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STUDY ON THE EFFECTS OF *LAVENDULA DENTATA* AND *MELALEUCA ARMILLARIS* ESSENTIAL OILS ON TWO LAB STRAINS BY A MULTIPARAMETRIC FLOW CYTOMETRY TECHNIQUE

VIŠEPARAMETARSKA PROTOČNA CITOMETRIJA KAO MOĆNO ORUĐE ZA PROCENU MEHANIZMA UTICAJA ESENCIJALNIH ULJA NA LABORATORIJSKI UREĐAJ ZA CEĐENJE

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SUMMARY

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 (EOs) and their components are known to exhibit wide range of antimicrobial activities namely against pathogens. Nevertheless, there is no data concerning the examination of their effects on beneficial normal digestive microflora or on the starter cultures largely used in many industries. This work describes, for the first time, the use of a multiparametric flow cytometry technique to assess the mode of action, at the single cell level, of Melaleuca armillaris and Lavandula dentata EOs against two lactic acid bacteria (LAB) strains. Using an automated microtiter assay (Bioscreen C), bacteriostatic or bactericidal effects were noticed depending on the studied strain and on the applied concentration of each of the EOs. The mathematical modelling of the kinetics showed that in presence of increasing concentrations of a given EO, the lag phases of growth were extended (1.55% to 94.3%) 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 done 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 each of the EOs, was evaluated by quantifying the relative percentages of each subpopulation during the cultivation period (3 days). Results displayed disparate patterns of subpopulations which reveal dynamic changes in cells behaviour.

***Key words**: Melaleuca armillaris, Lavandula dentata, Essantial Oils, Lactic Acid Bacteria, flow cytometry.*

INTRODUCTION

Plants, herbs, spices and their derived EOs and isolated compounds are known to retard or inhibit the growth of bacteria, yeast and moulds [1], although many of them are not yet completely exploited. The antimicrobial action of EOs in food systems is well known in the literature. Their use can be considered an additional intrinsic important factor to increase the safety and shelf life of foods. Furthermore, the possible modes of action of EOs, as antimicrobial agents, have been reported in various reviews. However, the mechanisms of action have not been completely elucidated. Over the years, several methods have been developed to measure viability and vitality of microbes under various stresses: 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 insult is assessed by classical Colony Forming Units (CFUs), a binary logic is imposed for the determination of viability [2]. Live cells will be those that managed to replicate under the particular experimental conditions, while all the others will be presumed dead [3]. 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, dormant cells etc, cannot be easily traced or even detected by traditional CFUs [3]. For all these reasons, fluorescence techniques combined with direct optical detection methods for the rapid assessment of bacterial viability are increasingly being favoured. They are highly sensitive and have high time resolution [4]. Particularly, flow cytometry has been shown to be a powerful tool for analyzing populations rapidly on a cell-by-cell basis thus allowing multi-sample 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. The most widely used dyes for the labelling of cells are the esterase substrate cFDA and PI [4]. 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 damaged membranes, and are considered as stressed cells [5, 6].

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. It has been estimated that 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods [7]. It is well stated that LAB, amongst a large population of microorganisms, inhabit the human gastrointestinal tract (GIT) and form a closely integrated unit with the host. Many reports show the usefulness of LAB as probiotics for human and animals [8].

In light of these statements, works exploring the behaviour, even at *in vitro* level, of LAB amongst human gastrointestinal microflora, when incubated with EOs remained scarce. On the other hand, in any industrial process it is relevant the identification and quantification of different species and strains involved in the biotechnological transformation. It is also important to

monitor the likely presence or absence of microorganisms in the final product after the manufacture process. Based on these considerations, this work had focused on (i) the anti-LAB activities of *M. armillaris* and *Lavendula dentata* EOs in correlation to their chemical compositions (ii) the mode of action of both EOs against two LAB as assessed by a multiparametric flow cytometry technique.

