Antimicrobial activity of gaseous Citrus limon var pompia leaf essential oil against Listeria monocytogenes on ricotta salata cheese

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Abstract

Listeria monocytogenes is particularly challenging in the food industry since it can develop under conditions normally used for food preservation. Here we show that Citrus limon var pompia leaf essential oil (hereafter PLEO) has specific anti-Listeria activity on ricotta salata cheese via its gaseous-phase when stored at 5°C. First, the synergic effect of gaseous oil and refrigeration temperature was observed in vitro on L. monocytogenes strains treated for 3 h with gaseous PLEO and then stored at 5°C. Then, ricotta cheese was inoculated with L. monocytogenes strains, and subjected to hurdle technology with different concentrations of gaseous PLEO. Cell counts revealed that gaseous PLEO exerted bactericidal effect on L. monocytogenes 20600 DSMZ and bacteriostatic effect on the mix of L. monocytogenes strains. Scanning and transmission electron microscopy analyses of L. monocytogenes cells suggested that gaseous PLEO targets the bacterial cell wall and plasma membrane. Chemical analyses of the liquid and vapor phase of PLEO indicated linalyl acetate to be the predominant compound, followed by limonene and the two isomers of citral, while EO chemical composition, mainly in line with the previous findings, showed for first time, the presence of linalyl acetate. SPME coupled with gas chromatography confirmed the presence of all crude oil components in the headspace of the box.

Keywords	hurdle technology, Listeria monocytogenes; ricotta salata; EO gas; citral; refrigeration temperature

Corresponding Author	Severino Zara
Corresponding Author's Institution	University Of Sassari
Order of Authors	Francesco Fancello, Giacomo Luigi Petretto, Salvatore Marceddu, Tullio Venditti, Giorgio Pintore, Giacomo Zara, Ilaria Mannazzu, marilena budroni, Severino Zara

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Università degli Studi di Sassari
DIPARTIMENTO DI AGRARIA
Sezione Scienze e Tecnologie Ambientali e Alimentari

Viale Italia 39, 07100 Sassari
Tel. +39. 079 229386/17 Fax. +39. 079 229370
Partita IVA 00196350904

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Editorial office

Food Microbiology

Dear Editor,

We would like to submit the manuscript "***Antimicrobial activity of gaseous Citrus limon var Pompia leaf essential oil against Listeria monocytogenes on ricotta salata cheese***" by Fancello et al. as a Research Article to *Food Microbiology.*

Citrus limon var. *pompia* is a plant which grows in a sub-region of Sardinia, Italy, where it is cultivated predominantly in Siniscola (NU). Usually, the main component of the fruit used for EO extraction is the peel. Recently our group studied the potentiality of the Pompia EO extracted from leaf as antimicrobial against different foodborne pathogen bacteria and in particular against *Listeria monocytogenes* strains.

In this research we used the essential oil extracted from Pompia leaf in a gaseous form together with the refrigeration temperature in vivo on *ricotta salata* cheese artificially contaminated with different strains of *L. monocytogenes* used singularly or mixed. Results showed that the combined utilization of the essential oil and the refrigeration temperature has a strong bactericidal activity on *L. monocytogenses* strains used singularly and a bacteriostatic activity when used mixed. Interestingly, the gaseous EO showed no activity against resident lactic acid bacteria. The electron microscopy analysis also showed that the putative cell targets of the oil are probably the the wall and the plasma membrane. These results highlight the potential application of this essential oil used in its gaseous-phase as natural alternative to synthetic substances to enhance the microbial safety of cheese and foodstuff in general.

We consider that this study will be of interest for the scientific community working on applied microbiology. As such, it could merit publication on *Food Microbiology*, as research article.

We thank you in advance for your interest in our submission.

Sincerely,

Dr. Severino Zara PhD.

Highlights

The antimicrobial activity of gaseous PLEO is increased when combined with refrigeration temperature

The antimicrobial activity of the gaseous PLEO vary between single and mixed *Listeria* strains

Bacterial cell wall and plasma membrane are putative targets of the PLEO

Gaseous PLEO shows no activity against resident mesophilic and lactic bacteria

PLEO may be considered a natural alternative to synthetic antimicrobials

Antimicrobial activity of gaseous *Citrus limon* var *Pompia* leaf essential oil against *Listeria monocytogenes* on *ricotta salata* cheese

Francesco Fancello[1§], Giacomo L. Petretto[1,2§], Salvatore Marceddu[3], Tullio Venditti[3], Giorgio Pintore[2]*, Giacomo Zara[1], Ilaria Mannazzu[1], Marilena Budroni[1], Severino Zara[1]*

[1]Department of Agriculture, University of Sassari, Italy

[2]Department of Chemistry and Pharmacy, University of Sassari, Italy

[3]CNR – ISPA Institute of Sciences of Food Production, Sassari, Italy

§ These authors should both be considered first name in the author list

*Corresponding authors: szara@uniss.it (Severino Zara) and pintore@uniss.it (Giorgio Pintore)

