Food Chemistry 111 (2008) 707–718

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Mechanism of action of *Melaleuca armillaris* (Sol. Ex Gaertu) Sm. essential oil on six LAB strains as assessed by multiparametric flow cytometry and automated microtiter-based assay

El Akrem Hayouni [a,b,*], Marielle Bouix [c], Manaf Abedrabba [d], Jean-Yves Leveau [c], Moktar Hamdi [a]

[a] *Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de la Technologie (INSAT), BP. 676, 1080 Tunis, Tunisia*
[b] *Département des Industries Agro-alimentaires, Institut Supérieur des Etudes Technologiques de Zaghouan, 1121 Mograne, Tunisia*
[c] *AgroParisTech, INRA, UMR 782 Génie et Microbiologie des Procédés Alimentaires, CBAI, 78850 Thiverval-Grignon, France*
[d] *Unité de Recherche Physico-chimie et Moléculaire, IPEST, La Marsa, Tunisia*

ARTICLE INFO

Article history:
Received 11 November 2007
Received in revised form 2 March 2008
Accepted 20 April 2008

Keywords:
Melaleuca armillaris
Essential oil
LAB
Antibacterial
Automated microtiter assay
Multiparametric flow cytometry
cFDA
PI

ABSTRACT

Little is known concerning the effects of essential oils (EOs) against lactic acid bacteria (LAB), either of the human gastrointestinal microflora or those involved in industrial processes. GC and GC/MS analysis of *Melaleuca armillaris* EO resulted in the identification of 68 compounds comprising 99.6% of the oil. Eucalyptol (1,8-cineole) was the major compound (68.9%) and the composition was largely dominated by the oxygenated monoterpenes fraction (77.3%). The anti-LAB activities of the EO were first checked by the disc diffusion assay. In a second phase, time-survival kinetics of each strain incubated with increasing concentrations of the EO (0.25, 2.5, 5 and 25 μg/ml) were established using an automated microtiter assay (Bioscreen C). Bacteriostatic or bactericidal effects were noticed depending on the studied strain and on the applied concentration of the EO. The mathematical modelling of the kinetics showed that in presence of increasing concentrations of *M. armillaris* EO, the lag phases of growth were extended (0.69%–97.5%) and both the growth rate and final cell density were reduced. Variations depending on the strain were noticed.

Live/dead assays of the multiparametric flow cytometry technique (combining carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) fluorescent probes) were performed by dual staining of each sample culture to differentiate viable, dead and stressed cells. The behaviour of each strain, in presence of increasing concentrations of *M. armillaris* EO, was evaluated by quantifying the relative percentages of each subpopulation throughout 3 days of culture. Results displayed disparate patterns of subpopulations which revealed dynamic changes in cell behaviour. This is probably due to disparate influences of the EO components on cellular physiological properties throughout the incubation period. This study proved that multiparametric flow cytometry was a convenient and rapid tool to evaluate the viability of LAB, and was well correlated with plate count results. Such study could be useful to understand how to fully take advantage of LAB as probiotics or as potential candidates to improve food hygiene and to assure food quality; namely when they are associated with natural preservatives such as EOs.

1. Introduction

Lactic acid bacteria (LAB) are found in many nutrient rich environments and occur naturally in various food products (dairy and meat products, vegetables) or are added as pure cultures to various food products (Carr, Chill, & Maida, 2002). It has been estimated that 25% of the European and 60% of many developing countries diets consist of fermented foods (Stiles, Penkar, Plockova, Chumchalova, and Bullerman, 2002). Besides, it is well known that LAB, among a large population of microorganisms, inhabit the human gastrointestinal tract (GIT) and form a closely integrated unit with the host called the intestinal microbiota. Many reports show the usefulness of LAB as probiotics for humans and animals (Hamilton-Miller, 2003).

Plants, herbs, spices and their derived essential oils (EOs) or isolated compounds are known to retard or inhibit the growth of bacteria, yeast and moulds (Burt & Reinders, 2003), although many of them are not yet completely exploited. The greatest use of EOs in the European Union is in food (as flavourings), perfumes (fragrances and aftershaves) and pharmaceuticals (for their functional properties). The antimicrobial action of EOs in food systems is well

* Corresponding author. Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de la Technologie, BP. 676, 1080 Tunis, Tunisia. Tel.: +216 974 71160; fax: +216 7266 0300.
E-mail address: a_hayouni@yahoo.com (E.A. Hayouni).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.04.044

known in the literature. Their use can be considered an additional intrinsic determinant to increase the safety and shelf life of foods (Nychas, 1995). Furthermore, the possible modes of action of EOs, as antimicrobials, have been reported in various reviews. However, the mechanisms of action have not been completely elucidated (Lambert, Skandamis, Coote, & Nychas, 2001). Over the years, several methods have been developed to measure viability and vitality of microbes under various stresses (Attfield, Kletsas, Veal, Van Rooijen, & Bell, 2000): plating, slide culture, vital stains, metabolic activity or other method to assess viability, and metabolic activity, cell components, fermentation capacity, acidification potential or oxygen uptake ability to assess vitality. Such methods are time-consuming and labour-intensive. When survival of a bacterial population after exposure to an environmental stress is assessed by classical colony forming units (CFUs), a binary logic is imposed for the determination of viability. Live cells will be those that managed to replicate under the particular experimental conditions, while all the others will be presumed dead (Kell & Young, 2000). Nowadays, it is well documented that under such conditions a population will exhibit cell subpopulations with phenotypes that most likely escape this two-value logic. Injured, viable but not culturable (or active but not culturable as otherwise stated), dormant cells etc., cannot be easily traced or even detected by traditional CFUs (Kell & Young, 2000). For all the reasons mentioned above, fluorescence techniques combined with direct optical detection methods for the rapid assessment of bacterial viability are increasingly being favoured (Bunthof, Bloemen, Breeuwer, Rombouts, & Abee, 2001; Ueckert et al., 1995). They are highly sensitive and have high time resolution (Breeuwer & Abee, 2000). Among these, flow cytometry has been shown to be a powerful tool for analyzing populations rapidly on a cell-by-cell basis thus allowing multisample processing. When fluorescence labelling of the cells is rapid, flow cytometry facilitates near real time monitoring, which is a very important feature for many industrial processes (Hewitt & Nebe-Von-Caron, 2001). The most widely used dye for the assessment of cell viability is the esterase substrate carboxyfluorescein diacetate (cFDA). Another probe used for mortality studies is the nucleic acid dye, propidium iodide (PI). It is excluded by cells having intact membranes, but can enter cells with compromised membranes (Breeuwer & Abee, 2000; Bunthof et al., 2001). Multiparametric flow cytometry that combines these two probes simultaneously is described as a powerful and sensitive tool for measuring the viability of stressed bacteria. Double stained cells correspond to cells having both enzymatic activity and compromised membranes, and are considered as stressed cells (Ben Amor et al., 2002). Nevertheless, this method has never been applied to the elucidation of LAB cells behaviour when exposed to EOs.

The genus *Melaleuca* approaches *Eucalyptus* in both abundance and diversity among Myrtaceae genera. Commercially useful EOs are sourced from the broadleaved *Melaleuca quinquenervia* (niaouli oil) and *Melaleuca cajuputi* (cajuput oil) and the small-leaved *Melaleuca alternifolia* (Southwell & Lowe, 1999). *Melaleuca armillaris* is one of the most widely cultivated *Melaleucas*. It is commonly known as the Bracelet Honey Myrtle and grows into large spreading shrub or small tree.

The use of herbs, spices and various plant extracts, namely EOs could interfere with the mechanisms involved in the LAB health-promoting activities. In light of this statement, no works have explored the behaviour, even at *in vitro* level, of LAB among human gastrointestinal microflora, when incubated with EOs. On the other hand, rational selection of a strain as a starter or adjunct starter culture requires knowledge of its basic physiology that will elucidate whether it will be able to withstand and perform under harsh environments encountered during food processing and storage (Van de Guchte et al., 2002). On the basis of these considerations, and taking into account that there is limited, if any, information relevant to the use of *M. armillaris* EO, even as a flavouring agent or as a preservative, this work had focused on (i) the anti-LAB activity of *M. armillaris* EO as correlated to its chemical composition, (ii) the use of an *in vitro* automated technique (Bioscreen) in order to establish the time-survival kinetics of these bacteria when incubated, during three days, with increasing concentrations of *M. armillaris* EO and (iii) in parallel, the mode of action of the EO against the six studied LAB was assessed by a multiparametric flow cytometry technique. Results obtained by such a technique were compared to those of the CFUs method.

2. Materials and methods

2.1. Collection of plant materials and isolation of the essential oil

The leaves of *M. armillaris* (Sol. Ex Gaertu) Sm. were collected, between March and May 2006, from the botanical garden of the National Institute of Agronomic Research (INRAT, Tunis). Specimens were identified by Dr. Nadia Ben Brahim at the Department of Botany, National Institute of Agronomic Research (INRAT, Tunis) and a voucher specimen was deposited at the Herbarium of the Department of Botany in the cited institute. The air-dried and finely grounded raw materials were submitted to water distillation for 4 h, using classic apparatus. The obtained essential oils were dried over anhydrous sodium sulphate and, after filtration, stored at 4–7 °C until use. Yield of 2.35% (v/w) was recorded.

2.2. Gas chromatography analysis/mass spectrometry analysis conditions

2.2.1. Gas chromatography analysis

The essential oil was analyzed using a Hewlett–Packard 5890 II GC equipped with flame ionization detector (FID) and HP-5 MS capillary column (5% phenyl/95% dimethylpolysiloxane: 30 m × 0.25 mm i.d., film thickness 0.25 μm) or HP-Innowax capillary column (Polyethyleneglycol: 30 m × 0.25mm i.d., film thickness 0.25 μm). Injector and detector temperature were set at 250 and 280 °C, respectively. Oven temperature was kept at 50 °C for 1 min then gradually raised to 250 °C at 5 °C/min and subsequently, held isothermal for 4 min. Nitrogen was the carrier gas at a flow rate of 1.2 ml/min. Diluted samples (1/100 in hexane, v/v) of 1.0 μl were injected manually and in the splitless mode. Quantitative data were obtained electronically from FID area percent data without the use of correction factors.

