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Effects of sub-lethal concentrations of hexanal and 2-(*E*)-hexenal on membrane fatty acid composition and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli*

Francesca Patrignani, Luciana Iucci, Nicoletta Belletti, Fausto Gardini, M. Elisabetta Guerzoni, Rosalba Lanciotti *

Dipartimento di Scienze degli Alimenti, Università degli Studi di Bologna, Via Fanin 46, 40127 Bologna, Italy

Abstract

In recent years, the interest in the possible use of natural alternatives to food additives to prevent bacterial and fungal growth has notably increased. Plants and plant products can represent a source of natural alternatives to improve the shelf-life and the safety of food. Some of these compounds, *i.e.* hexanal, hexanol, 2-(*E*)-hexenal and 3-(*Z*)-hexenol, produced throughout the lipoxygenase pathway have important roles in plant defence with a protective action towards microbial proliferation in wounded areas. Otherwise, hexanal and 2-(*E*)-hexenal have evidenced a marked antimicrobial activity against food spoilage and pathogenic microbial species both in model and real systems. The precise mechanisms of action of all these antimicrobial compounds are not yet clear. Because the usage of these compounds as antimicrobials in foods has to be supported by the comprehension of mechanisms of action of these compounds, the overall purpose of this work was to study the modifications of the cell membrane and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli* during the growth in the presence of sub-lethal doses of these two aldehydes. The results obtained evidenced that the tested molecules induced noticeable modifications of the composition of cell membrane and the volatile compounds produced during the growth. Although specific differences in relation to the species considered were identified, 2-(*E*)-hexenal and hexanal induced a marked increase of some membrane associated fatty acids, both linear and branched fatty acids as well as unsaturated fatty acids, and released free fatty acids.
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Keywords: Hexanal; 2-(*E*)-hexenal; Cell fatty acid composition; Volatile compounds

1. Introduction

In recent years, the interest in the possible use of natural alternatives to food additives to prevent bacterial and fungal growth has notably increased. Plants and plant products can represent a source of natural alternatives to improve the shelf-life and the safety of food. In fact, they are characterised by a wide range of volatile compounds, some of which are important flavour quality factors (Utama et al., 2002). A key role in the defence systems of fresh produce against decay microorganisms has been attributed to the presence of some of these volatile compounds (Ben-Yehoshua et al., 1998). Their ability to inhibit microorganisms is one of the reasons for the interest in them as components of biological means for prolonging the shelf-life of postharvest or minimally processed fruits and vegetables (Wilson and Winiewski, 1989). Moreover, plant volatiles have been widely used as food flavouring agents and most of them are generally recognized as safe (GRAS) (Newberne et al., 2000).

Some of these compounds, produced throughout the lipoxygenase pathway, have important roles in plant defence with a protective action towards microbial proliferation in

* Corresponding author. Dipartimento di Scienze degli Alimenti, Università degli Studi di Bologna, Piazza Goidanich, 60 — 47023 Cesena, Italy. Tel.: +39 0547 636132; fax: +39 0547 382348.
E-mail address: rosalba.lanciotti@unibo.it (R. Lanciotti).

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wounded areas (Casey et al., 1999). Moreover, aldehydes such as hexanal, and 2-(*E*)-hexenal have been demonstrated to possess a noticeable activity against several microfungal and gram-positive and gram-negative bacterial strains (Kubo et al., 1995, 1999, 2004; Gardini et al., 1997, 2002; Lanciotti et al., 1999, 2003, 2004; Corbo et al., 2000; Bisognano et al., 2001; Trombetta et al., 2002; Nakamura and Hatanaka 2002). Thus, these aldehydes might be good candidates for employment as antimicrobial agents against bacteria responsible for human infections or might find broad applications as food preservatives or might be a good alternative to other highly toxic disinfectants for hospital equipment. However, the practical exploitation as antimicrobials in foods has to be supported by the comprehension of mechanisms of action of these compounds. 2-(*E*)-hexenal is reported to act as a surfactant but likely permeates by passive diffusion across the plasma membrane. Once inside cells, its α, β-unsaturated aldehyde moiety 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). Although membrane functional proteins are generally supposed to be the potential targets toward which aldehydic antimicrobial agents are directed, other mechanisms of action/interaction can help to explain their antimicrobial activity (Denyer and Stewart, 1998). It is well known that when microbial cells are exposed to a sub-lethal stress, the cell membrane is able to change in order to cope with the new environment (Russell et al., 1995). In fact, the modification of membrane lipids is fundamental in maintaining both membrane integrity and functionality in the face of external stresses (Russell et al., 1995). Moreover, changes in fatty acid composition are reflected in modification of cell surface physical properties, such as phase transition temperature and microviscosity (Mejia et al., 1999). New findings relative to *Pseudomonas aeruginosa* have demonstrated that the fatty acid composition of the membrane phospholipids can activate the premature expression of a number of genes, including *rel*A and those involved in the quorum sensing system, resulting in a premature production of *N*-Acyl Homoserine Lactones signalling compounds (Baysse et al., 2005). This premature expression, which is independent of cell density, external stress exposure and nutrient starvation, provides further support for the concept that changes in the membrane structure can modulate stress-related gene expression. In this perspective, the overall purpose of this work was to study the change of the cell membranes fatty acid (FA) composition and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli* during the growth in the presence of sub-lethal doses of these two aldehydes.