Abstract

Contamination by *Listeria monocytogenes* is a particularly challenging problem in the food industry due to the ability of the bacterium to develop under conditions normally used for food preservation. Here, we show that the gaseous phase of *Citrus limon* var *pompia* leaf essential oil (hereafter PLEO) exerts specific anti-*Listeria* activity on *ricotta salata* cheese stored at 5 °C. The synergic effect of gaseous PLEO treatment and refrigeration was first confirmed *in vitro* on *L. monocytogenes* strains treated for 3 h with gaseous PLEO and then stored at 5 °C. Ricotta cheese was then inoculated with *L. monocytogenes* strains and subjected to hurdle technology with different concentrations of gaseous PLEO. Cell counts revealed gaseous PLEO to exert a bactericidal effect on *L. monocytogenes* 20600 DSMZ and a bacteriostatic effect on a mix of *L. monocytogenes* strains. Scanning and transmission electron microscopy analyses of *L. monocytogenes* cells suggested that gaseous PLEO targets the

bacterial cell wall and plasma membrane. Chemical analyses of the liquid and vapor phases of PLEO indicated linalyl acetate to be the predominant compound, followed by limonene and the two isomers of citral, whereas EO composition analysis, although generally in line with previous findings, showed the presence of linalyl acetate for the first time. SPME coupled with gas chromatography confirmed the presence of all crude oil components in the headspace of the box.

Keywords: hurdle technology, *Listeria monocytogenes*; *ricotta salata*; EO gas; citral; refrigeration temperature

1. Introduction

Microbial food spoilage is due to the uncontrolled growth of undesired microorganisms that cause food deterioration through the production of toxins and off-flavors and that may also be the source of food borne illness. In industrialized countries, more than 30 % of people are affected each year by foodborne disease that can largely be attributed to the contamination of food or drinking water (WHO, 2002; Nedorostova et al., 2009). Although chemical antimicrobial agents have been employed in food processing for decades with the aim of reducing the effect of microbial contamination, there is now increasing demand for natural food ingredients and additives, including natural antimicrobial compounds (Sofos et al., 1998). Thus, to keep up with consumer trends, naturally derived substances, such as plant essential oils (EOs), as well as plant secondary metabolites are being considered as alternatives to synthetic antimicrobials, (Jun et al., 2013; Sofos et al., 1998; Tyagi and Malik, 2012; Seo et al., 2015).

The *European Pharmacopoeia* (2008) defined essential oils (EOs) as an "odorous product, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating". Since the chemical composition of EOs vary according to the raw plant material, even in the same botanical species, the antimicrobial effect varies significantly with respect to plant origin. In addition, pathogens are able to develop resistance to specific compounds, whereas it may be more difficult for them to develop resistance to a complex mixture of compounds, as in the case of EOs (da Silva Luz et al., 2012).

Although the activity of EOs and their components has been evaluated *in vitro* against a number of foodborne microorganisms, fewer studies have been carried out on the application of EOs as antimicrobial agents in real food systems. The general conclusion emerging from the literature is that higher concentrations of EOs are required when working with a real food system in order to achieve the same effect observed under the same conditions in *in vitro* assays (Perricone et al. 2015).

The protocols developed to study the preservative effect of EOs in food systems can be divided into 3 main groups: i) direct contact with a solution of EOs and Tween®; ii) inclusion of the EOs in edible

films or nano-carriers able to interact directly with both the atmosphere and the food; iii) vapor treatment of food with gaseous EOs.

Gaseous EOs (EO gases) are not added directly onto foods, but can be used as primary antimicrobial agents in food packaging materials and also as sanitizing agents for food surfaces and food-contact surfaces. The advantage of using EO gases is that they often cause minimal alterations to food aroma and flavor because there is minimal penetration into the subsurface area (Goñi et al., 2009; Tyagi et al., 2012). Some EO gases have been found to exert greater antimicrobial activity against foodborne pathogens and spoilage bacteria when compared with EO in liquid phase (Tyagi and Malik, 2010). Devices have also been proposed for studying the antimicrobial activity of EO vapor or other volatile compounds applied directly onto the food matrix, such as compact chambers made from acrylic materials able to contain the food and produce EO vapor or gas (Venditti et al. 2009; Tyagy and Malik 2012; Petretto et al. 2013, Ladu et al. 2015, Lee et al. 2018).

Our research group recently studied the chemical composition and the *in vitro* antimicrobial activity of the EO extracted from the leaves of a particular variety of Sardinian lemon, namely *Citrus limon* var. *pompia* Camarda (Fancello et al 2016). Our *in vitro* experiments against several strains of *Listeria monocytogenes* showed its EO to exert antilisterial activity.

L. monocytogenes can be present in many environmental niches (Vivant et al., 2013) given its widespread distribution and ability to grow in a broad range of conditions (Ferreira et al., 2014), including the harsh conditions that may be encountered in food. *L. monocytogenes* has been isolated from a variety of raw and processed food matrices, including milk and dairy products, meat and egg products, seafood, vegetables, and other ready-to-eat foods (Ferreira et al., 2014). Dairy products have been associated with approximately half of all reported outbreaks of listeriosis in Europe and the United States (CDC, 2016; Filipello et al., 2017).