MATERIALS AND METHODS

Collection of plant materials and chemical compositions of the EOs

The leaves of *Melaleuca armillaris Sol.* ex Gaertn. Sm. and *Lavendula dentata* L. were collected from the botanical garden of the National Institute of Agronomic Research (INRAT, Tunis). The air-dried and finely ground raw materials were submitted to water distillation for 4 h, using Clevenger-type apparatus. The obtained EOs were dried over anhydrous sodium sulphate and, after filtration, stored at 4 to 7°C until use. Yields of 2.35% ± 0,103 and 0.89% ± 0.03 were recorded for *M. armillaris* and *L. dentata*, respectively. The chemical compositions of the EOs were determined using a Hewlett–Packard 5890 II GC (Minnesota, USA) equipped with flame ionization detector (FID) and HP-5 MS capillary column and using also a Hewlett–Packard 5890 II GC, equipped with a HP 5972 mass selective detector and a HP-5 MS capillary column (Minnesota, USA).

Antimicrobial Activity

Microbial strains and culture conditions

M. armillaris and *L. dentata* EOs were individually tested against two LAB: *Lactobacillus delbrueckii subsq. Bulgaricus* CNRZ 208T and *Lactobacillus sakeii* 23 K. These strains were kindly provided by Pr. Bouix, M. (AgroParistech, France) and Pr. Zagorec, M. (INRA, France), respectively. MRS broth and agar were from DIFCO Laboratories (Lawrence, USA).

Inhibition of bacterial strains by EOs 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 of a 100-well microtiter plates (Honeycomb Format 100-well Multi Well Plates). The stock solution (100 µg/ml) of the EO was diluted, in DSMO (0.1% as highest final concentration), 50 µl volumes were dispensed into the wells. Appropriate final concentrations were obtained in designed wells (25, 5, 2.5 and 0.25µg/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 (at 42°C for *L. delbrueckii* and at 30°C for *L. sakeii*.) for 3 days in a plate reader (Multiskan Ascent Microtiterplate Reader, LabSystems, Bioscreen C, Finland) with absorbance readings (600 nm) taken every 30 min. Software Biolink, integrated to the plate reader allowed an automated data recording (28800 values of absorbencies from two plates used each time).

Flow cytometry study

Fluorescent probes and staining protocols

Carboxyfluorescein diacetate was used to assess LAB viability, whereas the nucleic acid dye propidium iodide made it possible to quantify damaged and dead cells. The carboxyfluorescein (cF) has a maximum excitation wavelength of 492 nm and an emission wavelength of 517 nm in green. 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, 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. 10 µl of cFDA were then added and incubation took place for 10 min. After short centrifugation at 14.000g 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 analysed by flow cytometry and used as negative controls.

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 carboxyfluorescein (FL1 channel), a 670 nm long pass filter to collect the red fluorescence of propidium iodide (FL2 channel) and a 585 nm band-pass filter (564–606 nm) to collect the yellow-orange fluorescence (FL3 channel). The flow cytometry analyses were performed by using logarithmic gains and specific detectors settings. A combination of FSC and SSC was used to discriminate bacteria from background and a threshold was set on the FSC signal.

Data were collected and analyzed with CellquestPro software (Becton–Dickinson, France). 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 auto-fluorescence. Data were analyzed with the aid of statistical tables, which indicated the percentages of stained cells determined by each detector.

RESULTS AND DISCUSSION

Chemical composition of the essential oils

GC and GC-MS analysis (data not shown) resulted in the identification of 68 and 61 compounds representing 99.63% and 99.01% of *M. armillaris* and *L. dentata* EO, respectively. As usually occurs in the Myrtaceae family; 1,8-cineole (eucalyptol) was dominant among the major components (> 1%) of the oil (68.92%), followed from far away by α-pinene (7.40%) which is closely followed by borneol (6.16%). Whereas, the major components in *L. dentata* EO were linalool (19,79%), α-cadinene (10.69%) and limonene (9.54%). furthermore, these EOs showed a completely different chemical patterns since *M. armillaris* EO

is largely dominated by the oxygen-containing monoterpenes: omt (77.29%) then came at lower rate the monotrepene hydrocarbons components: mt (15.28%). The sesquiterpenes molecules were less represented with 6.11% for the sesquiterpenes hydrocarbons (st) and only 0.95% of oxygen-containing sesquiterpenes (ost). Meanwhile, the first three fractions were approximately equivalent in *L. dentata* EO (32.71% omt, 30.18% st and 28.79% mt).