2. Materials and methods

2.1. Strains and culture conditions

The strains *L. monocytogenes* Scott A, *S. aureus* F1, *S. enteritidis* E5 and *E. coli* 555, belonging to the strain collection of the Dipartimento di Scienze degli Alimenti of Bologna University, were employed. The strains were maintained on Brain Heart Infusion, BHI (Oxoid, Basingstoke, UK) added with 1.5% Agar (Oxoid). Before the experiments the strains were sub-cultured in BHI for 36 h at 37 °C until the end of exponential phase was reached.

2.2. Cell treatment

The inoculation of the tested strains was performed in 2.0 L conical flasks (12 for each strain) containing 1.5 L of BHI (Oxoid) at level of about 3.0 Log CFU ml^{-1}. Immediately after the inoculations the samples were supplemented with 1000 ppm of ethanol (3 flasks for each strain), 150 ppm of hexanal (3 flasks for each strain) or 20 ppm of 2-(*E*)-hexenal (3 flasks

Table 1
Membrane fatty acid composition of *Listeria monocytogenes* Scott A in relation to the stress conditions applied

	Total fatty acids (%)																			CL[a]	UL[b]
	C_{11i}	C_{11a}	$C_{12:0}$	$C_{14:0}$	C_{14i}	$C_{15:0}$	C_{15i}	C_{15a}	C_{16i}	$C_{16:0}$	$C_{17:0}$	C_{17i}	C_{17a}	$C_{18:0}$	$C_{18:1\ cis\ 9}$	$C_{18:1\ cis11}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$		
Control	–[c]	–	6.6	8.0	4.5	18.6	14.4	16.8	–	15.1	8.2	–	–	3.3	4.5	–	–	–	–	15.2	0.05
Ethanol[d]	–	–	–	3.0	3.3	0.3	16.2	21.1	1.8	8.4	0.7	2.4	2.1	20.6	3.4	0.7	1.0	1.6	13.4	16.6	0.10
Ethanol+ hexanal[e]	15.8	6.1	–	2.9	–	–	9.1	17.3	–	20.3	–	2.1	3.2	6.3	1.9	12.3	2.8	–	–	15.1	0.12
Ethanol+2-(*E*)-hexenal[f]	–	–	–	16.9	–	–	7.8	10.3	–	36.0	0.4	0.9	2.1	26.0	3.9	1.0	0.5	–	–	16.9	0.08

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%.

[a] Mean chain length calculated as (FAP*C)}/100 (where FAP is the percentage of fatty acid and C the number of carbon atoms).

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

[c] Undetectable level.

[d] Concentration employed 1000 ppm.

[e] Concentration employed 150 ppm.

[f] Concentration employed 20 ppm.

Table 2
Free fatty acid relative percentages (calculated with respect to the total fatty acid methyl esters) of *Listeria monocytogenes* Scott A in relation to the stress conditions applied