Considering its physico-chemical properties, *ricotta salata* cheese is prone to contamination of pathogenic and spoilage microorganisms (Casti et al., 2016). According to Spanu et al. (2012), *ricotta salata* cheese can become easily contaminated by *L. monocytogenes* due to its frequent manipulation

during the production process, posing serious challenges to *L. monocytogenes* growth control. A multi-state outbreak of listeriosis in the United States linked to *ricotta salata* imported from Italy was recently recorded. *Ricotta salata* cheese is usually stored at refrigeration temperature and its shelf-life may vary from three weeks up to several months (Spanu et al., 2015). It is often used in salads as well as many other dishes and may be sliced, crumbled or grated.

In the present work, a mild hurdle strategy was used to test the growth of *L. monocytogenes* inoculated onto *ricotta salata* cheese using *Pompia* leaf essential oil and citral gases at refrigeration temperature.

2. Material and Methods

2.1. Plant material and extraction of EO

Three samples of *Pompia* leaf were collected in May-June from the Sardinian town of Siniscola, Italy. A sample of leaves weighing 500 g was suspended over 1.000 mL water and subjected to steam distillation for 2 h using a Clevenger type apparatus (European Pharmacopoeia 2002). The yield of EOs ranged between 0.43 % (v/w) and 0.52 % (v/w), calculated from the dry weight. Extraction was carried out in triplicate, and the obtained EOs were collected separately, dried over anhydrous sodium sulfate (Na_2SO_4), and then stored under a nitrogen atmosphere at 4 ºC in amber glass vials until analyzed**.**

2.2. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC-MS analysis was carried out using an Agilent 7890 GC equipped with a Gerstel MPS autosampler, coupled with an Agilent 7000C MSD detector. The chromatographic separation was performed on a HP-5 capillary column (30 m × 0.25 mm, film thickness 0.17 μm) (Agilent). The following temperature program was used: 60 ºC hold for 3 min, then increased to 210 ºC at a rate of 4 ºC/min, then held at 210 ºC for 15 min, then increased to 300 ºC at a rate of 10 ºC/min, and finally held at 300 ºC for 15 min. Helium was used as the carrier gas at a constant flow of 1 mL/min. The data were analyzed using a MassHunter Workstation B.06.00 SP1, with identification of the

individual components (Table 1) performed by comparison with the co-injected pure compounds and by matching the MS fragmentation patterns and retention indices to the built-in libraries, literature data, or commercial mass spectral libraries (NIST/EPA/NIH 2008; HP1607 purchased from Agilent Technologies).

2.3. Gas Chromatography-Flame Ionization Detector (GC-FID) analysis

The GC analysis of the EOs was carried out using an Agilent 6890N instrument equipped with an FID and an HP-5 capillary column (30 m × 0.25 mm, film thickness 0.17 μm). The column temperature was held at 60 ºC for 3 min, then increased to 210ºC at a rate of 4 ºC/min and held at 210ºC for 15 min, then increased to 300 ºC at a rate of 10ºC/min, and finally held at 300 ºC for 15 min. The injector and detector temperatures were both 250 °C. Helium was used as carrier gas at a flow rate of 1 mL/min. The quantification of EO compounds was carried out using the internal standard method and involved injecting a solution of EOs diluted in hexane (dilution ratio 1:200). A calibration curve was constructed for each standard compound. When standards were unavailable, quantification was performed using the calibration curve for a compound of the same class of volatile (monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenates sesquiterpenes) present in the EO. The results are expressed as mg per mL of distilled EO (Petretto et al. 2016).

2.4. SPME conditions

The isolation of box headspace volatile compounds was carried out using a manual 100 μm PDMS/DVB/CAR (Polydimethylsiloxane/Divinylbenzene/Carboxen) coated fiber (Supelco, Sigma Aldrich, St. Louis, Mo., U.S.A.), which was conditioned prior to use according to manufacturer instructions. After 80 and 180 min of equilibration time, the conditioned fiber was injected through the septum and suspended in the headspace of the box device. The fiber was exposed for 2 min in order to extract the volatiles. The fiber was then retracted, removed from the box, and placed

immediately into the injector of the GC. Thermal desorption was performed inside the injector at a temperature of 250 °C for 5 min in splitless injection mode. Prior to and after each analysis, the fiber underwent a further bake-out step for 5 min at 250 °C.

2.5. Bacterial strains and culture conditions.

L. monocytogenes 20600 DSMZ (serovar 1/2a), obtained from DSMZ (*Deutsche SammLung von Mikroorganismen und Zellkulturen*, i.e., German Collection of Microorganisms and Cell Cultures), and two wild strains, *L. monocytogenes* B STAA and *L. monocytogenes* E STAA, previously isolated from *ricotta salata* cheese were used. The latter two strains belong to serogroup 3 (1/2b, 3b e 7) (data not shown). All strains were stored at -80 °C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with glycerol (20 % v/v) and subcultured twice in BHI broth at 37 °C for 18 h to reach the early stationary phase (~9 log CFU/mL).