2.2.2. Gas chromatography analysis/mass spectrometry analysis

Analysis of the oil was performed using a Hewlett–Packard 5890 II GC, equipped with a HP 5972 mass selective detector and a HP-5 MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). For GC/MS detection, an electron ionization system, with ionization energy of 70 eV, a scan time of 1.5 s and mass range 40–300 amu, was used. Helium was the carrier gas at a flow rate of 1.2 ml/min. Injector and transfer line temperatures were set at 250 °C and 280 °C, respectively. Oven program temperature was the same as with GC analysis. Diluted samples (1/100 in hexane, v/v) of 1.0 μl were injected manually and in the splitless mode. Mass spectrometer ChemStation Software was used to handle mass spectra and chromatograms. The identification of the compounds was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library) or with authentic compounds and confirmed by comparison of their retention indices, either with those of authentic compounds or with data published in the literature as described by Adams (2001). Further confirmation was done from Kovats Retention Index data generated from a series of alkanes

retention indices (relative to C_9–C_{28} on the HP5 and HP-Innowax columns).

2.3. Antimicrobial activity

2.3.1. Microbial strains and culture conditions

Melaleuca armillaris EO was individually tested against a panel of six LAB: *Lactobacillus pentosus, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus delbrueckii* subsq. *bulgaricus* CNRZ 208T and *Lactobacillus sakeii* 23 K. The three first strains were isolated and identified in our laboratory, the fourth and the fifth strains were kindly provided by Prof. Bouix, M. (AgroParistech, France) and Prof. Zagoreć, M. (INRA, France). The bacteriological agar was from Biokar Diagnostics (Beauvais, France). MRS broth and agar were from DIFCO Laboratories.

2.3.2. Antimicrobial screening using disc diffusion method

The paper disc diffusion method was employed for the determination of antimicrobial activities (NCCLS (National Committee for Clinical Laboratory Standards), 1999). Briefly, suspension in MRS of the tested microorganism (0.1 ml of 10^6–10^7 cells per ml) was spread on the solid MRS media plates. Paper discs (6 mm in diameter) were impregnated with 20 μl of the oil and placed on the inoculated plates. These plates, after remaining at 4 °C for 2 h, were incubated at 37 °C for the four first strains, at 42 °C for *L. delbrueckii* and at 30 °C for *L. sakeii*. Incubation period was 24 h for all strains. The diameter of the inhibition zones were measured in millimetres. The antibiotic oxytetracyclin (100 μg/disc) was used as positive control. All the tests were performed in duplicate and repeated twice.

2.3.3. Inhibition of bacterial strains by M. armillaris EO as assessed by automated microtiter-based assay

Overnight cultures of the test organisms were diluted to approximately 1×10^6 cfu/ml in MRS broth and 150 μl volumes added to each well of a 100-well microtiter plates (Honeycomb Format 100-well Multi Well Plates). The stock solution (100 μg/ml) of the *M. armillaris* EO was diluted, in DSMO (0.1% as highest final concentration), and 50 μl volumes were dispensed into the wells. Appropriate final concentrations were obtained in designed wells (25, 5, 2.5 and 0.25 μg/ml). All tests were conducted in duplicate and controls included as appropriate with one growth control (MRS + DMSO) and one sterility control (MRS + DMSO + test oil). The negative controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution (0.1%).

Microtiter plates were incubated at the optimum growth temperature for the test organism for 3 days in a plate reader (Multiskan Ascent Microtiterplate Reader, LabSystems, Bioscreen C, Finland) with absorbance readings (600 nm) taken every 30 min. A software Biolink, integrated to the plate reader allowed an automated data recording.

2.3.4. Measurement of cultirability

Culturability of cells was evaluated by plate counts and compared with cytometric results. After serial dilutions in physiological water, cells were plated onto solid MRS plates and incubated at corresponding optimal temperatures for 48 h under anaerobic conditions (Genbox 96124, bioMérieux, Marcy l'Etoile, France). Each result was the geometrical mean of at least three counts.

2.4. Flow cytometry study

2.4.1. Fluorescent probes and staining protocols

Carboxyfluorescein diacetate (cFDA) was used to assess LAB viability. It is a non-fluorescent precursor that readily diffuses across intact cell membranes and yields the positively charged green fluorescent compound, carboxyfluorescein (cF: it has a maximum excitation wavelength of 492 nm and an emission wavelength of 517 nm), upon hydrolysis by non specific cellular esterases. Labelled cells are characterized by efficient enzymatic activity and are considered as viable cells. The nucleic acid dye, propidium iodide (PI), made it possible to quantify damaged and dead cells. PI binds to DNA to form a red fluorescent DNA-complex with a maximum excitation wavelength of 535 nm and an emission wavelength of 617 nm. Stained cells correspond to cells having compromised or destroyed membranes and are considered as dead.

Before staining, cell suspensions of a given strain were diluted in Chemsol B4 buffer (AES-Chemunex, Combourg, France) to reach approximately 10^6 cells/ml. One milliliter of the diluted suspension was supplemented with 10 μl of cFDA (0.0217 mM in acetone, Invitrogen-Molecular Probes, Eragny-sur-Oise, France) and incubated for 10 min at optimal growth temperature for viability assessment, or with 10 μl of PI (1.496 mM in distilled water, Sigma–Aldrich, Lyon, France), and incubated for 30 min at optimal growth temperature for membrane integrity assessment. Live/dead assays were done by dual staining of each sample to differentiate viable, dead and stressed cells. The same dye concentrations were used. The diluted suspension was first incubated with 10 μl of PI for 20 min. Ten microliters of cFDA were then added and incubation took place for 10 min. After short centrifugation at 14,000*g* for 1 min (Microfuge E., Beckman Coulter, Roissy, France), cell pellets were resuspended in 1 ml of Chemsol B4 buffer before analysis by flow cytometry. Unstained cells which were preliminary not treated or incubated, during a fixed period with the EO, were analyzed by flow cytometry and used as negative controls.

2.4.2. Flow cytometry conditions and data analyses

Flow cytometry analyses were performed with a FAC Scan flow cytometer (Becton–Dickinson, Le Pont de Claix, France). FACSFlow solution (Becton–Dickinson) was used as sheath fluid and the cytometer was adjusted to count 10,000 fluorescent events. The FACScan was equipped with an air-cooled argon ion laser, emitting at 488 nm, and five band-pass filters: a forward-angle light scatter (FSC) combined with a diode collector, a side-angle light scatter (SSC) and three fluorescence signals, collected with photomultiplier tubes, a 530 nm band-pass filter (515–545 nm) to collect the green fluorescence of cF (FL1 channel), a 585 nm band-pass filter (564–606 nm) to collect the yellow–orange fluorescence (FL2 channel) and a 670 nm long pass filter to collect the red fluorescence of PI (FL3 channel). The flow cytometry analyses were performed by using logarithmic gains and specific detectors settings. Measurement on a log scale pulls cells of similar size together, so that the resulting dot plot is more compact. Thus, it is easier to draw the gating regions to discriminate between viable and dead cells. Moreover, a combination of FSC and SSC was used to discriminate bacteria from the background and a threshold was set on the FSC signal. Data were collected and analyzed with CellquestPro software (Becton–Dickinson). The subpopulations were identified using dot plots. Gates were defined in the dot plots of FSC, SSC, green fluorescence and red fluorescence, thus allowing the software to separate the different events. Before analysis, detectors were adjusted and compensation setting was performed on a sample with unstained cells. The corresponding signal on the dot plots was set in the lower left quadrant in order to eliminate cellular autofluorescence. Data were analyzed with the aid of statistical tables, which indicated the percentages of stained cells determined by each detector.

2.5. Statistical analysis

Diameters of inhibition zones were expressed as means ± SE of four values. In the automated microtiter assay, values of optical

density (OD) were expressed as average of two replicate growth curves. Growth curves were fitted with the function of Baranyi, Roberts, and McClure (1993) to estimate the main growth parameters namely the growth specific rate (μ), lag time (λ). The program also calculates the goodness of fit (R^2) for each curve performed. Statistical comparisons between variables (e.g. inhibition zones diameters) were performed with Student's *t*-test. The relationship between flow cytometry analyses and plate count determinations was established from a large number of measurements of viable and stressed cells as a function of culturability measurements. In this case, data analysis was carried out using the correlation and regression in the EXEL program. Differences were considered significant at $p \leqslant 0.05$.

3. Results and discussion

3.1. Chemical composition of the essential oil

The air-dried and finely grounded leaves of the plant were subjected to hydrodistillation using classic apparatus yielding 2.35% EO. This yield is close to results recorded for *Melaleuca ericifolia* Sm. (2.58%) (Lee, Annis, Tumaalii, & Choi, 2004). In the same work, we noticed yields (on dry basis) ranging between 0.29% and 7.59% from leaves and twigs of several *Melaleuca* species. Particularly, a yield of 4.66% for *M. armillaris* (Sol. Ex Gaertu.) Sm. was recorded.

GC and GC/MS analysis resulted in the identification of 68 compounds, representing 99.6% of the oil (Table 1). As usually occurs in the Myrtaceae family; 1,8-cineole (eucalyptol) was dominant among the major components (>1%) of the oil (68.9%), followed by α-pinene (7.40%), which is closely followed by borneol (6.16%), β-pinene (2.94%), β-myrcene (2.79%), β-elemene (1.74%) and aromadendrene (1.43%). As we can see, the oil composition is largely dominated by the oxygen-containing monoterpenes (77.3%) followed by the monotrepene hydrocarbons components (15.3%). The sesquiterpenes molecules were less abundant with 6.11% for the sesquiterpenes hydrocarbons and only 0.95% of oxygen-containing sesquiterpenes.