	Free fatty acids (%)																							CL [a]	UL [b]
	$C_{10:0}$	C_{11i}	C_{11a}	$C_{12:0}$	$C_{13:0}$	$C_{14:0}$	C_{14i}	$C_{15:0}$	C_{15i}	C_{15a}	C_{16i}	$C_{16:0}$	$C_{16:1cis9}$	$C_{17:0}$	C_{17i}	C_{17a}	$C_{17:1cis9}$	$C_{18:0}$	$C_{18:1\ cis9}$	$C_{18:1\ cis11}$	$C_{18:2}$	$C_{18:3}$	C_{20}		
Control	– [c]	–	–	–	–	8.3	1.5	0.9	15.7	17.3	–	16.5	–	0.6	9.3	13.3	1.0	8.0	1.3	0.9	0.5	0.5	4.0	16.0	0.06
Ethanol [d]	–	–	–	–	1.3	8.5	–	–	15.4	16.8	4.9	12.9	–	0.6	6.66	8.9	–	9.2	4.3	1.0	2.4	0.4	6.4	16.2	0.11
Ethanol+hexanal [e]	0.4	62.1	1.9	0.3	1.1	2.4	–	0.7	3.0	4.4	0.8	6.1	–	0.3	2.27	3.2	–	4.2	0.3	2.6	1.4	2.0	–	12.8	0.12
Ethanol+2-(*E*)-hexenal [f]	0.9	–	–	3.0	–	3.3	–	0.8	5.5	8.1	–	25.8	3.2	1.0	2.80	5.1	0.5	34.6	0.7	3.0	1.3	–	–	16.5	0.10

The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%.
[a] Mean chain length calculated as {Σ(FAP*C)} / 100 (where FAP is the percentage of fatty acid and C the number of carbon atoms).
[b] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100.
[c] Undetectable level.
[d] Concentration employed 1000 ppm.
[e] Concentration employed 150 ppm.
[f] Concentration employed 20 ppm.

for each strain) solubilized in the same amount of ethanol (1000 ppm). Hexanal, 2-(*E*)-hexenal and ethanol (all purchased from Sigma Aldrich, Steinheim, Germany) were previously sterilized through filtration (Millex-GS, Millipore, 0.22 μm, Molsheim, France). Inoculated flasks without any additions were used as controls. The incubation was performed overnight at 37 °C.

The evaluation of the viable cell numbers were performed plating the appropriate decimal serial dilutions onto Plate Count Agar (Oxoid). The incubation was performed at 37 °C for 48 h.

2.3. Fatty acid analysis

Late exponential phase cells were harvested by centrifugation (5000 $g\times 10$ min) and washed twice with 59 mM Tris HCl pH7, frozen in liquid nitrogen and stored at −80 °C until analyses. Lipid extraction and membrane fatty acid analyses were performed according to the method of Suutari et al. (1990). The relative percentages of the fatty acids were determined from the peak areas of the methyl esters using a DP 700 integrator (Spectra Physics). The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%.

For GC analyses, a Carlo Erba HRGR 5660 MEGA Series gas chromatograph (Carlo Erba Instruments) equipped with a flame-ionization detector and a 30 m × 0.32 mm i.d. fused silica capillary column coated with a 0.2 μm film of Carbowax (Supelco) as stationary phase was used. The conditions were as follows: injector temperature, 220 °C; detector temperature, 220 °C; carrier gas (He) flow rate, 3 ml min^{-1}; splitting ratio, 1:100 (v/v). The oven temperature was programmed from 60 to 220 °C at 4 °C min^{-1}. For peak identification, standard solution (Supelco) and GC/MS were used. For this a Fision HRGC MEGA Series gas chromatograph (Fision Instruments) equipped with a split–splitless injector and connected to a spectrometer (Carlo Erba QMD 1000; Carlo Erba Instruments) was used. A fused silica capillary column with a 0.10 μm methyl silicon (Chrompack) stationary phase was used. The carrier gas was He. The oven temperature was programmed from 60 to 220 °C at 4 °C min^{-1}.

2.4. GC–Mass Spectrometry (MS)-Solid Phase Microextraction (SPME) analysis

In the late exponential phase for each strain and each growth condition aliquots of cultural media were sterilely taken for SPME analyses. A divinylbenzene-poly(dimethylsiloxane)-coated stable flex fiber (65 μm) and a manual SPME holder (Supelco Inc., Bellefonte, PA) were used in this study after preconditioning according to the manufacturer's instruction manual. Before each headspace sampling, the fiber was exposed to the GC inlet for 5 min for thermal desorption at 250 °C in a blank run. Five milliliters of the sample was placed in 10 mL vials, and the vials were sealed by PTFE/silicon septa. The samples were then equilibrated for 15 min at 70 °C. The SPME

Table 3
Membrane fatty acid composition of *Staphylococcus aureus* F1 in relation to the stress conditions applied