2.6. In vitro antimicrobial assay

The *in vitro* antilisterial activity of gaseous PLEO and citral was evaluated using the disc volatilization method (Nedorostova et al., 2009) at five different concentrations (ranging from 0.001 to 0.168 µL/cm^3 for PLEO, and from 0.005 to 0.076 µL/cm^3 for citral). First, Petri dishes with exactly 20 mL of BHI agar were inoculated with 100 µL suspensions of 10^6 CFU/mL *L. monocytogenes*. Sterile paper disks (Whatman No. 1, diameter 55 mm) were then dosed with PLEO (10.4, 5.2, 2.6, 1.3, 0.65 µL) or citral (6.8, 3.4, 1.7, 0.85, 0.425 µL), and then taped to the cover of each Petri dish. In accordance with Nedorostova et al., (2009), the two lowest doses of PLEO (1.3, 0.65 µL) were diluted in diethyl ether 1:10 to facilitate the oil's solubilization. Then, plates were sealed with parafilm© to prevent gas leakages and incubated at 37 °C for 24 h. After incubation, the minimum inhibitory concentrations (MICs) were recorded. The MICs were expressed as microliters of PLEO or citral per volume of atmosphere above the inoculated agar surface and defined as the lowest

concentration which clearly produced a visible inhibition zone. Each test was performed in triplicate and the experiments were repeated twice.

2.7. Evaluation of the effect of gaseous PLEO treatment and refrigeration temperature

Twelve BHI agar Petri dishes for each strain were individually inoculated singularly with of one the three strains of *L. monocytogenes* or with the mix of these strains, with a final concentration of ~10^3 cells of *L.monocytogenes per* plate. For the treatment, 6 inoculated Petri dishes were placed inside a 20-liter sealed polyethylene box, previously sterilized by an overnight UV treatment, equipped with a circulation fan, to facilitate the circulation of the oil gas inside the box, and a heating system, to obtain the gradual evaporation of PLEO, both of which were connected to a power supply - see paragraph 2.9 - (Venditti et al., 2009; Ladu et al., 2015). The inoculated plates were incubated at 25 °C for 3 h in the presence of 0.5 ml of PLEO. The other 6 plates (used as positive control) were incubated at same conditions without the gas treatment. After three hours, treated and positive control plates were incubated at 37 °C and at 5 °C. The growth of *L.monocytogenes* strains were monitored for 2 and 10 days for plates incubated at 37 °C and 5 °C respectively.

2.8. L. monocytogenes determination on ricotta salata cheese.

Ricotta salata cheeses were purchased in 300-gram portions from a local retail grocer (Sassari, Italy), transported under controlled temperature to the laboratory and analyzed. The presence of *L. monocytogenes* strains was ascertained according to standard methods (EN ISO 11290-1:2017).

2.9. Inoculation of ricotta salata cheese with L. monocytogenes and EO vapor treatments.

For the artificial inoculation of the *ricotta salata* cheese, two different culture suspensions were prepared using: 1) an 18-hour culture of *L. monocytogenes* 20600 DSMZ; and 2) an 18-hour culture including the three strains used in this work (see above). Each inoculum was serially diluted in 0.1 % Buffered Peptone Water (BPW) to obtain a concentration of approximately 6 log CFU/mL for the

subsequent experiments. *Ricotta salata* cheeses were aseptically cut into 10 g slices, transferred into sterile Petri dishes, and inoculated with 100 µL of the previously prepared inocula. *L. monocytogenes*-inoculated *ricotta salata* cheese slices were then placed inside a 20-litres sealed polyethylene box equipped with a circulation fan and a heating system, both of which were connected to a power supply (Figure S1) (Venditti et al. 2009; Ladu et al., 2015). Samples were incubated at room temperature (20±2 °C) for 3 h in the presence of PLEO or citral gases, used separately, at two different concentrations (0.5 mL and 1.0 mL). In order to obtain their gradual evaporation, EOs were pipetted into a heatproof glass vessel, already placed on a hot plate, while the circulation of the oils inside the box was obtained by the attached fan. After the gaseous treatments, the exact quantity of liquid PLEO and citral still present in the glass vessel containing the EOs was measured in order to estimate the concentration of EO evaporated. Specifically, the residual oil was weighed, the weight subtracted from the initial weight, and the result divided by the volume (cm^3) of the polyethylene box. Post-treatment, the *ricotta salata* slices were picked up using sterile disposable tweezers and placed in sterile Petri dishes that were then sealed with parafilm© and incubated for 30 d at 5 °C. *Ricotta salata* slices inoculated but not treated and incubated at 5 °C for 30 d were used as positive control (PC). Three slices were analyzed for each sampling at each time point: at the beginning of the experiment (T0), after 15 d (T15), and after 30 d (T30). All experiments were repeated twice. The enumerations of *L. monocytogenes* were conducted according to standard methods EN ISO 11290-2:2017.

2.10. Survival of natural flora in ricotta salata *cheese after the gaseous EO treatments*

The effects of PLEO and citral gases and control on the natural mesophilic bacteria population and lactic acid bacteria of *ricotta salata* cheese at the end of the experiment were estimated by putting the cheeses in a sterile stomacher bag (filter 0.5 mm pore size) (VWR, Milan) and homogenized for 1 min in 90 mL BPW. Total mesophilic bacteria count (TMC) was enumerated in Plate Count Agar (PCA, Microbiol, Cagliari, IT) and incubated at 30 °C for 48 h; whereas lactic acid bacteria (LAB)

were enumerated in MRS (De Man, Rogosa, Sharpe) agar (Microbiol, Cagliari, Italy) after incubation at 30 °C for 48 h.