Although, there are a lot of studies concerning *Melaleuca alternifolia* (tea tree), reports about *Melaleuca armillaris* remain scarce. Lee et al. (2004) reported a similar pattern being the major components: 1,8-cineole (42.77%), terpinene-4-ol (15.97%), α-thujene (2.07%), γ-terpinene (8.86%), α-terpineol (4.56%), limonene (4.54%), sabinene (3.55%), and α-pinene (2.52%). In another work, it was reported that the EO of *M. ericifolia* contained methyl eugenol (96.84%) as a major constituent, whereas *Melaleuca leucadendron* was rich in 1,8-cineole (64.30%). In parallel, the EO of *M. armillaris* was rich in 1,8-cineole (33.93%) followed by terpinen-4-ol (18.79%), whereas *Melaleuca styphelioides* was rich in caryophyllene oxide (43.78%) and (−) spathulenol (9.65%) (Farag et al., 2004). Such discrepancy in the composition could be understood since several authors have documented significant species with specific variations in the concentrations of detected compounds and/or presence of others in high concentrations. Moreover, the EO composition of *M. arimillaris*, as occurs with other medicinal and aromatic plants, is highly influenced by genetic and environmental factors (climatical, seasonal, geographical, and geological) (Perry et al., 1999).

3.2. Antibacterial activity

3.2.1. Inhibition of bacterial strains as assessed by the paper disc diffusion assay

The *in vitro* antimicrobial activities of *M. armillaris* EO against the studied LAB strains were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameters. Results indicate that EO of *M. armillaris* exhibited a great potential of antibacterial activity against all tested LAB. Nevertheless, these strains were not inhibited at equal rates since it is clear that *L. casei* was the most sensitive bacterium, with a diameter of the inhibition zone of 23.83 mm ± 1.04. *L. sakeii* came in the second rank, with 16.83 mm ± 1.26, closely followed by *L. delbrueckii* (15.33 mm ± 1.53). *Lactobacillus planatrum* was less sensitive to the EO with an inhibition zone diameter of 13.5 mm ± 1.32. On the other hand, among the studied strains, *L. pentosus* and *L. rhamnosus* were the most resistant to the volatiles. Corresponding diameters of their inhibition zones were 11.50 mm ± 0.87 and 10.33 mm ± 0.58, respectively.

There are few works on biological activities of *M. armillaris* EO, Farag et al. (2004) found that the EOs of four *Melaleuca* species (*M. armillaris* among them) possessed antimicrobial and antifungal activities. *M. ericifolia* exhibited the highest inhibitory effects against *Bacillus subtilis* and *Aspergillus niger*. The volatile oil of *M. armillaris* was found to be more effective as a virucidal (up to 99%) than that of *M. leucadendron* (92%) and *M. ericifolia* (91.5%). Nevertheless, as far as our literature could survey, there are no reports dealing with antibacterial activities of *M. armillaris* EO against LAB.

On correlating the anti-LAB activities to the chemical composition of the EO, it is difficult to associate such activities to a specific compound, due to their complexity and variability. The high antibacterial activities of *M. armillaris* EO could be attributed to its particular chemotype characterized by an abundance of eucalyptol and other oxygenated monoterpenes. The polarity of such molecules allowed them a better solubility in aqueous media and then a better diffusion in the agar. Moreover, the synergistic effects of these major chemicals with each other and minor constituents of the EO, as well as strain to strain variation (Marino, Bersani, & Comi, 2001), should be taken into consideration when comparing the effectiveness of *M. armillaris* EO in inhibiting the LAB growth.

3.2.2. Inhibition of bacterial strains as assessed by microdilution automated microtiter assay

The absorbance readings obtained from inhibitory assays were plotted against time to obtain the growth curves of the test organisms (Fig. 1 panels A–F). Individual inhibitory effects of *M. armillaris* EO were checked based on the analysis of three criteria: (a) the increase in lag phase, (b) the reduction in culture density at 72 h, and (c) the residual viability at 72 h.

Growth curves were fitted with the function of Baranyi et al. (1993) to estimate the main growth parameters namely the growth specific rate (μ), lag time (λ). The program also calculates the goodness of fit (R^2) for each curve performed. The increase in lag phase was normalised by expressing it as a percentage of the experiment running time (Fig. 2). The second criterion, reduction in culture density, was determined by subtracting the $OD_{600\ nm}$ of the control culture (untreated sample) and was normalised (ΔOD) by expressing it as a percentage of the $OD_{600\ nm}$ of the control culture (Table 2). The third factor, residual viability, was determined at 72 h by streaking out a loopful of each test culture (treated sample) onto appropriate bacteriological agar and incubating for 48 h at the corresponding optimum growth temperature (Table 2). As it appears in Fig. 1, *M. armillaris* EO exhibited a strong antibacterial activity against all tested LAB strains. This remained true no matter what the level of concentration assayed is. Nonetheless, when tested against *L. casei*, at 0.25 μg/ml, the EO had no antibacterial effect and this was the only exception (Fig. 1, panel C). Using the growth kinetics of each of the LAB strains, when exposed to increasing concentrations of *M. armillaris* EO (Fig. 1), and data concerning their growth parameters reported in Fig. 2 (relative to the extension of lag phases) and Table 2, one can do comparative analysis of their respective behaviour and make some interesting

Table 1
Chemical composition (%[a]) of the essential oil isolated from the leaves of *Melaleuca armillaris* (Sol. Ex Gaertu) Sm

No.	Compound	KI[b]		(%[a])	Method of identification[c]
		(BP-1)	(BP-20)		
1	Hexanal	801	1084	tr	MS,KI
2	2-Hexanal	860	1216	tr	MS,KI
3	Tricyclene	930	1015	tr	MS,KI
4	α-Thujene	935	1020	0.22	MS,KI
5	α-Pinene	940	1028	7.40	MS,KI
6	α-Fenchene	949	1059	0.09	MS,KI
7	Camphene	952	1071	tr	MS,KI
8	Sabinene	976	1124	0.40	MS,KI
9	β-Pinene	979	1113	2.94	MS.KI
10	β-Myrcene	991	1161	2.79	MS,KI
11	α-Phellandrene	1005	1168	0.17	MS,KI
12	δ-3-Carene	1012	1145	tr	MS,KI
13	α-Terpinene	1019	1186	0.31	MS,KI
14	p-Cymene	1025	1269	tr	MS,KI
15	β-Phellandrene	1032	1210	tr	MS,KI
16	1.8-Cineole	1033	1213	68.92	MS,KI
17	γ-Terpinene	1063	1245	0.98	MS,KI
18	2.5-Dimethylsterene	1071	1437	tr	MS,KI
19	α-Terpinolene	1089	1286	tr	MS,KI
20	Linalool	1098	1548	0.51	MS,KI
21	α-Thujone	1117	1431	0.30	MS,KI
22	Camphor	1145	1517	0.93	MS,KI
23	Borneol	1169	1712	6.16	MS,KI
24	Terpinen-4-ol	1179	1600	tr	MS,KI
25	p-Cymen-8-ol	1186	1848	tr	MS,KI
26	α-Terpineol	1191	1693	tr	MS,KI
27	Myrtenol	1200	1791	tr	MS,KI
28	Myrtenal	1209	1624	tr	MS,KI
29	Fenchyl acetate	1221	1473	0.06	MS,KI
30	Carveol	1228	1804	tr	MS,KI
31	Linalyl acetate	1262	1554	tr	MS,KI
32	Bornyl acetate	1288	1586	tr	MS,KI
33	4-Terpenyl acetate	1340	1695	tr	MS,KI
34	α-Cubebene	1352	1459	tr	MS,KI
35	α-Terpenyl acetate	1353	1695	0.40	MS,KI
36	α-Ylangene	1372	1467	0.89	MS,KI
37	α-Copaene	1378	1489	0.18	MS,KI
38	α-Bourbobene	1380	1544	0.07	MS,KI
39	β-Elemene	1388	1587	1.74	MS,KI
40	β-Cubebene	1390	1543	tr	MS,KI
41	Longifolene	1403	1574	0.13	MS,KI
42	α-Gurjenene	1409	1510	0.18	MS,KI
43	β-Gurjenene	1420	1677	tr	MS,KI
44	β-Caryophyllene	1427	1595	0.12	MS,KI
45	α-Cedrene	1433	1587	0.07	MS,KI
46	Aromadendrene	1438	1615	1.43	MS,KI
47	α-Humulene	1460	1668	tr	MS,KI
48	allo-Aromadendrene	1464	1638	0.48	MS,KI
49	β-Muurolene	1476	1668	tr	MS,KI
50	Germacrene-D	1484	1703	tr	MS,KI
51	β-Selinene	1488	1724	tr	MS,KI
52	α-Murrolene	1499	1711	0.16	MS,KI
53	β-Bisabolene	1508	1729	0.29	MS,KI
54	*cis* Calamenene	1512	1808	0.06	MS,KI
55	γ-Cadinene	1516	1756	tr	MS,KI
56	δ-Cadinene	1523	1755	0.15	MS,KI
57	Cadina-1.4-diene	1528	1774	0.14	MS,KI
58	α-Cadinene	1538	1756	tr	MS,KI
59	Elemol	1540	2069	tr	MS,KI
60	α-Calacorene	1545	1912	tr	MS,KI
61	Germacrene-B	1555	1813	tr	MS,KI
62	β-Calacorene	1560	1921	tr	MS,KI
63	β-Caryophyllene oxide	1588	1976	tr	MS,KI
64	T-Cadinol	1638	2164	tr	MS,KI
65	T-Muurolol	1642	2187	tr	MS,KI
66	α-Muurolol	1645	2192	0.35	MS,KI
67	α-Cadinol	1654	2200	0.60	MS,KI
68	Neophytadiene	1827	1933	tr	
Identified components (%)				99.63	
Grouped components					
Monoterpene hydrocarbons				15.28	
Oxygen-containing monoterpenes				77.29	
Sesquiterpene hydrocarbons				6.11	

(*continued on next page*)

Table 1 (*continued*)

No.	Compound	KI[b]		(% [a])	Method of identification [c]
		(BP-1)	(BP-20)		
Oxygen-containing sesquiterpenes				0.5	
Oil yield (%) (v/w)				2.35	

tr: traces (<0.1%).