	Total fatty acids (%)																			CL[a]	UL[b]
	$C_{10:0}$	$C_{11:0}$	$C_{12:0}$	$C_{14:0}$	C_{14i}	$C_{15:0}$	C_{15i}	C_{15a}	$C_{16:0}$	C_{16i}	$C_{17:0}$	C_{17i}	C_{17a}	$C_{18:0}$	$C_{18:1\ cis9}$	$C_{18:1\ cis11}$	C_{19i}	C_{19a}	$C_{20:0}$		
Control	–[c]	–	1.5	4.5	–	1.4	16.2	19.0	17.1	–	4.4	1.6	4.2	18.1	–	–	–	–	12.0	16.4	–
Ethanol[d]	–	–	1.2	2.5	4.9	0.3	15.6	18.2	7.3	–	0.6	1.1	0.7	22.0	0.5	1.7	–	1.9	22.0	16.9	0.02
Ethanol+ hexanal[e]	3.0	7.9	2.5	3.9	2.5	0.8	7.1	8.5	9.3	1.6	–	0.8	0.8	15.7	1.5	11.2	2.7	1.5	18.6	16.5	0.12
Ethanol+2-(*E*)-hexenal[f]	–	–	0.7	3.7	2.9	0.5	6.9	8.7	27.7	–	0.7	0.7	0.8	30.1	5.8	0.7	–	1.6	10.5	16.9	0.06

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%.
[a] Mean chain length calculated as (FAP*C)}/100 (where FAP is the percentage of fatty acid and C the number of carbon atoms).
[b] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100.
[c] Undetectable level.
[d] Concentration employed 1000 ppm.
[e] Concentration employed 150 ppm.
[f] Concentration employed 20 ppm.

fiber was exposed to each sample for 5 min by manually penetrating the septum, and, finally, the fiber was inserted into the injection port of the GC for 5 min sample desorption. GC–MS analyses were carried out on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode (ionization voltage, 70 eV). A Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm i.d., 1.2 µm df) was used (Chrompack, Middelburg, The Netherlands). The temperature program was 50 °C for 2 min, then programmed at 1 °C min^{-1} to 65 °C and finally at 5 °C min^{-1} to 220 °C, which was maintained for 22 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively.

Injections were performed with a split ratio of 1:20 and helium (1 mL min^{-1}) as the carrier gas. The compounds were identified by use of the National Institute of Standards and Technology-United States Environmental Protection Agency-National Institute of Health (1998) and according to the Registry of Mass Spectral Data (1998) mass spectra libraries as well as literature MS data and, whenever possible, co-injections with authentic chemical compounds.

2.5. Thiobarbituric acid reactive substance(TBAR) analysis

The TBARs and cell protein contents were estimated according to Guerzoni et al. (1997).

3. Results

3.1. Cell fatty acid changes induced by sub-lethal concentrations of hexanal and 2-(E)-hexenal

The presence of the tested molecules did not significantly affect the growth of *L. monocytogenes*, Scott A, *S. aureus* F1, *S. enteritidis* E5 and *E. coli* 555. In fact, after an overnight incubation they attained levels higher than 7.50 Log CFU ml^{-1} independently of the supplementation with ethanol, hexanal and 2-(*E*)-hexenal (data not shown). By contrast, these molecules affected both membrane associated and released fatty acids (FA) of the strains tested. Table 1 shows the FA composition of the late exponential phase cells of the *L. monocytogenes* Scott A in relation to the stress conditions applied. The main FAs detected in the control cells were $C_{14:0}$, $C_{16:0}$, $C_{15iso,}$ $C_{15:0}$ and C_{15ante}. The sub-lethal chemical stresses adopted, and particularly

Table 4
Membrane fatty acid composition of *Salmonella enteritidis* E5 in relation to the stress conditions applied

	Total fatty acids (%)													CL[a]	UL[b]
	C_{11i}	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	C_{16i}	$C_{16:0}$	$C_{16:1\ cis9}$	C_{17cyc}	$C_{18:0}$	$C_{18:1\ cis9}$	$C_{18:1\ trans}$	$C_{18:2}$	$C_{19\ cyc}$		
Control	–[c]	0.4	4.3	0.6	–	35.0	6.7	21.5	0.8	15.5	0.9	–	14.1	16.9	0.23
Ethanol[d]	–	–	7.4	–	–	48.3	4.7	20.3	2.2	4.8	3.2	0.5	8.5	16.6	0.13
Ethanol+hexanal[e]	0.4	0.3	4.2	0.1	0.9	37.9	5.0	21.4	1.0	12.2	1.3	0.2	14.0	16.8	0.19
Ethanol+2-(*E*)-hexenal[f]	–	0.2	5.0	–	1.0	37.0	4.2	18.0	6.9	16.4	1.8	0.4	9.1	16.1	0.23

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%.
[a] Mean chain length calculated as (FAP*C)}/100 (where FAP is the percentage of fatty acid and C the number of carbon atoms).
[b] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)]/100.
[c] Undetectable level.
[d] Concentration employed 1000 ppm.
[e] Concentration employed 150 ppm.
[f] Concentration employed 20 ppm.

