared to the control. On the contrary, thyme EO and citral significantly affected the cell loads of *E. coli* after the treatment. In fact, the cell loads of *E. coli* treated with thyme EO resulted significantly lower compared to the control after 30 min of exposure, while in case of citral exposure, the *E. coli* cell loads resulted significantly lower for the whole period considered (2 h) after the treatment compared to control samples.

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 4). 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 2-(E)-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.

A Venn diagram was constructed to investigate the distribution of differentially expressed genes in relation to the stress applied (Fig. 1) (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.

The most significantly (p -value lower than 0.01) up- or down-regulated genes are reported in Table 5 and they were further considered for the discussion. While the all genes resulted up- or down regulated are reported in Table S1. 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*, *fabD*, *fabI*, *accB*, *accC*, *accD* was evidenced for all the tested antimicrobials. These changes were accompanied by a down-regulation, in the presence of carvacrol and 2-(E)-hexenal, of *glpC* and *glpD*. 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*) and heat shock genes (*ibpA* and *ibpB*) in particular in the presence of thyme EO and citral. 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 2-(E)-hexenal and of *grcA* for all three antimicrobials. In addition, we observe a repression of the genes involved in glycerol metabolism *glpC* and *glpD* upon treatment of the cells with 2-(E)-hexenal, thyme EO and carvacrol.

The *ompX* gene, was up-regulated upon treatment of the cells with all tested compounds. The exposure to citral increase the expression of *tolC* gene of 5.96 times. Finally, an increase in expression of ribosomal subunit genes (*rps*, *rpm* and *rpl*) was evident after all three chemical stresses applied here.

Discussion

The MIC and MBC values were affected by the substance and the inoculum level of *E. coli*. On the other hand, the effects of inoculation level on MIC and MBC values of EOs and their components were described previously (Burt, 2004). The low MIC values observed by using carvacrol and thyme EO are in agreement with other studies (Bagamboula *et al.*, 2004; Klein *et al.*, 2013).

The exposure to sublethal concentrations of the natural antimicrobials resulted in a significant modification of the membrane fatty acid composition. On the other hand, it is well known that one of the main targets of EOs is the cytoplasmic membrane (Burt, 2004; Nazzaro *et al.*, 2013). The main fatty acids detected in this work are in agreement with literature (Di Pasqua *et al.*, 2006; Siroli *et al.*, 2015b). The treatment resulted in an increase of UFAs. On the other hand, UFAs 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). Also, Patrignani *et al.* (2008) and Siroli *et al.* (2015b) observed a significant increase of UFAs in cell membranes of *E. coli* grown in the presence of sublethal concentrations of natural antimicrobials including carvacrol, E-(2)-hexenal, citral and thyme EO. 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 between anabolic and catabolic pathways under stress conditions, since the desaturase of many microorganisms are oxygen dependent and, consequently, reduce the O₂ vapour pressure and its reactivity within cell membrane (Dodd *et al.*, 1997; Chatterjee *et al.*, 2000; Guerzoni *et al.*, 2001).

The observed increase of CL and cyclic fatty acids represent a mechanism adopted by several microorganisms to counteract the fluidizing effect of UFAs and to maintain the suitable fluidity of the membrane (Zhang and Rock, 2008; Royce *et al.*, 2015). Moreover, the cyclopropanic fatty acid synthesis, in addition to the effect on membrane fluidity, is reported to play a pivotal role in the stress response and survival of Gram-negative bacteria to several stringent conditions including the presence of antimicrobials substances (Zhang and Rock, 2008; Siroli *et al.*, 2015b). The modulation of 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).