[a] % percent peak area of essential oil components.

[b] KI: Kovats indices on non polar HP-5 column in reference to *n*-alkanes.

[c] Components were identified on KI and GC/MS (gas chromatograph coupled with mass spectrometry) and listed according to their elution on polar column BP-20 (30 m).

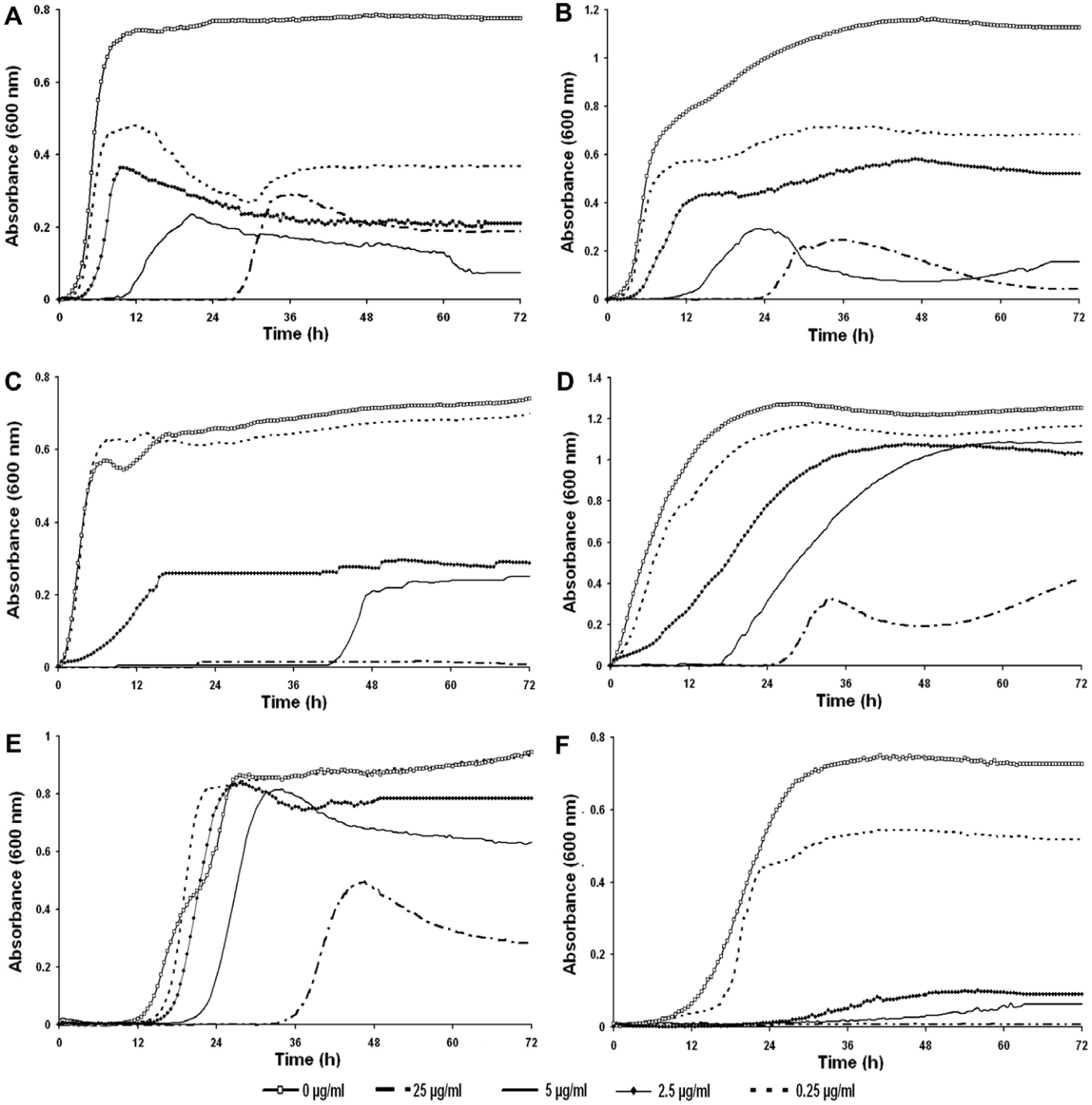

Fig. 1. Time-survival kinetics of the six LAB strains in absence or in presence of 0.25, 2.5, 5 and, 25 μg/ml of *M. armillaris* EO. Cultures were performed in MRS broth. Panel A: *L. pentosus* (37 °C), Panel B: *L. plantarum* (37 °C), Panel C: *L. casei* (37 °C), Panel D: *L. rhamnosus* (37 °C), Panel E: *L. sakeii* 23 K (30 °C), and Panel F: *L. delbrueckii subsq. Bulgaricus* CNRZ 208T (42 °C).

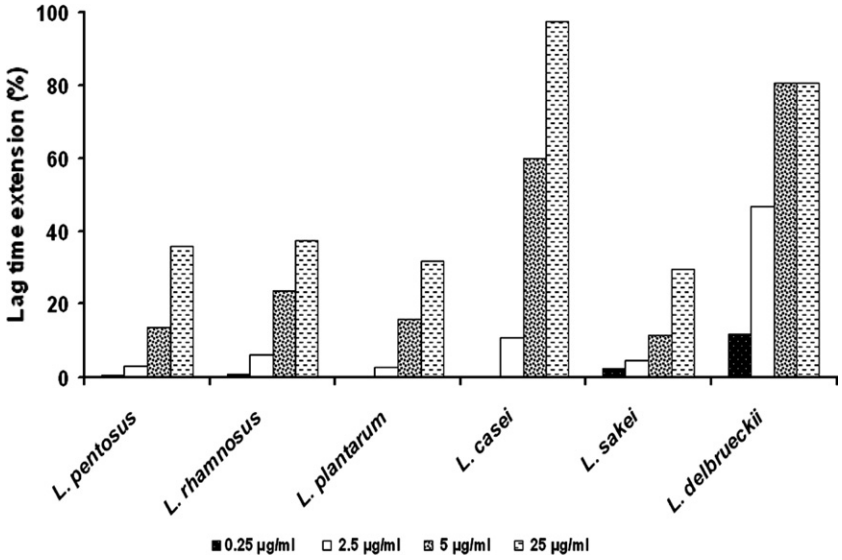

Fig. 2. Extensions in the lag phases of growth of the studied LAB cells when incubated with increasing concentrations of *M. armillaris* EO. Lag phases (λ) were first estimated using the model of model of Baranyi et al. (1993), and then they were normalised by expressing them as a percentage of the running time of the experiment (72 h). For example, if the control and test cultures took 2 and 10 h, respectively, to reach a ΔOD of 0.1, the increase in lag phase of 8 h was represented as 11.1% of 72 h.

Table 2
Growth parameters estimated using the model of Baranyi et al. (1993), for the cultures of the six LAB strains in MRS broth without and with increasing concentrations of *M. armillaris* EO

	0 µg/ml	0.25 µg/ml			2.5 µg/ml			5 µg/ml			25 µg/ml		
	µ	µ	ΔOD	V^a	µ	ΔOD	V^a	µ	ΔOD	V^a	µ	ΔOD	V^a
L. pentosus	0.177 (0.992)	0.123 (0.989)	52.5	82 1299	0.105 (0.995)	73.1	76 1189	0.021 (0.911)	90.6	61 1177	0.054 (0.937)	75.7	62 1143
L. rhamnosus	0.148 (0.988)	0.074 (0.971)	6.9	88 1267	0.04 (0.962)	17.7	82 1398	0.037 (0.953)	13.2	75 1375	0.048 (0.965)	65.7	63 1205
L. plantarum	0.139 (0.991)	0.123 (0.993)	51.8	98 1555	0.055 (0.992)	53.8	70 1323	0.027 (0.982)	86.2	58 1222	0.037 (0.972)	96	53 1056
L. casei	0.153 (0.989)	0.152 (0.987)	5.3	62 1733	0.022 (0.987)	61.2	93 1371	0.041 (0.965)	66.2	62 1304	NG	98.7	84 1222
L. sakei	0.117 (0.978)	0.15 (0.973)	1	82 1567	0.146 (0.899)	16.9	72 1565	0.095 (0.897)	32.9	61 1341	0.066 (0.952)	70	57 1211
L. delbrueckii	0.064 (0.967)	0.056 (0.982)	28.5	79 1538	0.004 (0.898)	87.5	67 1319	NG	91.3	59 1241	NG	99	50 1309

In the last columns (V) is given the relationship between the decrease in the percentage of viable cells (V, %) assessed by PI/cFDA double staining and the decrease of the bacterial concentration (N in CFU/ml) measured by plate counts. Data shown are the average of two replicate growth curves. µ: Maximum specific growth rate (h^{-1}). Value between brackets represents the coefficient of determination (R^2) for fit of the model data. ΔOD: reduction in culture density after 72 h of incubation with the EO. NG: no growth within 3 days.