Study of the inhibition of bacterial strains 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-D). Individual inhibitory effects of each of the studied EOs were checked using 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 [9] to estimate the main growth parameters namely the growth specific rate (μ), lag time (λ).

As it appears in Fig. 1, both EOs exhibited a strong antibacterial activity against the tested LAB strains at each of the applied concentrations. Nonetheless, when tested against *L. sakeii*, at 0.25μg/ml and 2.5μg/ml, *L. dentata* EO had no bactericidal effect and this was the only exception. Even though, an activation of this strain was noticed during the first twelve hours of incubation with 0.25 or 2.5 μg/ml of *L. dentata* EO (Fig.1B). Using the growth kinetics of each of the LAB strains, when exposed to increasing concentrations of *M. armillaris* or *L. dentata* EO (Fig.1), and data concerning their growth parameters as determined by the function of Baranyi (data not shown), one can do comparative analysis of their respective behaviour and make some interesting observations. The most sensitive strain was *L. delbrueckii*, since no growth was noticed after three days of incubation along with 25 or 5 or 2.5 μg/ml of each of the EOs (Fig.1: panels A-D). In the case of *L. sakeii*, both EOs showed no absolute growth inhibition but showed considerably prolonged lag periods with a reduction of the final absorbencies. Overall, bacteriostatic or bactericidal effects were noticed depending on the studied strain as well as on the concentration of each of the tested EOs. According to these results, it is obvious that the relative extension of the lag phases was dose-dependant. Nevertheless, such correlation varied from strain to strain and is closely dependant on the chemical composition of the EO. In all cases the relative extensions of the lag-phases ranged between 1.55% and 94.3%, except for *L. sakeii*, when an early activation of the cells was noticed at 0.25 and 2.5 μg/ml of *L. dentata* EO. Concerning the reduction of the culture density, there was a proportional evolution of this parameter to the increase of the concentration. In all cases, these reductions ranged between 10.7% and 100% (data not shown). Similar observations were mentioned by Olasupo [10] when they studied the activity of natural antimicrobial compounds against *E. coli* and *S. 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 optical density (OD) measurements appear to be suitable for this kind of 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 many factors. Especially, 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 which may also affect absorbance measurements in growth media [11].