The transcriptomic analyses of genes involved in fatty acids biosynthesis confirmed a perturbation of this metabolism after the one-hour exposure to the tested substances. The data obtained are in agreement and confirm the outcome of the analyses of fatty acids composition of the cell membranes. In fact, the *E. coli* accBCD genes, involved in 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.*, 2013) as well as the *fab* genes that encode enzymes responsible for a series of condensation, reduction, and dehydration reactions,

followed by elongation (My *et al.*, 2015) resulted up-regulated. The up-regulation of these genes might be related to the increase of unsaturated, branched and cyclic fatty acids biosynthesis observed in the samples treated with the natural antimicrobials. In addition, genes involved in glycerophospholipid metabolism resulted downregulated. 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, 1995). GlpD is an aerobic glycerol 3-phosphate dehydrogenase catalyzing the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate (Varga and Weiner, 1995). These data suggest a modification of the glycerophospholipid content of the cell membrane, on the other hand, is widely reported as a survival strategy adopted by gram-negative bacteria to survive under stress conditions (Dalebroux, 2017; Keller *et al.*, 2015).

A response of *E. coli* to the stresses applied 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 bacterial phage shock protein (Psp) stress response functions is responsible for repairing and manage 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) under stress conditions. It is well reported that the *pspA* operon expression is induced by heat shock, osmotic shock, ethanol, defects in protein transport across the cytoplasmic membrane and by the overproduction of some integral membrane proteins, including secretins (reviewed by Model *et al.*, 1997, Darwin, 2005, Joly *et al.*, 2010). Though, *pspA* is involved in two different aspects of the Psp response: negative regulation of *pspF* and an effector function of the Psp response (Joly *et al.*, 2010). It is reported that Psp effectors *pspA* and *pspD* in increased concentrations may counteract the proton motive force dissipation induced in the cell grown under stress conditions (Joly *et al.*, 2010; Jovanovic *et*

al., 2006). The data obtained in this study are in agreement with literature data that showed a correlation between the induction of *pspA* and modifications of the membrane fatty acid composition following treatment with psp-inducing agent ethanol or other chemical stresses (Chiou et al., 2004 Joly et al., 2010).

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 suggesting that natural antimicrobials might cause an oxidative stress to the cell. 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 antimicrobials tested resulted in an up-regulation of the gene *frmA* and *grcA*. The first encodes a glutathione-dependent formaldehyde dehydrogenase and the enzyme is part of aldehyde detoxification pathways (Mills *et al.*, 2009), its up-regulation is a probable attempt of the cell to inactivate the added aldehydes citral and 2-(E)-hexenal. Previous reports have shown that several microorganisms can detoxify citral and 2-(E)-hexenal by transforming them into alcohols (Siroli *et al.*, 2015b). 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 *grcA* 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 *grcA* 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 by Cox *et al.* (1998). In the present work, the *glpD* gene, that encodes aerobic glycerol 3-phosphate dehydrogenase, a respiratory enzyme, resulted down-

regulated and suggests a shift occurs from respiration to fermentation when the cells are faced with 2-(E)-hexenal, thyme EO and carvacrol.

The data obtained suggests a perturbation of outer membrane composition of *E. coli*. In fact, *ompX*, that resulted up-regulated, plays a key role in the down-regulation of porins in the outer membrane (OM) of *E. coli* in response to environmental stresses that induce its overproduction (Dupont *et al.*, 2007). Moreover, in case of citral exposure, an up-regulation of *tolC* was observed. The function of this gene is the efflux transmembrane activity, and it plays a key role in the export of antibiotics and other toxic compounds from the cells (Zakharov *et al.*, 2012). 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.

The resulted up-regulation of ribosomal subunit genes, is in agreement with literature data. 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 (Bailey *et al.*, 2009). 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 energy metabolism, protection against oxidative stress and, fatty acid biosynthesis. Although, the key role of membrane unsaturated fatty

acids in the response mechanisms of *E. coli* to natural antimicrobials was previously documented, the correlation between the transcriptome modification induced by short term exposure and the changes of *E. coli* cell membrane fatty acid composition represents the novelty of the present research, contributing to increase the EOs applications as food preservatives. In conclusion, 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.