2.11 Scanning and Transmission electron microscopy.

For the SEM morphologic investigation, samples of *L. monocytogenes* cells treated with 0.5 mL gaseous PLEO by the volatilization method and untreated cells were fixed for 3 h with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed in 1.0 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h. Samples were then dehydrated in an acetone series (ascending acetone concentrations) and critical point dried using a Polaron Jumbo critical point drier with carbon dioxide (CO_2) as medium. Dried samples were mounted on aluminum stubs and sputter coated with gold/palladium in an Edwards S150A Sputter Coater unit and examined using a Zeiss DSM 962 scanning electron microscopy.

For TEM investigations, samples of *L. monocytogenes* cells treated with gaseous 0.5 mL PLEO by the volatilization method and untreated cells were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 3 h and post-fixed in osmium tetroxide (OsO_4) 1 % for 1 h. Specimens were then dehydrated in an acetone series, embedded in Agar 100 epoxy resin, and ultra-thin sections were cut with a diamond knife on an RMC MT-7 ultramicrotome and stained with uranyl acetate and lead citrate. Samples were examined in a Zeiss EM 109 transmission electron microscope operating at 50 kV.

2.12 Statistical analyses

All microbial counts were log transformed to obtain a normal distribution. Data were analyzed by analysis of variance (ANOVA). When a significant effect was observed ($P < 0.05$), the differences between means were separated using the Tukey–Kramer multiple comparisons test. The mean TMC and LAB counts for control and treated samples were compared using Student's t-tests. SPSS software, version 19.0, was used to conduct the statistical analyses.

3. Results

3.1 Chemical composition of PLEO

The qualitative GC-MS analysis of EO extracted from *Pompia* leaves revealed some different results compared with literature data. In particular, the total ion chromatogram (TIC) obtained by a full scan analysis showed the presence of 27 compounds, of which terpinen-4-ol, linalyl acetate, and some sesquiterpene derivatives were not reported in similar studies from the literature (Flamini et al. 2019; Fancello et al. 2016). Quantitative determinations of EOs are not easy to perform since the whole range of required standards is not often available. Nonetheless, it is well accepted in the literature to use a limited number of calibration curves obtained by GC-FID analysis with standards for the same classes of compound as the target analytes (Bicchi et al 2008). By applying this method to *Pompia* leaf EO, it was possible to confirm that the main bulk of components that differentiated our results from those reported in the literature (Flamini et al. 2019; Fancello et al 2016) are related to compounds detected in trace quantities, with the exception of linalyl acetate, which had a concentration of almost 300 mg/mL of EO. By contrast, Fancello et al. (2016) detected D2-carene in a non-negligible amount (about 22 mg/mL), although this compound was not detected in the present study. The acetic ester of linalool was the main compound detected in the EO of the present study, followed by limonene detected at a concentration of about 257 mg/mL. Neral and geranial – the two Citral isomers – were detected at lower concentrations compared with those reported in our earlier publication (Fancello et al., 2016), precisely 87 and 98 mg/mL, respectively, compared with 173 and 214 mg/mL. A direct comparison of quantitative data with those obtained by Flamini et al. (2019) was not possible since different quantification methods were used.

3.2. Chemical composition of gaseous PLEO

The fumigation of PLEO was carried out by adding a measured amount of the EO into a Petri dish using the device previously described by Petretto et al. (2013) and the qualitative chemical analysis

of the compounds released into the headspace was carried out by HS-SPME/GC-MS. The chemical analyses were carried out at two different times and the results are reported as relative percentages obtained by peak area normalization (Table 2). In agreement with our previous study (Petretto et al. 2013), the concentration of the more volatile components is greater in the vapor phase with respect to the pure oil; they include the components that elute in the first part of the chromatogram (i.e., those with smaller retention times), such as limonene, myrcene, and other monoterpene compounds. By contrast, the less volatile compounds, such as neral, geranial, linalyl acetate, and the sesquiterpene fraction were present at lower concentrations in the vapor phase with respect to the pure oil. These results were also confirmed by the qualitative analysis of the residue (Figure 1) obtained by rinsing the Petri dishes with hexane once HS-SPME/GC-MS was complete. In addition to some decomposition derivatives, the residue was characterized by higher amounts of neral, geranial, and linalyl acetate, as well as lower amounts of limonene, sabinene, and the other more volatile components of PLEO.

3.3. MIC of PLEO and citral gases

The MICs of PLEO and citral gases, determined using the disc volatilization method, showed a strain dependent behavior. While the MIC of PLEO gas (0.086 µL/cm^3) was the same for all three *L. monocytogenes* strains tested, differences were observed in relation to citral gas sensitivity: the MIC of citral gas on *L. monocytogenes* 20600 DSMZ was 0.014 µL/cm^3, whereas it was 0.028 µL/cm^3 for the other two *L. monocytogenes* strains.

3.4. Determination of synergic antimicrobial effect between gaseous PLEO treatment and refrigeration temperature.

The treatment at different temperatures showed opposite results. At 37 °C no differences were observed in the growth of *L. monocytogenes* treated with gaseous PLEO respect to the control plate, except for a slight growth delay on the first day of incubation. On the contrary, when treatment with

gaseous PLEO was followed by incubation at 5 °C, a marked growth delay of *Listeria* was observed, even after 10 days of incubation. (Figure S2). In particular the growth of the strain *L. monocytogenes* E STAA was completely inhibited by the treatment and the following incubation at 5 °C, while *L. monocytogenes* B STAA was the less sensitive to the treatment. These results showed that refrigeration temperature and gaseous PLEO have a synergic effect.