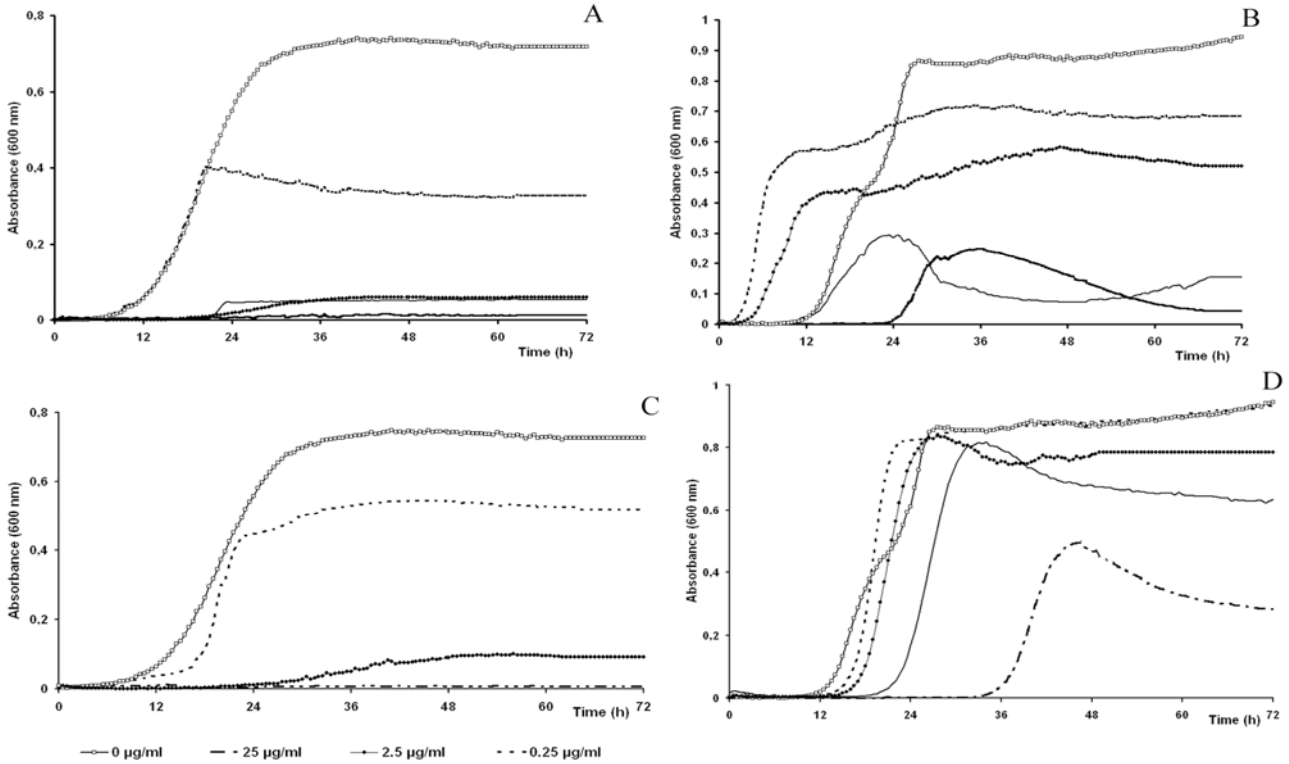


Fig.1. Time-survival kinetics of the two LAB strains in absence or in presence of 0.25 μg/ml, 2.5 μg/ml, 5 μg/ml and, 25 μg/ml of M. armillaris (panels C and D) or L. dentata (panels A and B) EOs. Cultures were performed in MRS broth. Panels A and C: L. delbrueckii subsq. Bulgaricus CNRZ 208T (42°C); Panels B and D: L. sakeii 23 K (30°C)

Sl. 1. Kinetika opstanka dve bakterije LAB roda sa prisustvom i bez prisustva 0,25 μg/ml, 2,5 μg/ml, 5 μg/ml i 25 μg/ml , M. armillaris (slikes C i D) ili L. dentata (slike A i B) EOs. Kulture su izvedene iz MRS. Slike A i C: L. delbrueckii subsq. Bulgaricus CNRZ 208T (42°C); Slike B i D: L. sakeii 23 K (30°C)

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 can not be easily traced or even detected, in a given biotope [3]. 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 [12].

On the basis of these considerations and in order to provide some insights into the mechanisms of action of the components of each of the studied EOs on LAB cells, multiparametric flow cytometry technique was exploited to assess the evolution of the physiological states of the tested LAB cells depending on the increasing concentrations (0.25; 2.5; 5 and 25 µg/ml) of each EO. In that respect, a kind of "behaviour kinetic" of each of the studied LAB strains was performed. Incubations were conducted under slow agitation (200 rpm) at the optimal temperature for each of the strains. Thus, in order to allow the differentiation of subpopulations in a sample containing one of the LAB strains incubated with *M. armilaris* or *L. dentata* EO, 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.

Fig. 2 (A-L) shows the evolution of the physiological states of the studied LAB cells when they were continuously incubated with 5 µg/ml (Fig.2: panels E, I, G, K) or with 25 µg/ml (Fig.2: panels F, J, H and L) of each EO after 48 h of incubation. Single and double staining were performed as described in Materials and Methods. As illustrated in Fig.2, 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, thus allowing penetration of the PI probe.