Table 5
Membrane fatty acid composition of *Escherichia coli* 555 in relation to the stress conditions applied

	Total fatty acids (%)													CL[a]	UL[b]
	C_{11i}	$C_{12:0}$	$C_{14:0}$	C_{16i}	$C_{16:0}$	$C_{16:1\ cis9}$	$C_{17:0}$	C_{17cyc}	$C_{18:0}$	$C_{18:1\ cis9}$	$C_{18:1\ trans}$	$C_{18:2}$	C_{19cyc}		
Control	–[c]	0.5	3.3	0.6	36.4	3.3	0.2	26.0	0.9	–	1.0	10.9	16.7	16.93	0.26
Ethanol[d]	–	0.6	3.9	0.8	38.9	2.9	–	25.0	1.0	9.8	1.1	–	15.9	16.86	0.14
Ethanol+hexanal[e]	4.5	0.6	2.6	0.7	34.6	4.8	–	22.5	0.8	16.2	0.8	–	11.9	16.63	0.22
Ethanol+2-(*E*)-hexenal[f]	–	0.5	3.4	10.4	35.8	2.8	0.1	20.6	1.7	9.3	2.1	13.5	–	16.65	0.41

The fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%.
[a] Mean chain length calculated as (FAP*C)} / 100 (where FAP is the percentage of fatty acid and C the number of carbon atoms).
[b] Unsaturation level calculated as [percentage monoenes+2(percentage dienes)+3(percentage trienes)] / 100.
[c] Undetectable level.
[d] Concentration employed 1000 ppm.
[e] Concentration employed 150 ppm.
[f] Concentration employed 20 ppm.

hexanal, resulted in the marked increase of C_{17iso}, C_{17ante}, $C_{16:0}$, $C_{18:0}$, $C_{18:1cis11}$ and $C_{18:2}$. Moreover, the supplementation with hexanal and 2-(*E*)-hexenal decreased the relative percentages of C_{14iso}, $C_{15:0}$, C_{15iso} and $C_{18:1cis9}$. Only in the presence of hexanal fatty acids having 11 carbon atoms were detected. In Table 2 the relative percentages of the free fatty acids (FFAs) in relation to the chemical stress conditions applied to *L. monocytogenes* are shown. Hexanal remarkably increased the relative percentages of medium chain branched FAs ($C_{11:0}$–$C_{13:0}$), particularly C_{11iso}. Moreover an increase of unsaturated FAs such as $C_{18:1cis11}$ and $C_{18:3}$ was observed. The exposure to 2-(*E*)-hexenal increased the relative percentages of saturated FAs such as $C_{16:0}$ and $C_{18:0}$. Both hexanal and 2-(*E*)-hexenal significantly reduced the proportions of iso and anteiso branched chain $C_{15:0}$ and $C_{17:0}$, which predominated the FAs of the control and of the cells exposed to ethanol.

The fatty acid composition of *S. aureus* F1 is shown in Table 3. The exposure to hexanal solubilized in ethanol caused the appearance of medium chain FAs such as $C_{10:0}$ and $C_{11:0}$ and long chain FAs, *i.e.* C_{19iso}, C_{19ante} and $C_{20:0}$. Moreover, a marked reduction of the relative percentages of iso and anteiso branched FA having 15 and 17 carbon atoms counteracted by the increase of $C_{18:1cis9}$ and $C_{18:1\ cis11}$ was observed under this growth condition. Also the growth in the presence of 20 ppm of 2-(*E*)-hexenal provoked the diminution of the relative percentages of iso and anteiso branched FA having 15 and 17 carbon atoms and the increase of $C_{18:1cis9}$. However, in the presence of 2-(*E*)-hexenal a relevant increase of $C_{16:0}$ and $C_{18:0}$ was observed. The FFA relative percentages of *S. aureus* F1 in relation to the growth conditions adopted indicated that the presence of hexanal and 2-(*E*)-hexenal increased the proportions mainly of $C_{14:0}$, $C_{15:0}$, $C_{18:1cis\ 9}$, $C_{18:1cis11}$, C_{19ante} (data not shown).