[a] Relationship ($V = a + b \cdot \ln(N)$) between PI/cFDA double staining and plate counts were established for cultures after 72 h of incubation with the EO. Left and wright values represent the slope and the intercept, respectively.

observations. The most sensitive strain was *L. delbrueckii*, since no growth was noticed after 3 days of incubation along with 25 or 5 or 2.5 µg/ml of the EO (Fig. 1, panel F). *L. casei* came immediately after, since no growth was observed at 25 µg/ml during 72 h of incubation. So, in these cases the EO showed absolute growth inhibition with a considerably prolonged lag period compared to the lag phases estimated without the EO. Bacteriostatic or bactericidal effects were noticed depending on the studied strain as well as on the concentration of the EO.

According to results reported in Fig. 2, it is obvious that the relative extension of the lag phases was dose dependant. Nevertheless, such correlation varied from strain to strain. Hence, *M. armillaris* EO affects differently the growth and multiplication behaviour of the studied LAB. Indeed, one can notice that at 0.25 µg/ml EO, there was a weak relative extension of the lag phase, being inferior to 1% for *L. pentosus*, *L. rhamnosus*, *L. plantarum* and *L. casei*. Meanwhile, there was a 2.5% and 11.8% relative extension in the lag phase for *L. sakeii* and *L. delbrueckii*. In addition, when incubated at 2.5 µg/ml EO, the relative extension in lag phase was higher in the case of *L. delbrueckii* (46.7% which implies a delay of approximately 44 h) when compared to the other strains, namely *L. casei* (10.7% or a delay of 7.7 h). It is also important to mention that this extension was higher than those recorded with all the other strains (except *L. casei*) exposed, even at 25 µg/ml of *M. armillaris* EO. In addition, the longer recorded lag phases were obtained with *L. casei* when exposed to 25 µg/ml EO (97.5%); then came *L. delbrueckii* for which 80.56% extension in lag phase was noticed at 5 or 25 µg/ml EO. The above-mentioned results clearly show a close behaviour of *L. pentosus*, *L. rhamnosus*, *L. plantarum* and *L. sakeii* since there is no statistically significant difference between the percentages of their lag phase extensions. Meanwhile, when compared to these strains, *L. casei* and *L. delbrueckii* showed a disparate behaviour. Besides, Valero and Salmerón (2003) had studied the effects of the EOs of some spices and herbs at their MIC level, on *Bacillus ceureus* in tyndallized carrot broth. They observed that after the lag phase, once the growth and multiplication of the bacteria had started, the presence of these EOs at the concentrations tested had practically no effect on the growth rate. Such results were not noticed in this study since the recorded growth rates, were less than those of the controls, no matter were the strain and the concentration used (Table 2). The only exception was noticed when the EO was used, at 0.25 µg/ml against *L. casei* (the same rate as the control was obtained: 0.153 h^{-1}). Moreover, it is noteworthy that the growth rates were greater when the EO was applied at 0.25 and 2.5 µg/ml against *L. sakeii*, when compared to the control, even though; weak increases of 2.5% and 4.58% were

achieved in both cases. Concerning the reduction of the culture density, there was a proportional evolution of this parameter to the increase of the concentration. Nevertheless, this observation was not true in the case of *L. casei* which showed a reduction of the culture density of 90.6% and 75.7% at 5 μg/ml and at 25 μg/ml, respectively. The same observation was noticed for *L. rhamnosus*, which showed a reduction in the culture density higher at 2.5 μg/ml than at 5 μg/ml (17.7% and 13.2%, respectively).

Thus, it clearly appears that in presence of *M. armillaris* EO, the lag phases of growth were extended and both the growth rate and final cell density were reduced with increasing concentrations of EO. Variations depending on the considered strain were noticed. Similar observations were mentioned by Olasupo, Fitzerald, Gasson, and Narbad (2003) when they studied the activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica*. Indeed, they found that at the sub-MIC levels, the lag phase of growth was extended and both growth rate and final cell density were reduced with increasing concentrations of diacetyl.

Although automated OD measurements appear to be suitable for such a study since estimation of growth kinetics in mathematical modelling is a simple task, it is not a "perfect" method as it does not take into account several factors. Specifically, turbidometry detects only the upper part of microbial growth curves, and requires calibration in order to correlate the results with viable counts obtained on agar media (Skandamis, Koutsoumanis, Fasseas, & Nychas 2001). In addition, the physiological state of the cells (injured or healthy), the state of oxidation of the essential oil, as well as inadequate dissolution of the compound may also affect absorbance measurements in growth media (Nychas et al., 1995).

3.3. Flow cytometry investigation

Traditionally, the assessment of antimicrobial activity of EOs has been based on the evaluation of reduction in CFUs or by the broth dilution method. Nevertheless, it is well established that with such methods subpopulations cannot be easily traced, or even detected, in a given biotope (Kell & Young, 2000). Additionally, attempts to circumvent the length of time to yield results with plate counts (24 h to 1–2 weeks for a colony to become visible) with bulk measurements like optical density (OD), respiration level and ATP concentration to assess viability in most cases failed. Furthermore, one of the major drawbacks of CFUs that is particularly relevant is the inability to determine whether any colony formed on a plate derived from a single cell or a clump of cells (Breeuwer & Abee, 2000; Nebe-von-Caron, Stephens, Hewitt, Powell, & Badley, 2000).

On the basis of these considerations and in order to provide some insights into the mechanisms of action of *M. armillaris* EO components on LAB cells, the multiparametric flow cytometry technique was exploited. This more sensitive and rapid test was useful for providing information about the cellular physiological state and its evolution (eventually its deterioration) during incubation with the EO. Thus, the evolution of the physiological states of the six tested LAB cells was established depending on the increasing concentrations (0.25, 2.5, 5 and 25 μg/ml) of *M. armillaris* EO and the period of incubation (time). In that respect, a kind of "behaviour kinetic" of each of the studied LAB strains, when continuously incubated during 72 h in presence of a given concentration of the EO, was obtained. Incubations were conducted under slow agitation (200 rpm) at the optimal temperature for each of the strains.

To the best of our knowledge, this multiparametric flow cytometry technique has been applied to study *L. plantarum* survival under thermal stress (Ueckert et al., 1995), *Bifidobacterium lactis* and *Bifidobacterium adolescentis* under bile salt stress (Ben Amor et al., 2002) and *Oenococcus oeni* under ethanol stress (Graca da Silveira et al., 2002). Recently, Rault, Béal, Ghorbal, Ogier, and Bouix (2007) used this test in order to assess the viability of four strains of *L. delbrueckii* after freezing and during frozen storage. The multiparametric flow cytometry assay had never been used to assess the behaviour of LAB in presence of EOs. Thus, in order to allow the differentiation of subpopulations in a sample containing one of the LAB strains incubated with *M. armilaris* EO at a given concentration, dead/live assays were conducted with PI/cFDA dual staining. Simultaneous probing of the cells within a sample with different fluorescence probes has the advantage of combining the principles imposed by each of the probes for the characterization of the population on a cell-by-cell basis. In the live/dead two-colour flow cytometric assay presented here, cells were assessed for enzymatic activity by cFDA and for membrane integrity in parallel by retention or leakage of cFDA and extrusion or intake of PI.

As an illustration, Fig. 3 (A–M) shows the evolution of the physiological state of one of the studied LAB (*L. plantarum*) cells when they were continuously incubated with 0.25 μg/ml (Fig. 3, panels D–H) or with 5 μg/ml (Fig. 3, panels I–M) of *M. armillaris* EO depending on the period of incubation (sampling were performed at 4, 8, 14, 48 and 72 h). Single and double staining were performed as described in Section 2. As illustrated in Fig. 3, cF and PI-labeled populations were spatially discriminated in dot plots of FL1 (cF fluorescence: *x*-axis) and FL2 (PI fluorescence: *y*-axis). Four kinds of subpopulations were observed with different staining characteristics. The first one was located in the lower left quadrant, it corresponded to unstained debris. The PI-labeled cells appeared on the FL2 detector in the upper left quadrant; they corresponded to dead cells. The cF-labeled cells were grouped in the lower right quadrant and included viable cells. Finally, a double stained population was observed in the upper right quadrant; it represented injured cells, which still displayed an esterase activity (cF-labeled), but with a damaged membrane, that allowed penetration of the PI probe.

According to Fig. 3 (panels A and B), unstained samples (negative controls) of *L. plantarum* cells (which were amended or not with the EO) showed a neglected artefact since 99.94% and 99.97% of the cells did not show any exogenous staining. This result proved the very low rate of contamination by any spontaneous fluorochromes or by exogenous stained particles or debris. On the contrary, for stained samples we noticed a variation, depending on the time, among the lower left subpopulation (unstained debris). Indeed, this population accounted for less than 3.5% regardless of the EO concentration and the culture period. Nevertheless, some exceptions were noticed namely at greater EO concentration. The highest amounts were obtained with all tested strains when incubated with 25 μg/ml of the EO or with *L. delbrueckii*, *L. casei* and *L. sakeii* incubated with 5 μg/ml of the EO (data not shown). In all cases, the percentages of the unstained subpopulation did not exceed 7.69%. It is noteworthy that similar results, concerning the variation among the percentage of this subpopulation, were obtained with the other studied LAB strains (data not shown). Such variations could be explained since we knew that applications of the technique to such specific bacteria seem to be hampered, to some extent, by the fact that many bacterial species exist or are able to easily form clumps or interlaced cell chains. Particularly, it is well known that *Lactobacilli* exhibit a planar division with cells remaining attached to each other, thus, producing chains characteristic for the genus. Consequently, the formation during the incubation period of a group of cells attached to each other would provide a single cumulative signal resulting from the contribution of each cell to it, thus decreasing accuracy and many times producing misleading results. This phenomenon was avoided in many works by using sonication treatment of the cells (Braga, Bovio, Culici, & Dal Sasso, 2003; Nebe-von-Caron et al., 2000). Indeed, sonication caused single cells to appear homogenously in the samples as observed under microscope and as demonstrated by