3.5. Determination of the evaporation of PLEO and citral gases inside of the polyethylene box

The concentration of PLEO that evaporated inside the box in 3 h at room temperature, from the initial 1 mL and 0.5 mL doses was estimated by subtracting the weight of oil still present in the vessel post treatment from its initial weight, corresponding to 0.043 μL/cm^3 and 0.021 μL/cm^3 respectively. These values were thus considered as the concentrations of gaseous PLEO inside the container and they corresponded to half and a quarter of the MIC, respectively (previously determined by comparison with the disc volatilization method). Less evaporation occurred from 1 mL and 0.5 mL of liquid citral, giving final gaseous concentrations of 0.028 μL/cm^3 and 0.014 μL/cm^3 respectively, also corresponding to half and a quarter of the MIC.

3.6. Antimicrobial activity of gaseous PLEO against L. monocytogenes *strains*

The effects of two concentrations of gaseous PLEO on *L. monocytogenes* 20600 DSMZ strain inoculated onto *ricotta salata* cheese were evaluated as described in the paragraph 2.9 of the materials and methods (Figure 2A). Specifically, the activities of a 0.5 mL (low dose, LD) or 1 mL (high dose, HD) dose of PLEO were assessed. The positive control for bacterial growth (from here on, PC) was represented by *L. monocytogenes* 20600 DSMZ inoculated onto ricotta cheese in the absence of PLEO. Under these PC conditions, *L. monocytogenes* 20600 DSMZ reached a cell density of about 7 $\log_{10}$ CFU/g. The antilisterial effect of PLEO was both time and concentration dependent. Thus, the *L. monocytogenes* 20600 DSMZ viable count was significantly ($p<0.001$) reduced in the presence of a HD of PLEO gases (Figure 2A). The effect was noticeable after 15 days of incubation, with

significant reductions ($p<0.001$) of about 3 and 4 log units with respect to PC at T0 and at T15, respectively. At T30, the viable count was 1.69 $\log_{10}$ CFU\g, about 5.5 $\log_{10}$ CFU/g lower with respect to that of *L. monocytogenes* 20600 DMSZ measured in the PC sample at T30. *L. monocytogenes* 20600 DMSZ showed less susceptibility at the LD of PLEO gas, reaching a significantly lower value ($p<0.001$) of 6.08 $\log_{10}$ CFU/g at T30, a log unit lower than the PC count (7.29 $\log_{10}$ CFU/g) at the same sampling time.

The effect of gaseous PLEO treatment on the growth of the mix of the three strains of *L. monocytogenes* on *ricotta salata* cheese was less pronounced with respect to that observed on *L. monocytogenes* 20600 DSMZ alone (Figure 2B). Even in this experimental trial, the concentration of gaseous PLEO was the main factor that influenced the bacterial growth. In particular, the HD of PLEO gas caused a significant decrease in the mixed strain count ($p<0.001$) after 15 d of incubation (4.08 $\log_{10}$ CFU/g) compared with the T0 count (5.14 $\log_{10}$ CFU/g). After a further 15 d incubation (i.e., T30), we observed a significant rise ($p<0.001$) in the colony count, which reached 5.41 $\log_{10}$ CFU/g, but this count was still significantly lower ($p<0,001$) than the equivalent PC count (6.07 $\log_{10}$ CFU/g). At the LD, gaseous PLEO had a bacteriostatic effect on the growth of the *L. monocytogenes* mix of strains in *ricotta salata* cheese. After 15 d of incubation we observed a very slight decrease in the *L. monocytogenes* mix CFU/g. The CFU/g tended to increase significantly ($p<0.001$) after 30 days of incubation, but was still significantly different ($p<0.001$) to that in the PC.

3.7. Antimicrobial activity of gaseous citral against L. monocytogenes *strains*

The effect of gaseous citral on *L. monocytogenes* 20600 DMSZ in *ricotta salata* cheese was lower with respect to that exerted by PLEO gases, as showed in Figure 3A. At the HD, the effect on *L. monocytogenes* 20600 DMSZ growth was significant ($p<0.001$), with a reduction of about 2 $\log_{10}$ following 15 d of incubation (3.42 $\log_{10}$ CFU/g) with respect to T0 (5.29 $\log_{10}$ CFU/g), and dropping to 2.56 $\log_{10}$ CFU/g at T30. On the contrary, at the LD of citral gas, the viable counts were very similar to those observed following treatment with the LD of PLEO gas.

The antimicrobial efficacies of HD and LD citral gases against the mix of the three *L. monocytogenes* are presented in Figure 3B. The HD of citral gases significantly reduced ($p<0.001$) the CFU/g of the *L. monocytogenes* mix at T15, which remained constant until the end of experiment (T30) and was significantly different ($p<0.001$) from the PC count (4.58 $\log_{10}$ CFU/g vs. 6.31 $\log_{10}$ CFU/g, respectively). Similar results were observed for the LD of citral. Finally, it seems that the mix of three *L. monocytogenes* strains proliferated more slowly on *ricotta salata* cheese (PC) with respect to *L. monocytogenes* DSMZ alone, obtaining a CFU concentration just above 6 $\log_{10}$ CFU/g at T30.