Conflict of Interest

No conflict of interest declared.

References

- Abdollahzadeh, E., Rezaei, M. and Hosseini, H. (2014). Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control *Listeria monocytogenes* inoculated in minced fish meat. *Food Control* **35**, 177-183. doi: 10.1016/j.foodcont.2013.07.004
- Bagamboula, C.F., Uyttendaele, M. and Debevere, J. (2004). Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*. *Food Microbiol* **21**, 33–42. doi:10.1016/S0740-0020(03)00046-7
- Bailey, A.M., Constantinidou, C., Ivens, A., Garvey, M.I., Webber, M.A., Coldham, N., Hobman, J.L., Wain, J., Woodward, M.J. and Piddock, L.J.V. (2009). Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. *J Antimicrob. Chemother* **64**. doi:10.1093/jac/dkp320

- Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. (2008). Biological effects of essential oils--a review. *Food Chem Toxicol* **46**, 446–75. doi:10.1016/j.fct.2007.09.106
- Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods--a review. *Int J Food Microbiol* **94**, 223–53. doi:10.1016/j.ijfoodmicro.2004.03.022
- Cameron, A., Frirdich, E., Huynh, S., Parker, C.T. and Gaynor, E.C. (2012). Hyperosmotic Stress Response of *Campylobacter jejuni*. *J Bacteriol* **194**, 6116–6130. doi:10.1128/JB.01409-12
- Chatterjee, M.T., Seunath, A., Khalawan, S.A. and Curran, B.P.G. (2000). Cellular lipid composition influences stress activation of the yeast general stress response elements (STRE). *Microbiology* **146**, 877–884.
- Chueca, B., Pagán, R. and García-Gonzalo, D. (2014). Differential mechanism of *Escherichia coli* Inactivation by (+)-limonene as a function of cell physiological state and drug's concentration. *PLoS One* **9**, e94072. doi:10.1371/journal.pone.0094072
- Chung, H.J., Bang, W. and Drake, M.A. (2006). Stress Response of *Escherichia coli*. *Compr Rev Food Sci Food Saf* **5**, 52–64. doi:10.1111/j.1541-4337.2006.00002.x
- Cosentino, S., Barra, A., Pisano, B., Cabizza, M., Pirisi, F.M. and Palmas, F. (2003). Composition and Antimicrobial Properties of Sardinian Juniperus Essential Oils Against Foodborne Pathogens and Spoilage Microorganisms. *J Food Prot* **66**, 1288–1291. doi:10.4315/0362-028X-66.7.1288
- Cox, S.D., Gustafson, J.E., Mann, C.M., Markham, J.L., Liew, Y.C., Hartland, R.P., Bell, H.C., Warmington, J.R. and Wyllie, S.G. (1998). Tea tree oil causes K⁺ leakage and inhibits respiration in *Escherichia coli*. *Lett Appl Microbiol* **26**, 355–358. doi:10.1046/j.1472-765X.1998.00348.x
- Dalebroux, Z.D. (2017). Cues from the membrane: Bacterial glycerophospholipids. *J*

Bacteriol 199, 00136-17. doi: 10.1128/JB.00136-17

Darwin, A.J. (2007) Regulation of the Phage-Shock-Protein Stress Response in *Yersinia enterocolitica*. In: Perry R.D., Fetherston J.D. (eds) The Genus *Yersinia*. Advances In Experimental Medicine And Biology, **603**. Springer, New York, NY doi:10.1007/978-0-387-72124-8-14

Di Pasqua, R., Hoskins, N., Betts, G. and Mauriello, G. (2006). Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J Agric Food Chem* **54**, 2745-2749.