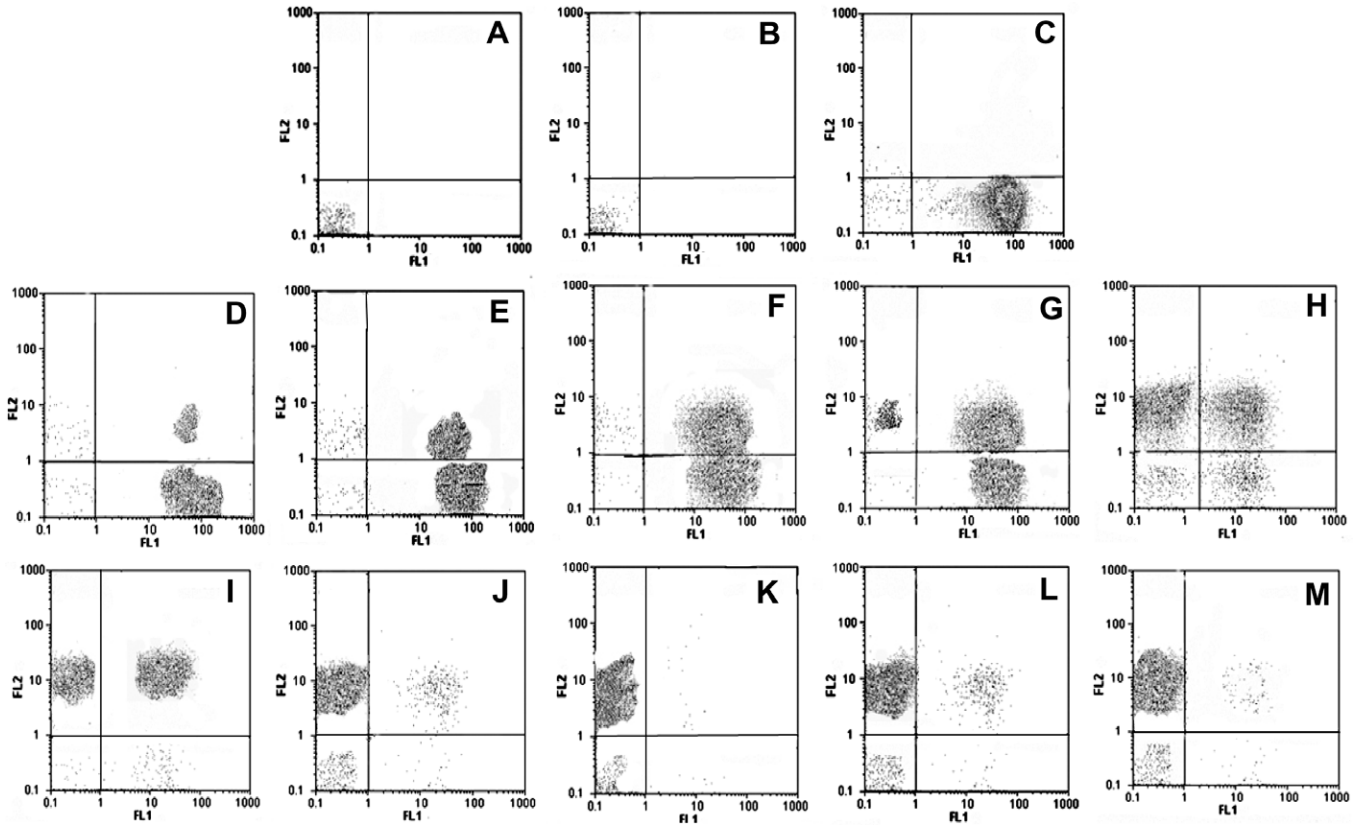

Fig. 3. Multiparametric flow cytometry dot plots of FL1 (Fluorescence collected at 525 nm) vs. FL2 (Fluorescence collected at 620 nm) of *L. plantarum* to assess the effect of different concentrations of *M. armillaris* essential oil and incubation period on esterase activity and membrane integrity. Panels A and B: unstained cells which were exposed or not to *M. armillaris* EO, respectively. Panel C: Freshly harvested mid-log phase cells which were stained with cFDA. Panels D–H: cells were continuously exposed to 0.25 μg/ml of *M. armillaris* EO (sampling and PI/cFDA double staining were performed after 4, 8, 14, 48 and 72 h, respectively). Panels I–M: cells were continuously exposed to 5 μg/ml of *M. armillaris* EO (sampling and PI/cFDA double staining were performed after 4, 8, 14, 48 and 72 h, respectively).

flow cytometric analysis of the cell populations based on their light scattering properties (forward scatter vs. side scatter) (Papadimitriou, Pratsinis, Nebe-von-Caron, Kletsas, & Tsakalidou, 2006). However, in our study cultures amended with the EO, were gently agitated and no sonication was used in further steps.

Additionally, the excellent physiological state of the cells, when sampled at the mid-log phase, was demonstrated by the single use of a cFDA probe. Fig. 3C shows that 97.87% of cells were detected as viable (fluorescent intensity higher than 10^2). Similar results were recorded with all the other strains of the studied LAB (data not shown). Nevertheless, analysis of the 100% live cells sample (Fig. 3, panel C) exhibited an average of 1.43 ± 0.75% PI positive population, a fact previously observed also in *bifidobacteria* (Ben Amor et al., 2002). These results indicate that even in a presumed totally healthy sample as assessed by classical microbiology there are already some cells in "injured" or even permeabilized states.

Concerning the evolution of the physiological states of *L. plantarum* cells, during 72 h of culture under the EO stress (Fig. 3); we can state that at 0.25 μg/ml EO, the cells seemed not to be affected by such a concentration after 4 h of incubation (Fig. 3, panel D). Indeed, 97.52% of the cells were detected as viable (right lower quadrant) with a corresponding fluorescence intensity of approximately 10^2. In contrast, the percentage of stressed cells (right high quadrant) reached 10.16%. Nevertheless, no substantial dead cells were recorded at this stage of culture (1.65 ± 0.35%). Four hours later, the repartition of the physiological states of the observed subpopulations varied notably since the percentage of the viable cells decreased to 53.88%. However, the percentage of the stressed subpopulation increased by approximately 4 folds to reach 37.96%. Such high level of injured cells allowed us to say that *M. armillaris* EO exhibited an early bacteriostatic effect. Furthermore, as indicated before, the rate of unstained debris weakly increased reaching 3.39% instead of 2.32%. It is noteworthy that compared to samples, that were amended with the EO, the percentages of the unstained debris as recorded with the negative controls (culture of LAB without *M. armillaris* EO) did not follow the same evolution pattern. Indeed, the highest level of the left lower subpopulation was 2.86%, after 72 h of culture.

After 14 h of incubation (Fig. 3, panel F) the previous pattern drastically changed with the appearance of a high level of dead cells. Indeed approximately 10% of the cells were detected at the upper left quadrant. This bactericidal potential remained weak, but the percentage of the stressed cells increased again. At the same time the viable cells subpopulation decreased. We recorded approximately, equal amounts of viable and stressed cells: 43.59% and 42.49%, respectively. After 2 days of incubation (Fig. 3, panel G), the viable subpopulation of *L. plantarum* cells slowly fell. Hence, the percentage of dead cells became 1.5 folds greater than the recorded value at 14 h. Indeed 16.24% was recorded as the percentage of the PI-labelled subpopulation. Such a stationary behaviour is likely due to the development, by the cells, of some mechanisms of adaptation allowing them to withstand such stressful conditions. At this stage, the greatest percentage was recorded for the injured subpopulation (46.49%), closely followed by the viable subpopulation (32.29%). Furthermore, after 3 days of culture (Fig. 3, panel H), the viable subpopulation strongly decreased reaching only 3.53% (approximately 11 folds lower than the recorded value at 48 h). Meanwhile, the dead subpopulation cells dominated with a

recorded percentage of 62.56% and the stressed subpopulation was still represented with a rate of 17.59%. Such behaviour, after 3 days of incubation could additionally be explained by the deterioration of the medium conditions (namely pH fell and accumulation of cell wastes) which contributes to the lyses of the cells in addition to the EO action. It can be assumed, as described elsewhere, that not only the cell density but also the culture conditions in general contribute to a decrease of viability (Cox et al., 2000).

On the contrary, when used at 5 μg/ml, *M. armillaris* EO influenced differently the behaviour of *L. plantarum* cultivated cells. Indeed, the recorded percentages of the various detected subpopulations were widely different from those recorded previously; the most dominant event was the drastic viability loss. Thus, after 4 h of incubation, the culture was largely dominated by two subpopulations: the PI-labelled cells and the stressed cells with 58.85% and 34.76%, respectively vs. 2.94% of viable cells (Fig. 3, panel I). It is obvious, that at 5 μg/ml, *M. armillaris* EO exhibited an immediate bactericidal activity. Nevertheless, a respectable percentage of cultivated cells were capable to overcome the action exerted by the components of *M. armillaris* EO. These rescued cells were able to restart a moderate growth phase (with a growth rate of 0.027 h^{-1}). However, when the incubation was prolonged for 3 days the cells were strongly inhibited. The percentage of stressed cells decreased to reach 5.37%, 2.10%, then 1.23% and finally 0.17% after 8, 14 h, 2 and 3 days of culture, respectively. Meanwhile, after the same periods of incubation, the recorded percentages of the dead subpopulations were 77.26%, 80.75%, 81.26% and, 90.5%, respectively (Fig. 3, panels J–M). Based on these observations, we can state that the mode of action of *M. armillaris* EO components could involve a complex and interdependent phenomena.