3.8. Effect of gaseous PLEO and citral treatments on mesophilic and lactic acid bacteria

The treatment with gaseous PLEO and citral did not affect the TMC (total mesophilic bacteria) (8.02 $\log_{10}$ CFU/g *Vs* 8.03 $\log_{10}$ CFU/g, control and treated respectively) or LAB counts (7.17 $\log_{10}$ CFU/g *Vs* 7.47 $\log_{10}$ CFU/g) with no significant differences detected at T30 in their viable counts.

3.9. SEM and TEM analyses of PLEO gas-treated L. monocytogenes cells

The treatment of *L. monocytogenes* with gaseous PLEO *in vitro* showed a marked effect on cell morphology. SEM analysis showed that whereas untreated cells present intact, turgid, smooth surfaces (Figure 4A) and fibrillar structures (Zaamer et al., 2013), PLEO gas-treated cells (Figure 4B) showed no fibrillar structures. Moreover, PLEO gas-treated cells showed modifications in cell wall morphology and the presence of small bulges of coagulated structures – possibly proteins coming from the cell membrane and cytoplasm, as suggested by Koyama et al. (1997). To explore the ultra-structural modifications occurring in vapor-treated cells further, transmission electron microscopy was used. While *L. monocytogenes* cells showed a regular and delineated cell wall and plasma membrane with some dense structures well distributed through the cytoplasm (Figure 5A-B), most of the treated cells showed a thinner cell wall and white dense areas within the cytoplasm. Other cells showed internal damage and several deformities; in particular, an altered periplasmic space and dense

zones asymmetrically distributed throughout the cytoplasm (Figure 5C). Finally, some cells showed evident cell wall and plasma membrane ruptures (Figure 5D).

4. Discussion

Essential oils are considered to be powerful antimicrobial agents, with a plethora of potential applications in the food and food packaging industries, and as sanitizing agents for food surfaces. Moreover, there is growing evidence that EOs in the vapor phase are more effective antimicrobials than in the liquid phase (Tyagi and Malik, 2011; Lopez et al., 2005; Inouye et al., 2003). In the present work, we show that the MIC of PLEO gas was the same for all three tested strains of *L. monocytogenes*, while the MIC of citral gases varied among the strains, with the MIC for *L. monocytogenes* 20600 DSMZ being lower than that for the other two strains tested. These results are in accordance with those from other authors (Desai et al., 2012; Fancello et al., 2017; Yuan et al., 2017 Petretto et al., 2018), who have shown that the antimicrobial activity of EOs are species- and strain- dependent.

Moreover, we found that gaseous PLEO and citral were much more effective than their liquid phases (as reported by Fancello et al., 2016). We can postulate that gaseous PLEO and citral have different mechanisms of action in the vapor phase vs. liquid phase. Indeed, it has been previously demonstrated that essential oils used as vapors have better antimicrobial activities than the essential oils used in the liquid phase, probably as a result of better penetration and increased contact of the essential oil in the vapor phase (Tiagy et al., 2010 and 2011). Our results, in accordance with Inouye at al. (2001), also show that the vapors of aldehyde components, such as citral and cinnamaldehyde, were more active compared with the terpene ketone and terpene ether components, demonstrating that, in general, the antimicrobial activity of essential oils may depend to the volatility of the active compounds that makes the essential oils better antimicrobial agents in the vapor phase (Tyagi and Malik, 2012). Recently, Feyaerts et al. (2018) showed that the presence of vapor-phase-mediated antimicrobial activity could be predicted from the chemical class that predominates in the EOs, with aldehyde-,

phenol-, monoterpenol-, ether- and ketone-rich EOs showing the highest inhibitory activity. The greater antimicrobial activity of the vapor phase could also be explained by the fact that water reduces volatility, considering that components with hydroxyl groups may be more solvated and remain in the water phase (Sato el al., 2006). In relation to this, Boukhatem et al. (2014) stated that lipophilic molecules in the aqueous phase associate to form micelles and thus restrain the attachment of EOs to microorganisms. Moreover, we found that the strong antilisterial activity was dependent on the concentration of PLEO and citral used and on the type of inoculum (the 20600 DSMZ strain alone or mixed strains). Specifically, the antimicrobial activity of PLEO and citral gases was more effective when *ricotta salata* cheese was inoculated with just a single strain than with a mix of strains, and gaseous PLEO showed greater antimicrobial activity than pure citral gas, as already observed by other authors (Tyagi et al., 2012), thus indicating a synergistic or additive role of the EO components, enhancing the antimicrobial activity of the oil. Finally, we found that, the antilisterial activities of the gaseous PLEO tended to be more efficient when *L. monocytogenes* 20600 DSMZ cells were inoculated on *ricotta salata* slices than on BHI agar medium. This result lies in contrast with those of other studies where the activities of the EOs gases tended to be lower when used on contaminated food matrices (Lee et al., 2018), although Lorenzo-Leal et al. (2019) observed that the vapor from all-spice EO showed greater antimicrobial activity when assessed in alfalfa seeds than on laboratory media, in line with our findings.