S. enteritidis E5 modified the membrane FA composition in response to the stress conditions applied mainly increasing the relative percentages of unsaturated FAs such as $C_{18:1trans}$ and $C_{18:2}$ as well as of specific saturated FA, *i.e.* $C_{16:0}$ (Table 4). On the contrary, the cyclopropanic FA (CFA) relative percentages were not significantly affected by the adopted chemical stresses. The occurrence of specific branched FAs (*i.e.* C_{16iso} and/or C_{11iso}) was observed only in the presence of the tested aldehydes. The growth in the presence of the sub-lethal chemical stresses increased the relative percentages in the FFAs of $C_{16:0}$, $C_{18:1cis}$ and $C_{18:1trans}$ and $C_{18:2}$ (data not shown). Under the same growth conditions, the decrease of $C_{14:0}$ relative percentages was observed. Only in the FFAs of cells exposed during growth to hexanal, 11 carbon atom branched chain FA were detected (data not shown).

Also *E. coli* 555 modulated membrane FA composition in response to stress exposure (Table 5) decreasing the cyclopropanic FAs relative percentages and increasing those of specific unsaturated FAs such as $C_{18:1cis}$ and $C_{18:1trans}$. A relevant increase of C_{16iso} and $C_{18:2}$ coupled with the decrease under the detection limit of C_{19cyc} characterized the FA profiles of the cells subjected to 2-(*E*)-hexenal exposure, while C_{11iso} was detected only in the cells exposed to hexanal. The FFA profiles of *E. coli* 555 showed that the exposure to the two aldehydes provoked mainly a diminution of the relative percentages of CFA, $C_{18:1}$ (cis and trans isomers) and $C_{18:2}$ accompanied by the marked increase of $C_{16:0}$ as well as the appearance of $C_{20:0}$ (data not shown).

Table 6
Cellular thiobarbituric acid reactive substance (TBARS) detected in the four strains in relation to the stress conditions applied

	S. enteritidis E5	*E. coli* 555	*S. aureus* F1	*L. monocytogenes* Scott A
Control	2.03	1.71	2.16	2.89
Ethanol[a]	2.32	1.59	2.22	2.91
Ethanol+hexanal[b]	1.49	1.38	2.25	3.12
Ethanol+2-(*E*)-hexenal[c]	1.65	1.91	2.51	2.73

The values are expressed as malodialdehyde (μmol mg protein^{-1}). The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 5 and 7%.
[a] Concentration employed 1000 ppm.
[b] Concentration employed 150 ppm.
[c] Concentration employed 20 ppm.

Table 7
Volatile compounds (expressed as peak area × 10^6) detected for *Escherichia coli* 555 in relation to the different stress conditions applied

	Control	Ethanol [b]	Ethanol + hexanal [c]	Ethanol + 2-(*E*)-hexenal [d]
Ethanol	23	26	90	98
Disulphyde	9	7	10	–
Hexanal	– [a]	–	150	–
Benzene	25	9	7	14
1-butanol	6	4	–	–
p-xilene	17	28	36	44
Heptanal	5	1	–	–
1,2-dymethyl benzene	51	25	21	20
2-hydroxy-*2*-decenoic acid lactone	49	23	29	–
Acetic acid	3	2	47	–
Octanal	8	4	3	–
Isoamylic acid	–	–	–	7
Limonene	–	–	–	12
2-(*E*)-hexenal	–	–	–	34
2,5-dymethyl pirazine	64	47	34	57
Hexanol	7	4	755	150
2-(*E*)-hexen-1-ol	–	–	–	324
2-(*Z*)-hexen-1-ol	–	–	–	38
Nonanal	13	5	16	–
Hexanoic acid	–	–	7	–
Trisulphyde	–	3	7	–
Octanoic acid	–	–	3	–
Heptanol	–	–	3	–
3-ethyl-2,5-dymethyl pirazine	29	19	16	25
Decanal	8	–	10	8
Octanol	2	11	14	17
3-hexen-1-ol	–	–	222	–
Benzaldehyde	27	5	–	5
Undecan-5-ol	4	3	–	–
3-decen-2-one	9	7	–	8
2-(5*H*)-furanone	–	–	26	–
Nonanol	–	2	6	3
Octenal	–	–	665	6
Tiophene	–	–	615	–
2-furan methanol	7	3	–	4
1-decanol	–	109	–	–
Undecanol	–	3	–	–
3-cyclo hexenol	11	–	–	–
1-dodecanol	3	–	–	23

The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 5 and 10%.
[a] Undetectable level.
[b] Concentration employed 1000 ppm.
[c] Concentration employed 150 ppm.
[d] Concentration employed 20 ppm.