On comparing the evolution of the physiological states between the six used LAB, under *M. armillaris* EO stress, we summarized in Fig. 4 the evolution of the subpopulations cited above, depending on the concentration of *M. armillaris* EO, as realised for the six studied LAB, after 14 h of incubation. It is relevant as illustrated by this figure that significant differences were observed between the four subpopulations under *M. armillaris* EO stress, regardless of the considered strain. Moreover, it is important to note an increasing rate for the unstained as well as for the dead subpopulations, when concentrations increased. In parallel, the percentages of viable cells fell when concentrations increased. Such observations were the same for the six strains. For example, the percentage of the viable cells fell from 82.45% to 11.44%, when *L. pentosus* cells were incubated at 0.25 or 25 μg/ml of the EO, respectively. Also, the recorded percentages of viable cells were 64.21%, 18.67%, 8.45% and 5.54%, when *L. delbrueckii* cultures were performed in presence of 0.25, 2.5, 5 and 25 μg/ml of the EO, respectively. Consequently, the kinetic patterns of each of the detected subpopulations depend on the LAB strain, when increasing concentrations of the EO were used. Nonetheless, when considering the evolution of the stressed subpopulation, disparate patterns were noticed depending on the strain as well as the concentration of the EO. Indeed, when the EO was tested, at 2.5 or 5 or 25 μg/ml against *L. delbrueckii*; no significant differences were recorded concerning the stressed subpopulation. The same observation was done with *L. pentosus* and *L. casei*, when they were incubated with 5 or 25 μg/ml of the EO. In all other cases, the percentages of stressed subpopulation increased with the increase of the EO concentration. One exception to these observations was *L. planatrum* for which the percentage of the stressed cells, at 2.5 μg/ml of *M. armillaris* EO, was higher (38.99%) than the one recorded at 5 μg/ml of the EO (26.17%). Hence, the LAB studied herein showed obvious differences in their susceptibility to *M. armillaris* EO.

Based on the results presented above, the flow cytometric distribution patterns during the culture reveals dynamic changes in heterogeneous populations of viable cells which can be explained by the cellular physiological diversity occurring throughout the incubation period. Similar results were obtained by many works

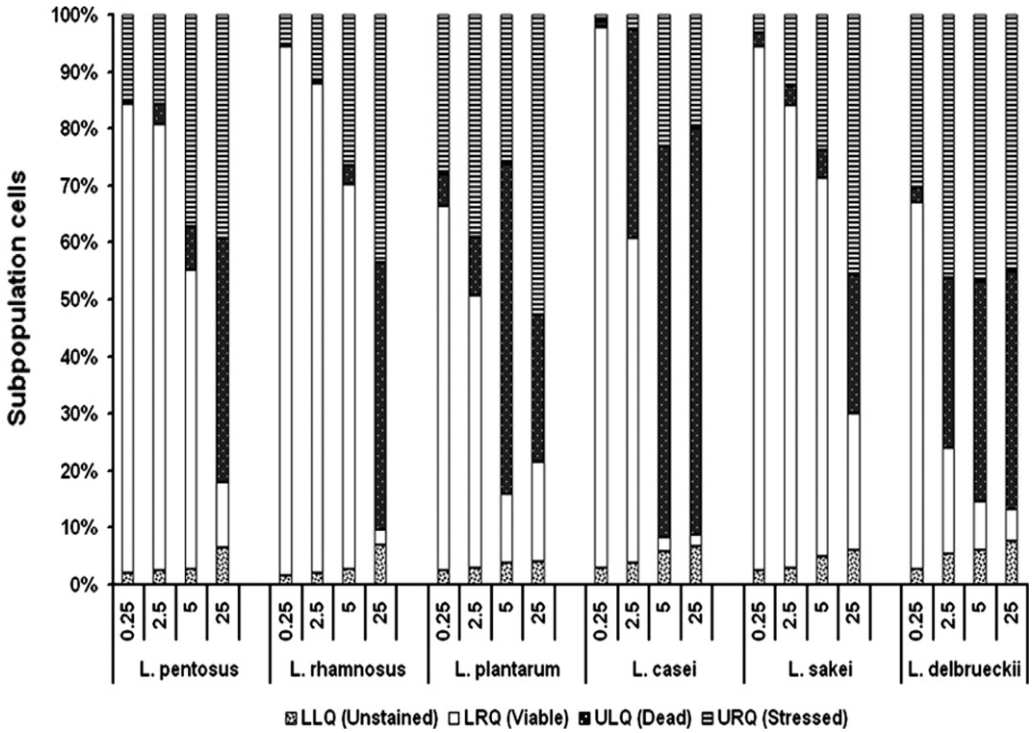

Fig. 4. The various distribution patterns (%) of the subpopulation cells (unstained, viable, dead and stressed) as obtained by PI/cFDA double staining of each of the six LAB strains after 14 h of incubation with increasing concentrations of *M. armillaris* EO. LLQ: lower left quadrant, LRQ: lower right quadrant, ULQ: upper left quadrant and URQ: upper right quadrant.

studying yeasts fermentation processes (Breeuwer et al., 1995). Nonetheless, in analysis of small bacteria (such as LAB), careful attention should be paid to optimisation of instrumental set up and to the quality of sheath and microbial suspension buffers. These results confirmed the fact that the activity of the EO was dose dependant, as found with the paper disc diffusion and the microdilution assays. Moreover, the interpretation of the kinetic behaviours of LAB cells, during incubation with *M. armllaris* EO, is a tough task (disparate behaviours were noticed between the strains and depending on the EO concentration as well as on time). This might be attributed to the fact that fluorescence staining depended on a multitude of intracellular events or properties (intracellular pH, esterase activity, probe efflux...) that could not be attributed solely to vitality, except during the early stage of culture. Thereby, flow cytometric analysis of microbial populations will always be subject to certain difficulties. These points should be borne in mind when using these methods for microbial strains.

In addition, the variations relative to the behaviour of each of the studied LAB under increasing concentrations of *M. armillaris* EO and depending on the period of incubation, could be understood when correlated to the chemical composition of the EO. Indeed, it is well established that considering the large number of different groups of components in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Carson, Mee, & Riley, 2002; Skandamis et al., 2001). Moreover, not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted. An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Knobloch, Weigand, Weis, Schwarm, & Vigenschow, 1986). Leakage of ions and other cell contents can then occur (Carson et al., 2002; Cox et al., 2000; Lambert et al., 2001; Skandamis et al., 2001; Ultee, Bennink, & Moezelaar, 2002). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death. Additionally, it is demonstrated that generally the EOs possessing the strongest antibacterial properties contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol (Farag, Daw, Hewedi, & El-Baroty, 1989; Lambert et al., 2001). Since the composition of *M. armillaris* EO was dominated by one major phenolic compound: 1,8-cineole, it seems reasonable to consider a similar mechanism of action as described for other phenol-rich EOs. Consequently, it could be hypothesized that eucalyptol destabilized the cytoplasmic membrane of the studied LAB, but at various extent. It could act as a proton exchanger with a decrease in the pH gradient resulting in a collapse of proton motive force and depletion of ATP and eventually cell death (Lambert et al., 2001). Such observations were previously described by Ultee et al. (2002) who showed that the hydroxyl group of carvacrol and the presence of a system of delocalized electrons are important for the antimicrobial activity of this compound. However, the postulated mode of action of *M. armillaris* EO could also be attributed to more complex phenomena involving the other components namely minor components: terpenes hydrocarbons. Such molecules interact with the cell membrane, where they dissolve in the phospholipidic bilayer and are assumed to align between the fatty acid chains. This distortion of the physical structure would cause expansion and destabilisation of the membrane, increasing membrane fluidity, which in turn would increase passive permeability.

As stated elsewhere, components of *M. armillaris* EO also could act on cell proteins embedded in the cytoplasmic membrane (Knobloch et al., 1986). Enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on these. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid–protein interaction; alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Juven, Kanner, Schved, & Weisslowicz, 1994). Taking into account the differences, between the six studied LAB, on the structural, biochemical, physiological and genetic bases; the above described events might occur differently. So that, the discrepancy between the kinetic behaviour of these strains when stressed by increasing concentration of *M. armillaris* EO could be explained, at least in part. Nevertheless, to fully master all the mechanisms, no matter are their nature, one should deeply correlate all these observations to the conditions of cultures. The discrepancy between the physiological behaviour of the studied LAB cells during exposure to increasing levels of M. *armillaris* EO should be correlated to the strength and the rapidity of these mechanisms, namely cell membrane disruption. At lower concentrations and even if the components of the EO do actually damage the outer membrane; it is probably of an insufficient magnitude to permit a drastic loss of cell content and integral lyses of the cells.

3.4. Relationship between the double staining flow cytometry technique and plate counts determination

The loss of viability observed by flow cytometry was compared with plate count results. Linear regressions were established between the percentages of viable cells (V, in%) obtained by PI/cFDA double staining flow cytometry analysis, and the bacterial concentration (N, in CFU/ml) obtained by plate counts $V = a + b \cdot \ln(N)$, where a and b were respectively, the intercept and the slope that characterized each strain. High values of intercept and slope corresponded to a good resistance of a strain to the action of *M. armillaris* EO. As summarized in Table 2, it appears that there is a high correlation between results obtained by both techniques. Nonetheless, few exceptions were noticed, concerning *L. pentosus* and *L. rhamnosus*, evoked above. Such observations could be understood since we knew that survival of cells after a perturbation expressed as a decline in numbers of CFUs is most probably an erroneous quantification of the real number of cells that survived or died. This is due to the distribution of dead cells in chains with live cells that will ultimately give rise to colonies (Braga et al., 2003). Consequently, it could be assumed that multiparametric flow cytometry was a convenient and meaningful tool for the assessment of LAB cells viability during exposure to a natural preservative such *M. armillaris* EO. Moreover, it was rapid since one analysis required approximately 30 min, instead of 2 days required for plate counts.