Dodd, C.E.R., Sharman, R.L., Bloomfield, S.F., Booth, I.R., and Stewart, G.S.A.B. (1997). Inimical processes: Bacterial self-destruction and sub-lethal injury. *Trends Food Sci Technol* **8**, 238-241. doi:10.1016/S0924-2244(97)01043-1

Dupont, M., James, C.E., Chevalier, J. and Pagès, J.-M. (2007). An early response to environmental stress involves regulation of OmpX and OmpF, two enterobacterial outer membrane pore-forming proteins. *Antimicrob Agents Chemother* **51**, 3190–8. doi:10.1128/AAC.01481-06

EFSA, (2010). Panel on Food Contact Materials, Enzymes, Flavouring and Processing Aids; Draft Guidance on the data required for the risk assessment of flavouring. *EFSA Journal* **8**, 1623. doi: 10.2093/j.efsa.2010.1623. Available online: www.efsa.europa.eu

FDA, (2017). Food and Drug Administration of the US, 21, CFR 184. Available online: <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?cfrpart=182&showfr=1>

Filannino, P., Di Cagno, R., Crecchio, C., De Virgilio, C., De Angelis, M. and Gobbetti, M. (2016). Transcriptional reprogramming and phenotypic switching associated with the adaptation of *Lactobacillus plantarum* C2 to plant niches. *Sci Rep* **6**, 27392. doi:

- Gianotti, A., Iucci, L., Guerzoni, M. E. and Lanciotti, R. (2009). Effect of acidic conditions on fatty acid composition and membrane fluidity of *Escherichia coli* strains isolated from Crescenza cheese. *Ann Microbiol* **59**, 603-610. doi: 10.1007/BF03175152
- Goeser, L., Fan, T.J., Tchaptchet, S., Stasulli, N., Goldman, W.E., Sartor, R.B. and Hansen, J.J. (2015). Small heat-shock proteins, IbpAB, protect non-pathogenic *Escherichia coli* from killing by macrophage-derived reactive oxygen species. *PLoS One* **10**, e0120249. doi:10.1371/journal.pone.0120249
- Guerzoni, M.E., Lanciotti, R. and Cocconcelli, P.S. (2001). Alteration in cellular fatty acid composition as response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology* **147**, 2255–2264.
- Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M. and von Wright, A. (1998). Characterization of the Action of Selected Essential Oil Components on Gram-Negative Bacteria. *J Agric Food Chem* **46**, 3590-95. doi: 10.1021/jf980154m
- Horstman, N.K. and Darwin, A.J. (2012). Phage shock proteins B and C prevent lethal cytoplasmic membrane permeability in *Yersinia enterocolitica*. *Mol Microbiol* **85**, 445–60. doi:10.1111/j.1365-2958.2012.08120.x
- Huvet, M., Toni, T., Sheng, X., Thorne, T., Jovanovic, G., Engl, C., Buck, M., Pinney, J.W. and Stumpf, M.P.H. (2011). The evolution of the phage shock protein response system: interplay between protein function, genomic organization, and system function. *Mol Biol Evol* **28**, 1141–55. doi:10.1093/molbev/msq301
- Hyldgaard, M., Mygind, T. and Meyer, R.L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front Microbiol* **3**, 12. doi:10.3389/fmicb.2012.00012

Jayasena, D.D. and Jo, C. (2013). Essential oils as potential antimicrobial agents in meat and meat products: A review. *Trends Food Sci Technol* **34**, 96–108. doi:10.1016/j.tifs.2013.09.002

Jovanovic, G., Engl, C., Mayhew, A.J., Burrows, P.C. and Buck, M. (2010). Properties of the phage-shock-protein (Psp) regulatory complex that govern signal transduction and induction of the Psp response in *Escherichia coli*. *Microbiology* **156**, 2920–32. doi:10.1099/mic.0.040055-0