3.2. Cell lipid oxidation changes induced by sub-lethal concentrations of hexanal and 2-(E)-hexenal

TBARS (expressed as μmol malondialdehyde mg protein^{-1}) are regarded as an approximate measure of lipid oxidation and have been used to study the extent of damage in the cells subjected during the growth to the chosen chemical stresses. The levels of TBARs in the four strains were not markedly affected by the stress conditions adopted (Table 6). In fact, the TBARs values recorded in the controls were similar to those detected in the cells subjected to stress exposures.

3.3. Volatile profile changes induced by sub-lethal concentrations of hexanal and 2-(E)-hexenal

The supplementation of the growth media with hexanal and 2-(*E*)-hexenal provoked marked modifications of the GC–MS-SPME profiles of the tested strains. As expected, the volatile profiles of the strains differed according to the species. With respect to the controls and the cultures added with ethanol, which were characterized by GC profiles accounting for a weak release of metabolites, the exposure to hexanal and 2-(*E*)-hexenal resulted in the release of enhanced amounts of several molecules. In particular, aldehydes, hydrocarbons, pyrazines and alcohols were the principal families of metabolites. As shown by Tables 7 and 8, the supplementation of the growth medium with hexanal and 2-(*E*)-hexenal resulted in the gram-negative species considered in the marked increase of hexanol and aldehydes (saturated or unsaturated). The occurrence of tiophenes as well as of the 2-(*E*)-hexenal detoxification products, such as 2-(*E*)-hexen-1-ol and 2-(*Z*)-hexen-1-ol, charaterized the SPME-GC profile of *E. coli* subjected to the unsaturated aldehyde exposure, while a not completely identified 2-*(5H)*-furanone was detected in volatile profile of

Table 8
Volatile compounds (expressed as peak area × 10^6) detected for *Salmonella enteritidis* E5 in relation to the different stress conditions

	Control	Ethanol [b]	Ethanol + hexanal [c]	Ethanol + 2-(*E*)-hexenal [d]
Ethanol	22	25	100	5
Hexanal	– [a]	–	163	–
2-pentyl furane	–	–	43	–
Acetic acid	–	–	14	–
Limonene	4	3	16	–
2-(*E*)-hexenal	–	–	–	28
2,5-dymethyl pirazine	45	9	60	40
Hexanol	2	4	675	29
Nonanal	10	7	85	28
Octanoic acid	4	5	13	4
Heptanol	3	4	–	–
3-ethyl-2,5-dymethyl pirazine	9	10	32	–
Decanal	–	–	8	3
Octanol	6	5	19	5
2-hexen-1-ol	–	–	3	–
Benzaldehyde	4	3	9	4
2-(5*H*)-furanon	–	–	11	–
Octenal	5	2	1110	8
1-decanol	–	–	87	24
Decenol	–	–	9	–
1-dodecanol	–	–	727	–

The results are means of three independent experiments. The coefficients of variability, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 5 and 10%.
[a] Undetectable level.
[b] Concentration employed 1000 ppm.
[c] Concentration employed 150 ppm.
[d] Concentration employed 20 ppm.

S. enteritidis grown in the presence of hexanal. In the GC–MS-SPME profile of The increase of hexanol and aldehydes was observed also in *L. monocytogenes* Scott A and *S. aureus* F1 (data not shown).

4. Discussion

Bacterial cells have developed efficient protection systems to cope with a variety of physicochemical unfavorable conditions and to adapt to the environmental stresses. In particular, fundamental for the microbial cells is to maintain membrane integrity and functionality in response to environmental stresses. In response to stresses, the glycerolphospholipids can alter their acyl chain structure by changing the ratio of saturation to unsaturation, cis to trans unsaturation, branched to unbranched structure and type of branching and acyl chain length (Russel, 1984). Different modulation mechanisms can be used by different strains of the same species also in relation to the physiological state of the cells (Rock and Cronan, 1996).