According to Fig. 2 (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. Additionally, the excellent physiological state of the cells, when sampled at the mid-log phases, was demonstrated by the single use of cFDA probe. Fig. 2 (panels C and D) shows that 97.87% and 96.67% of the cells were detected as viable (fluorescent intensity higher than 10^2) for *L. delbrueckii* and *L. sakeii*, respectively.

Concerning the evolution of the physiological states of both strains, after 48 h of incubation in presence of different concentrations (5 and 25 µg/ml) of each of the studied EOs, one can notice that *L. delbrueckii* cells were more sensitive than those of *L. sakeii* when incubated at 5 or 25 µg/ml of *M. armillaris* EO. Indeed, panel G of Fig.2 shows that 54.22% of *L. delbrueckii* cells were detected as stresssed cells (right high quadrant) and the percentage of dead cells was 9.22%. Wheras, these percentages were 21.13% and less than 2%, respectively for *L. sakeii* cells (panel E). Such observations remained true at 25 µg/ml since the percentage of the dead cells of *L. delbrueckii* increased to reach 89.3% versus 76.31% for *L. sakeii*. In this case, the percentages of stressed cells (of both strains) drastically fell to reach approximately 3.4% and 2.1%, respectively. Similar observations could be done when *L. dentata* EO was used. Indeed, at 5 µg/ml the stressed cells subpopulation of *L. delbrueckii* reached 45.14% versus 40.1% for *L. sakeii*. On comparing the effect of the EOs, it is abvious to notice an immediate bactericidal action on both strains when each of the EOs was applied at 25µg/ml. Indeed, the viable cells subpopulations drastically decreased along with the stressed cells. We recorded approximately, equal amounts of the dead cells in both cases.

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. 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 microdilution assays. Moreover, the interpretation of the kinetic behaviours of LAB cells, during incubation with these EO, is a tough task (disparate behaviours were noticed between the strains and depending on the EO and its concentrations). 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 each of the studied EOs 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 [11]. 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 [13]. Leakage of ions and other cell contents can then occur [14, 11, 15]. Additionally, it is demonstrated that generally the EOs possessing the strongest antibacterial properties (herein *M. armillaris* EO) contain a high percentage of phenolic compounds [16]. 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 [16]. However,

the postulated mode of action of each of the tested EOs 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 (probable dominant mechanism of action of *L. dentata* EO). Taking into account the differences, between the studied LAB, on the structural, biochemical, physiological and genetic bases; the above described events might occur differently. 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 an EO should be correlated to the strength and the rapidity of these mechanisms, namely cell membrane disruption.

CONCLUSION

The main scope of this work was to investigate the mode of action of two EOs when incubated in presence of two 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, 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 to combine different preservative factors in order to design an effective antimicrobial system for selected foods. 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 a given EO. Finally, this study proved that multiparametric flow cytometry made it possible to differentiate strains according to their susceptibility to EOs effects. Furthermore, 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.