Keating, D.H., Zhang, Y., Ong, I.M., McIlwain, S., Morales, E.H., Grass, J.A., Tremaine, M., Bothfeld, W., Higbee, A., Ulbrich, A., Balloon, A.J., Westphall, M.S., Aldrich, J., Lipton, M.S., Kim, J., Moskvina, O. V., Bukhman, Y. V, Coon, J.J., Kiley, P.J., Bates, D.M. and Landick, R. (2014). Aromatic inhibitors derived from ammonia-pretreated lignocellulose hinder bacterial ethanologenesis by activating regulatory circuits controlling inhibitor efflux and detoxification. *Front Microbiol* **5**, 402. doi:10.3389/fmicb.2014.00402

Keller, R., Ariöz, C., Hansmeier, N., Stenberg-Bruzell, F., Burstedt, M., Vikström, D., Kelly, A., Wieslander, A., Daley, D.O., and Hunke, S. (2015). The *Escherichia coli* envelope stress sensor CpxA responds to changes in lipid bilayer properties. *Biochemistry* **54**, 3670–3676. doi: 10.1021/acs.biochem .5b00242.

Khan, A., Ahmad, A., Akhtar, F., Yousuf, S., Xess, I., Khan, L.A. and Manzoor, N. (2011). Induction of oxidative stress as a possible mechanism of the antifungal action of three phenylpropanoids. *FEMS Yeast Res* **11**, 114-122. doi: 10.1111/j.1567-1364.2010.00697.x

Kitagawa, M., Matsumura, Y. and Tsuchido, T. (2000). Small heat shock proteins, IbpA and IbpB, are involved in resistances to heat and superoxide stresses in *Escherichia coli*. *FEMS Microbiol Lett* **184**, 165–171. doi: 10.1111/j.1574-6968.2000.tb09009.x

- Kitagawa, M., Miyakawa, M., Matsumura, Y. and Tsuchido, T. (2002). *Escherichia coli* small heat shock proteins, IbpA and IbpB, protect enzymes from inactivation by heat and oxidants. *Eur J Biochem* **269**, 2907–2917. doi: 10.1046/j.1432-1033.2002.02958.x
- Klein, G., Rüben, C. and Upmann, M. (2013). Antimicrobial activity of essential oil components against potential food spoilage microorganisms. *Curr Microbiol* **67**, 200–208. doi:10.1007/s00284-013-0354-1. doi: 10.1007/s00284-013-0354-1
- Kobayashi, R., Suzuki, T. and Yoshida, M. (2007). *Escherichia coli* phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. *Mol Microbiol* **66**, 100–109. doi:10.1111/j.1365-2958.2007.05893.x
- Kordali, S., Cakir, A., Ozer, H., Cakmakci, R., Kesdek, M. and Mete, E. (2008). Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish *Origanum acutidens* and its three components, carvacrol, thymol and p-cymene. *Bioresour Technol* **99**, 8788–95. doi:10.1016/j.biortech.2008.04.048
- Kuipers, O.P., de Jong, A., Baerends, R.J.S., van Hijum, S.A.F.T., Zomer, A.L., Karsens, H.A., den Hengst, C.D., Kramer, N.E., Buist, G. and Kok, J. (2002). Transcriptome analysis and related databases of *Lactococcus lactis*. *Antonie Van Leeuwenhoek* **82**, 113–22. doi: 10.1023/A:1020691801251
- Lanciotti, R., Gianotti, A., Patrignani, F., Belletti, N., Guerzoni, M. and Gardini, F. (2004). Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. *Trends Food Sci Technol* **15**, 201–208. doi:10.1016/j.tifs.2003.10.004
- Leite, M.C.A., Bezerra, A.P. de B., de Sousa, J.P., Guerra, F.Q.S., Lima, E. de O., Leite, M.C.A., Bezerra, A.P. de B., Sousa, J.P. de, Guerra, F.Q.S., Lima, E. de O., Guerra, F.Q.S., Lima, E. and de O. (2014). Evaluation of Antifungal Activity and Mechanism of Action of Citral against *Candida albicans*. *Evid Based Complement Alternat Med* **2014**, 378280. doi:10.1155/2014/378280