As expected, the four strains showed sound differences in the modulation of membrane FA composition. Although a uniform response was not identified, the comparison of the FA profiles of the different strains obtained in the different stress conditions evidenced the crucial role of specific unsaturated FAs. In fact, the increase of $C_{18:1}$ and $C_{18:2}$ relative percentages was observed both in the gram-positive and gram-negative tested strains. However in the gram-negative strains chemical stress response also the cis/trans isomerization seems to play a key role. In fact, the increase of $C_{18:1trans}$ was observed both in *E. coli* and *S. enteritidis* cells exposed to ethanol, hexanal and 2-(*E*)-hexenal. On the other hand the isomerization of double bonds is reported to confer membrane chemical stability and protection against toxic molecules (Härtig et al., 2005). Although the synthesis of CFA is reported to be increased by a change in environmental conditions, entry into the stationary growth phase and acidic conditions in several gram-negative strains (Grogan and Cronan, 1997; Bruce et al., 1995; Yuk and Marshall, 2004), the CFA relative percentages decreased both *E. coli* and *S. enteritidis* cells exposed to chemical stress considered. Hexanal and 2-(*E*)-hexenal displayed different effects on *L. monocytogenes* and *S. aureus*. In fact, while a significant increase of pair long chain saturated FA *i.e.* $C_{16:0}$, $C_{14:0}$, and $C_{18:0}$ were observed in the presence of 2-(*E*)-hexenal, hexanal strongly affected the biosynthesis of branched chain FAs (C_{15} and C_{17}) as demonstrated by the accumulation of precursors (Russell et al., 1995; Annous et al., 1997) such as C_{11iso} both in total FAs and FFAs. The increase of $C_{18:1}$ and $C_{18:2}$ chracterised also the FA profiles of the two gram-positive strains considered. The crucial role of unsaturated FAs in the microbial stress response mechanisms was previously reported by other Authors. In particular, Guerzoni et al. (2001) showed that the increase of an oxygen-consuming desaturase system, with a consequent increase in fatty acid desaturation, is a cellular response to environmental stresses able to protect the cells of *Lactobacillus helveticus* from toxic oxygen species and high temperatures. Also Chatterjee et al. (1997) and Chatterjee et al. (2000) correlated an increase in the presence of UFAs in yeast cells with a decrease in the responsiveness of the stress response promoter element (STRE)-driven gene to heat and salt stresses. In fact, yeast cells supplemented with linoleic acid required a further 6 °C temperature increase or 200 mM higher salt concentration to maximally induce stress response elements, demonstrating that unsaturation level influences the expression of STRE-driven genes. Moreover, in bacterial cells the interaction between stress response proteins and lipid membrane was described by Torok et al. (1997), who demonstrated the influence of composition and the physical state of the phospholipid bilayer on the binding of chaperonins (GroES–GroEL oligomers) to the cellular membrane.

Usually, 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). Epoxides of long chain fatty acids were detected in cell membrane of *L. helveticus* after 100 min exposure to sub-lethal combinations of temperature, NaCl, H_2O_2 and pH (Guerzoni et al., 2001). For this reason the TBARs were taken as a measure of the oxidative damage. However the TBARs values did not increase in the cells grown in the presence of ethanol, hexanal and 2-(*E*)-hexenal, due to the detoxification mechanisms of the growing cells. In fact, it is well known that the epoxides react readily with a great number of cell compounds, including proteins and DNA. Therefore, they have to be rapidly detoxified and eliminated by specific hydrolases or glutathione transferases (Swaving and de Bont, 1998). On the contrary, the growth in presence of the considered molecules resulted in a dramatic increase of the amounts in the GC-SPME profiles of hexanol, deriving from the detoxification of hexanal in the growth media, as well as of aldehydes, that are regarded as end products of the breakdown of peroxidated unsaturated fatty acids. Moreover, the occurrence of molecules such as tiophene and a not yet completely identified 2-*(5H)*-furanone, which are reported to be involved in the cell signalling mechanism (Ndagijimana et al., 2006) in some microorganisms, suggests that other more specific response mechanisms are involved.

In conclusion the findings of this work contribute to the comprehension of the membrane FA modulation mechanisms used by the different strains in relation to the exposure to sub-lethal concentrations of hexanal and 2-(*E*)-hexenal. However, a deeper investigation is necessary to clarify if the changes in membrane FA compositions induced by the two aldehydes are the consequences of or the trigger for stress-related gene expression.

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