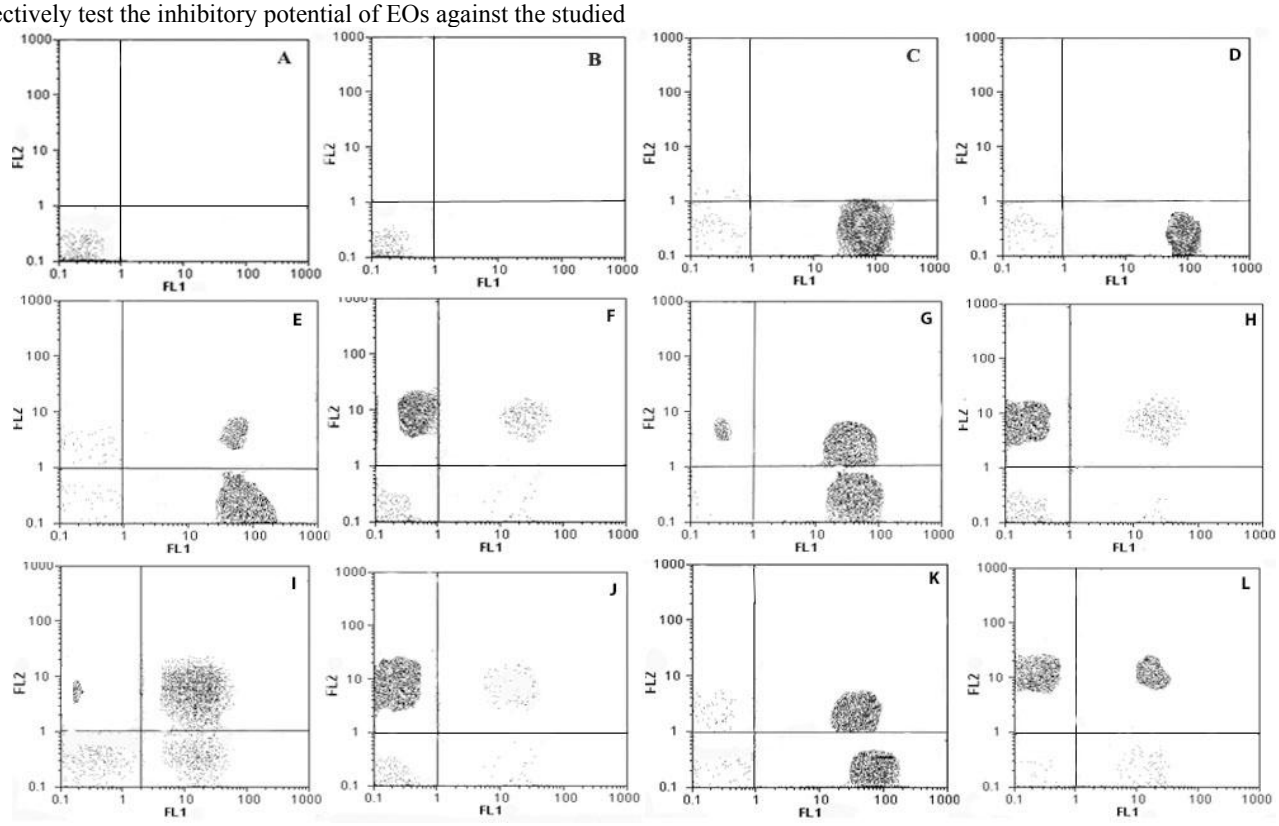


Fig.2. Multiparametric flow cytometry dot plots of FL1 (Fluorescence collected at 525 nm) vs. FL2 (Fluorescence collected at 620 nm) of two LAB to assess the effect of different concentrations of M. armillaris (panels E-H) and L. dentata (panels I-L) EOs after 48 h of incubation on esterase activity and membrane integrity. Panels A and B: unstained cells which were exposed or not to each of the tested EOs, respectively. Panels C and D: Freshly harvested mid-log phase cells of L. delbrukii and L. sakeii which were stained with cFDA, respectively. Panels E, I and G,H: L. sakeii and L. delbrukii cells were continuously exposed to 5 µg/ml of each of the EOs. Panels F, J and H, L: L. sakeii and L. delbrukii cells were continuously exposed to 25 µg/ml of each of the EOs.

Sl. 2. Dijagram citrometrijskog multiparametarskog protoka (fluroscencija računata na 525 nm) nasuprot FL2 (fluroscencije računate na 620 nm) za dve bakterije LAB roda sa ocenom efekta različitih koncentracija M. armillaris (panels E-H) i L. dentata (panels I-L) EOs posle 48 h inkubacije na aktivnost esterase i membranski integritet. Slike A i B: nestabilne ćelije koje su izlagane ili ne za svaki od testiranih EOs, ponaosob. Slike C i D: Sveže urbane ćelije u mid-log fazi L. delbrukii i L. sakeii koje su bojene sa cFDA, svaka ponaosob. Slike E, I i G,H: L. sakeii i L. delbrukii ćelije koje su neprekidno izlagne 5 µg/ml za svaki od EOs. Slike F, J i H, L: L. sakeii and L. delbrukii ćelije su nepreidno izlagane 25 µg/ml za svaki od EOs.

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