- Lucera, A., Costa, C., Conte, A. and Del Nobile, M.A. (2012). Food applications of natural antimicrobial compounds. *Front Microbiol* **3**, 287. doi:10.3389/fmicb.2012.00287
- Mills, T.Y., Sandoval, N.R. and Gill, R.T. (2009). Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. *Biotechnol Biofuels* **2**, 26. doi:10.1186/1754-6834-2-26
- My, L., Ghandour Achkar, N., Viala, J.P. and Bouveret, E. (2015). Reassessment of the Genetic Regulation of Fatty Acid Synthesis in *Escherichia coli*: Global Positive Control by the Dual Functional Regulator FadR. *J Bacteriol* **197**, 1862–72. doi:10.1128/JB.00064-15
- My, L., Rekoske, B., Lemke, J.J., Viala, J.P., Gourse, R.L. and Bouveret, E. (2013). Transcription of the *Escherichia coli* fatty acid synthesis operon FabHDG is directly activated by FadR and inhibited by ppGpp. *J Bacteriol* **195**, 3784–3795. doi:10.1128/JB.00384-13
- Nazzaro, F., Fratianni, F., L., Coppola, R. and De Feo, V. (2017). Essential oils and antifungal activity. *Pharmaceuticals (Basel)* **10**, E86. doi: 10.3390/ph10040086
- Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R. and De Feo, V. (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals (Basel)* **6**, 1451–74. doi:10.3390/ph6121451
- Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., der Giessen, J. van, Kruse, H., van der Giessen, J. and Kruse, H. (2010). Food-borne diseases - The challenges of 20years ago still persist while new ones continue to emerge. *Int J Food Microbiol* **139**, S3–S15. doi:10.1016/j.ijfoodmicro.2010.01.021
- Oliveros, J.C. (2015). Venny 2.1.0 An interactive tool for comparing lists with Venn's diagrams. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>.

- Oussalah, M., Caillet, S., Saucier, L. and Lacroix, M. (2007). Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. *Food Control* **18**, 414–420. doi:10.1016/j.foodcont.2005.11.009
- Patrignani, F., Iucci, L., Belletti, N., Gardini, F., Guerzoni, M.E. and Lanciotti, R. (2008). Effects of sub-lethal concentrations of hexanal and 2-(E)-hexanal on membrane fatty acid composition and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli*. *Int J Food Microbiol* **123**, 1–8. doi:10.1016/j.ijfoodmicro.2007.09.009
- Patrignani, F., Tabanelli, G., Siroli, L., Gardini, F. and Lanciotti, R. (2013). Combined effects of high pressure homogenization treatment and citral on microbiological quality of apricot juice. *Int J Food Microbiol* **160**, 273–281. doi:10.1016/j.ijfoodmicro.2012.10.021
- Picone, G., Laghi, L., Gardini, F., Lanciotti, R., Siroli, L. and Capozzi, F. (2013). Evaluation of the effect of carvacrol on the *Escherichia coli* 555 metabolome by using ¹H-NMR spectroscopy. *Food Chem* **141**, 4367–4374. doi: 10.1016/j.foodchem.2013.07.004
- Royce, L.A., Yoon, J.M., Chen, Y., Rickenbach, E., Shanks, J. V. and Jarboe, L.R. (2015). Evolution for exogenous octanoic acid tolerance improves carboxylic acid production and membrane integrity. *Metab Eng* **29**, 180–188. doi:10.1016/j.ymben.2015.03.014
- Sayings, H. (1999). Chapter 3. doi:10.1016/B978-1-85617-567-8.50011-1
- Severino, R., Ferrari, G., Vu, K.D., Donsì, F., Salmieri, S. and Lacroix, M. (2015). Antimicrobial effects of modified chitosan based coating containing nanoemulsion of essential oils, modified atmosphere packaging and gamma irradiation against *Escherichia coli* O157:H7 and *Salmonella Typhimurium* on green beans. *Food Control* **50**, 215–222. doi:10.1016/j.foodcont.2014.08.029