4. Conclusion

The growing interest in the substitution of traditional food preservatives, both antimicrobials and antioxidants, by natural ones has fostered the screening of plant materials. In this context, essential oils and their components are known to exhibit varying degrees of antimicrobial activity namely against pathogens. Nevertheless, there is no data concerning the examination of their effects on beneficial normal microbiota or on the starter cultures largely used in many industries. The main scope of this work was to investigate the mode of action of *M. armillaris* essential oil against six LAB strains. In that context, different but complementary techniques were used. Thus, the use of microtiter-based assay and automated data recording and processing enable us to rapidly and effectively test the inhibitory potential of EOs against the studied strains. Furthermore, from agar diffusion and flow cytometry methods we conclude that the activity of EO can vary with the

evaluation technique applied partly due to differences in the assay matrices, which point out the importance to verify the activity of EO in the food matrix to be protected and even in the digestive microbiota.

Furthermore, the use of a rapid and sensitive technique such as flow cytometry is advantageous for quickly generating a large amount of data. This technique can serve as powerful tool in optimally combining different preservative factors in order to design an effective antimicrobial system for selected foods. However, additional experiments are required to evaluate the antibacterial activity of EO in different food matrices and their influence on the sensory characteristics of food. The relevance of cFDA and PI to quantify bacterial viability was proven. When bacterial suspensions were simultaneously stained with these two florescent probes, three major subpopulations were identified: viable, dead and injured cells. The mode of action of the EO seems depending on the strain as well as on the concentration of the EO. This is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport and coagulation of cell contents. Different extent of permeabilization of the outer membranes could be the major cause of discrepancy between the behaviour of each of the studied bacteria when exposed to the EO as assessed by the flow cytometry method. Finally, this study proved that multiparametric flow cytometry was a convenient and rapid tool to evaluate the viability of LAB, and was well correlated with plate count results. Moreover, it made it possible to differentiate strains according to their susceptibility to EOs effects.

Acknowledgments

We are very grateful to Dr. Nadia Ben Brahim, head of the Department of Botany, National Institute of Agronomic Research (INRAT, Tunis, Tunisia) for identification of the plant. The authors equally thank Prof. Bouix, M. (AgroParistech, France) and Prof. Zagorec, M. (INRA, France) for providing us with microorganisms. This study is financially supported by the Tunisian Ministry of Higher Education, Scientific Research and Technology.

References

Adams, R. P. (2001). Identification of essential oils components by gas chromatography/quadrupole mass spectroscopy. Carol Stream, IL, USA: Allured Publishing Corporation.

Attfield, P. V., Kletsas, S., Veal, D. A., Van Rooijen, R., & Bell, P. J. L. (2000). Use of flow cytometry to monitor cell damage and predict fermentation activity of dried yeasts. *Journal of Applied Microbiology, 89*, 207–214.

Baranyi, J., Roberts, T. A., & McClure, P. J. (1993). A nonautonomous different equation to model bacterial growth. *Food Microbiology, 10*, 43–59.

Ben Amor, K., Breeuwer, P., Verbaarschot, P., Rombouts, F. M., Akkermans, A. D. L., & De Vos, W. M., et al. (2002). Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. *Applied and Environmental Microbiology, 68*, 5209–5216.

Braga, P. C., Bovio, C., Culici, M., & Dal Sasso, M. (2003). Flow cytometric assessment of susceptibilities of *Streptococcus pyogenes* to erythromycin and rokitamycin. *Antimicrobial Agents and Chemotherapy, 47*, 408–412.

Breeuwer, P., & Abee, T. (2000). Assessment of viability of microorganisms employing fluorescence techniques. *International Journal of Food Microbiology, 55*, 193–200.

Breeuwer, P., Drocourt, J-L., bunschoten, N., Zwietering, M.-H., Rombouts, F.-M., & Abee, T. (1995). Characterization of uptake and hydrolysis of fluorecein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*, which result in accumulation of fluorescent product. *Applied and Environmental Microbiology, 61*, 1614–1619.

Bunthof, C. J., Bloemen, K., Breeuwer, P., Rombouts, F. M., & Abee, T. (2001). Flow cytometric assessment of viability of lactic acid bacteria. *Applied and Environmental Microbiology, 67*, 2326–2335.

Burt, S. A., & Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against *Escherchia coli* O157:H7. *Letters in Applied Microbiology, 36*(36), 162–167.

Carr, F. J., Chill, D., & Maida, N. (2002). The lactic acid bacteria: A literature survey. *Critical Reviews in Microbiology, 28*, 281–370.

Carson, C. F., Mee, B. J., & Riley, T. V. (2002). Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. *Antimicrobial Agents and Chemotherapy, 46*(6), 1914–1920.

Cox, S. D., Mann, C. M., Markham, J. L., Bell, H. C., Gustafson, J. E., Warmington, J. R., et al. (2000). The mode of action of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology, 88*, 170–175.

Farag, R. S., Daw, Z. Y., Hewedi, F. M., & El-Baroty, G. S. A. (1989). Antimicrobial activity of some Egyptian spice essential oils. *Journal of Food Protection, 52*(9), 665–667.

Farag, R. S., Shalaby, A. S., El-Baroty, G. A., Ibrahim, N. A., Ali, M. A., & Hassan, E. M. (2004). Chemical and biological evaluation of the essential oils of different *Melaleuca* species. *Phytotherapy Research, 18*(1), 30–35.

Graca da Silveira, M., Vitoria San Romao, M., Loureiro-Dias, M. C., Rombouts, F. M., & Abee, T. (2002). Flow cytometric assessment of membrane integrity of ethanol stressed *Oenococcus oeni* cells. *Applied and Environmental Microbiology, 68*, 6087–6093.

Hamilton-Miller, J. M. (2003). The role of probiotics in the treatment and preservation of *Helicobacter pylori* infection. *International Journal of Antimicrobial Agents, 22*, 360–366.

Hewitt, C. J., & Nebe-Von-Caron, G. (2001). An industrial application of multiparmeter flow cytometry: Assessment of cell physiological state and its application to the study of microbial fermentations. *Cytometry, 44*, 179–187.

Juven, B. J., Kanner, J., Schved, F., & Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *Journal of Applied Bacteriology, 76*, 626–631.

Kell, D. B., & Young, M. (2000). Bacterial dormancy and culturability: The role of autocrine growth factors. *Current Opinion in Microbiology, 3*, 238–243.

Knobloch, K., Weigand, H., Weis, N., Schwarm, H.-M., & Vigenschow, H. (1986). Action of terpenoids on energy metabolism. In E. J. Brunke (Ed.), *Progress in essential oil research: 16th international symposium on essential oils* (pp. 429–445). Berlin: De Gruyter.

Lambert, R. J. W., Skandamis, P. N., Coote, P., & Nychas, G.-J. E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology, 91*, 453–462.

Lee, B.-H., Annis, P. C., Tumaalii, F., & Choi, W.-S. (2004). Fumigant toxicity of essential oils from the Myrtaceae family and 1,8-cineole against 3 major stored-grain insects. *Journal of Stored Products Research, 40*, 553–564.

Marino, M., Bersani, C., & Comi, G. (2001). Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceae* and *Compositae*. *International Journal of food Microbiology, 67*, 187–195.

NCCLS (National Committee for Clinical Laboratory Standards) (1999). *Performance standards for antimicrobial susceptibility testing*. 9th International Supplement, M100-S9. Wayne, PA.

Nebe-von-Caron, G., Stephens, P. J., Hewitt, C. J., Powell, J. R., & Badley, R. A. (2000). Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *Journal of Microbiological Methods, 42*, 97–114.

Nychas, G. J. E. (1995). Natural antimicrobials from plants. In G. W. Gould (Ed.), *New methods of food preservation* (pp. 58–89). London: Blackie Academic and Professional.

Olasupo, N. A., Fitzerald, D. J., Gasson, M. J., & Narbad, A. (2003). Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* servovar *Typhimurium*. *Letters in Applied Microbiology, 36*, 448–451.

Papadimitriou, K., Pratsinis, H., Nebe-von-Caron, G., Kletsas, D., & Tsakalidou, E. (2006). Rapid assessment of the physiological status of *Streptococcus macedonicus* by flow cytometry and fluorescence probes. *International Journal of Food Microbiology, 111*, 197–205.

Perry, N. B., Anderson, R. E., Brennan, N. J., Douglas, M. H., Heaney, A. J., & McGimsey, J. A., et al. (1999). Essential oils from Dalmation sage (*Salvia officinalis* L.): Variations among individuals, plant parts, seasons and sites. *Journal of Agricultural and Food Chemistry, 47*, 2048–2054.

Rault, A., Béal, C., Ghorbal, S., Ogier, J.-C., & Bouix, M. (2007). Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. *Cryobiology, 55*, 35–43.

Skandamis, P., Koutsoumanis, K., Fasseas, K., & Nychas, G.-J. E. (2001). Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. *Italian Journal of Food Science, 13*(1), 65–75.

Southwell, I., & Lowe, R. (1999). Tea tree: The genus *Melaleuca*. In R. Hardman (Ed.). *Medicinal and aromatic plants-industrial profiles* (Vol. 9). Amsterdam: Harwood Academic Press.

Stiles, J., Penkar, S., Plockova, N., Chumchalova, J., & Bullerman, L. B. (2002). Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. *Journal of Food Protection, 65*, 1188–1191.

Ueckert, J., Breeuwer, P., Abee, T., Stephens, P., von Caron, G. N., & Steeg, P. F. (1995). Flow cytometry applications in physiological study and detection of foodborne microorganisms. *International Journal of Food Microbiology, 28*, 317–326.

Ultee, A., Bennink, M. H. J., & Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology, 68*(4), 1561–1568.

Valero, M., & Salmerón, M. C. (2003). Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. *International Journal of Food Microbiology, 85*, 73–81.

Van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S. D., & Maguin, E. (2002). Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek, 82*, 187–216.