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- Siroli, L., Patrignani, F., Serrazanetti, D.I., Tabanelli, G., Montanari, C., Tappi, S., Roccuoli, P., Gardini, F. and Lanciotti, R. (2014). Efficacy of natural antimicrobials to prolong the shelf-life of minimally processed apples packaged in modified atmosphere. *Food Control* **46**, 403–411. doi:10.1016/j.foodcont.2014.05.049
- Siroli, L., Patrignani, F., Serrazanetti, D.I., Tappi, S., Roccuoli, P., Gardini, F. and Lanciotti, R. (2015a). Natural antimicrobials to prolong the shelf-life of minimally processed lamb's lettuce. *Postharvest Biol Technol* **103**, 35–44. doi:10.1016/j.postharvbio.2015.02.016
- Siroli, L., Patrignani, F., Gardini, F. and Lanciotti, R. (2015b). Effects of sub-lethal concentrations of thyme and oregano essential oils, carvacrol, thymol, citral and trans-2-hexenal on membrane fatty acid composition and volatile molecule profile of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteritidis*. *Food Chem* **182**, 185–192. doi:10.1016/j.foodchem.2015.02.136
- Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, 3. doi: 10.2202/1544-6115.1027
- Suutari, M., Linkkonen, K. and Laakso, S. (1990). Temperature adaptation in yeast: The role of fatty acids. *J Gen Microbiol* **136**, 1469-1474.
- Tabanelli, G., Patrignani, F., Gardini, F., Vinderola, G., Reinheimer, J., Grazia, L. and Lanciotti, R. (2014). Effect of a sublethal high-pressure homogenization treatment on the fatty acid membrane composition of probiotic lactobacilli. *Lett Appl Microbiol* **58**, 109–117. doi:10.1111/lam.12164
- Tajkarimi, M.M., Ibrahim, S.A. and Cliver, D.O. (2010). Antimicrobial herb and spice compounds in food. *Food Control* **21**, 1199–1218. doi:10.1016/j.foodcont.2010.02.003
- Varga, M.E. and Weiner, J.H. (1995). Physiological role of GlpB of anaerobic glycerol-3-

phosphate dehydrogenase of *Escherichia coli*. *Biochem Cell Biol* **73**, 147–53.

- Wagner, A.F., Schultz, S., Bomke, J., Pils, T., Lehmann, W.D. and Knappe, J. (2001). YfiD of *Escherichia coli* and Y06I of bacteriophage T4 as autonomous glycyl radical cofactors reconstituting the catalytic center of oxygen-fragmented pyruvate formate-lyase. *Biochem Biophys Res Commun* **285**, 456–62. doi:10.1006/bbrc.2001.5186
- Wyborn, N.R., Messenger, S.L., Henderson, R.A., Sawers, G., Roberts, R.E., Attwood, M.M. and Green, J. (2002). Expression of the *Escherichia coli* yfiD gene responds to intracellular pH and reduces the accumulation of acidic metabolic end products. *Microbiology* **148**, 1015–1026. doi:10.1099/00221287-148-4-1015
- Zakharov, S.D., Sharma, O., Zhalnina, M., Yamashita, E. and Cramer, W.A. (2012). Pathways of colicin import: utilization of BtuB, OmpF porin and the TolC drug-export protein. *Biochem Soc Trans* **40**, 1463-1468. doi: 10.1042/BST20120211
- Zhang, Y.-M. and Rock, C.O. (2008). Membrane lipid homeostasis in bacteria. *Nat. Rev Microbiol* **6**, 222–233. doi:10.1038/nrmicro1839

TABLES

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of citral, 2-(E)-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
2-(E)-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

Table 2. Relative percentages of saturated, unsaturated and branched fatty acids as well as their mean chain length (CL) and unsaturation level (UL) of the total membrane fatty acids in *E. coli* K12 MG1155 in relation to the exposure to the antimicrobial used

	<i>saturated fatty acids</i>	<i>unsaturated fatty acids</i>	<i>branched fatty acids</i>	<i>cyclic fatty acids</i>	UL ^a	CL ^b
	%	%	%	%		
Control EtOH 1%	96.51 ^a	3.50 ^a	4.66 ^a	3.80 ^a	0.03	1642.6
Citral 500 mg/L	95.35 ^a	4.70 ^a	18.33 ^c	11.80 ^c	0.06	1726.8
Carvacrol 60 mg/L	89.99 ^b	10.00 ^b	5.38 ^a	6.60 ^b	0.14	1662.4
(E)-2-hexenal 175 mg/L	90.96 ^{ab}	9.00 ^b	5.45 ^a	9.50 ^{bc}	0.11	1727.2
Thyme EO 125 mg/L	91.82 ^{ab}	8.18 ^b	11.39 ^b	6.89 ^b	0.10	1726.4

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%. Means followed by different letters are significantly different ($p < 0.05$).

^a Unsaturation level calculated as $[\text{percentage monoenes} + 2(\text{percentage dienes}) + 3(\text{percentage trienes})]/100.0$

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

Table 3. Effect of natural antimicrobials addition on the growth of *E. coli*. *E. coli* K12 growth, expressed as log CFU/mL, 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.

	log CFU/mL			
	0 h	0.5 h	1 h	2 h
Control Eth1%	7.34±0.15 ^a	7.89±0.10 ^a	8.15±0.31 ^a	8.19±0.16 ^a
Citral 500ppm	7.34±0.15 ^a	7.25±0.07 ^c	7.24±0.29 ^b	7.42±0.34 ^b
T-2-Hexenal 175ppm	7.34±0.15 ^a	7.65±0.17 ^a	7.61±0.32 ^{ab}	7.93±0.18 ^{ab}
Carvacrol 60ppm	7.34±0.15 ^a	7.58±0.12 ^{ab}	7.70±0.21 ^{ab}	7.97±0.15 ^{ab}
Thyme EO 125 ppm	7.34±0.15 ^a	7.37±0.11 ^{bc}	7.63±0.67 ^{ab}	7.69±0.40 ^{ab}

Values are reported with ±standard deviation. Means followed by different letters are significantly different (p<0.05).

Table 4. Number of significantly (p<0.01) up- or down regulated genes in *E. coli* K12 MG1155

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

Table 5 Genes up- or down regulated in *E. coli* K12 after treatment with citral (500 mg/l), 2-(E)-hexenal (175 mg/l), carvacrol (60 mg/l) and thyme EO (125 mg/l) (p<0.01) involved in the main metabolic pathways.

Gene	Fold Change				description
	Carvacro l	Thyme EO	Citral	2-(E)- hexenal	
<i>Ribosome</i>					
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 (rpsl)	2.26	2.49	2.65	1.44	30S ribosomal subunit protein S9 [b3230]
b1717 (rpml)	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]
<i>Glycerophospholipid metabolism</i>					
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]
<i>Fatty acid biosynthesis</i>					
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]
<i>Energetic metabolism</i>					
b1651 (gloA)	*	1.72	*	2.87	lactoylglutathione lyase [b1651]
b2579 (grcA)	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]
<i>Purine, pyrimidine metabolism and transcription</i>					
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 and detoxification 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	10.72	*	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]
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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

Supporting information legend

Table S1) All genes resulted up- or down regulated in *E. coli* K12 after treatment with citral (500 mg/l), 2-(E)-hexenal (175 mg/l), carvacrol (60 mg/l) and thyme EO (125 mg/l) ($p < 0.01$).

Figure legends

Figure 1) Distribution of differentially expressed genes in antimicrobial-treated *E. coli* K12. The Venn diagram (Oliveros *et al.*, 2015) reports the numbers of unique and common differentially expressed genes.