High doses of EOs are generally required to inhibit microbial growth; thus a combination of EOs with other preservation techniques should be used rather than using them alone in order to reduce the required dose of EO (Frankova et al., 2014).

For this reason, the mild hurdle approach to fight the growth of *L. monocytogenes* in *ricotta salata* cheese using gaseous PLEO in combination with refrigeration was first tested *in vitro*, by comparing the effect at 5 °C with an incubation at 37 °C. The *in vitro* results confirmed the synergic effect of the gaseous oil combined with the refrigeration temperature. Once applied to the *in vivo* tests, results of the mild hurdle technology, showed that at 5 °C the antimicrobial activity of gaseous PLEO was very

strong with a significant reduction in terms of CFU counts of a single strain (or mix of strains) of *L. monocytogenes* expressed as log units after 15 and/or 30 days of incubation compared with the control.

We might therefore postulate that the use of mild hurdle technology, such as storage at 5 °C post treatment, favors bacterial cell damage and importantly prevents the cells from repairing and reforming their damaged cellular structures. In the case of our 3-strain mix, the lower anti-bacterial efficacy of the PLEO and citral gases on inoculated r*icotta salata* cheese could be due to the cross-resistance effect of the different *L. monocytogenes* strains (Giaouris et al., 2015; Kadam et al, 2013**)**. The efficacy of the mild hurdle technology used in this work has also been confirmed by other authors (Guevara et al., 2015; Ngang et al., 2014). Their studies showed that the combination of moderate heat with carvacrol and thymol or curcuma had a synergistic effect, leading to inactivation values that were three- or four-fold faster than when heat was used alone. Recent findings have suggested that cellular damage can already occur at sub-lethal concentrations of citral (Siroli et al., 2016); indeed, the sub-MIC concentration used in our work was capable of causing damage to the bacterial cells, and the post-treatment low storage temperature could hinder cellular repair. Interestingly, the continuous exposure of *L. monocytogenes* to citral does not induce the development of antimicrobial resistance (Apolonio et al., 2014).

The SEM images of the untreated samples revealed the presence of fibrillar structures, probably caused by the shrinkage of the exopolymeric matrix. These fibrillar structures were not, on the other hand, observed in PLEO-treated samples, perhaps due to the activity of the EO vapor against the matrix structure. Moreover, small bulges of coagulated structures were visible in the PLEO-treated cells, which were probably proteins extruded from the cell membrane and cytoplasm, (Koyama et al., 1997). These putative proteins may have been pushed out through holes produced in the cell wall by the oil vapors. A recent interesting work by Tyagi and Malyk (2012) showed a similar modification in cell morphology resulting from the use of gaseous *lemon* grass oil.

The data obtained by SEM and TEM reinforce the idea that the PLEO could target the cell wall and the plasma membrane, which could explain the bulges of coagulated structures. Indeed, similar results were found by: Gustafson et al. (1998), who treated *E. coli* with tea tree oil; Rasooli et al. (2006), who treated *L. monocytogenes* with thymus; and Becerril et al. (2007), who treated *E. coli* with cinnamon and oregano and with lemon grass (Tyagi and Malyk, 2012).

The chemical analysis of the EO employed in this study highlighted some differences compared with results reported in literature. With the exception of linalyl acetate, the differences regard minor compounds. The variability in chemical composition of the EO could be related to climatic conditions (Melito et al. 2016), geographical position (Petretto et al. 2018), and the harvesting period (Bennaoum et al. 2017).

5. Conclusions

In this work, we showed that different strains of *L. monocytogenes* can be inactivated by PLEO when used as a gas in artificially contaminated *ricotta salata* cheese. Moreover, we proved that the antimicrobial activity can be enhanced by mild hurdle technology, in the form of refrigeration. This combination of methods allowed us to significantly reduce the presence of *L. monocytogenes* in the *ricotta salata* cheese when the 20600 DSMZ strain was used alone, even at half MIC, and it resulted in bacteriostatic activity when all three *Listeria* spp. strains were used together. In the latter case, a possible cross-protection effect between the strains could have caused the reduced antimicrobial activity of the EO. Electron microscopy analyses implicated the putative targets of the EO to be the cell wall and the plasma membrane. Finally, it is important to underline that treatment with the gaseous EO vapor had no significant effect on the *ricotta salata* cheese's resident lactic acid bacteria. In summary, these results highlight the importance of investigating the effects of PLEO gas on different pathogen strains, used singularly or mixed, especially because it has been shown that more than one strain can reside in a specific ecological niche. Our results also highlight the potential

application of gaseous PLEO as an effective natural alternative to synthetic substances to enhance the microbial safety of *ricotta salata* cheese and other foodstuffs, while maintaining high cheese quality.

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References

Anonymous, 2017. EN ISO 11290-1, Microbiology of the Food Chain — Horizontal Method for the Detection and Enumeration of Listeria monocytogenes and of *Listeria* spp. — Part 1: Detection Method. International Organization for Standardization, Geneva

Anonymous, 2017. EN ISO 11290-2, Microbiology of the Food Chain — Horizontal Method for the Detection and Enumeration of *Listeria monocytogenes* and of *Listeria* spp. — Part 2: Enumeration Method. International Organization for Standardization, Geneva.

Apolonio, J., Faleiro, M.L., Miguel, M.G., Neto, L., 2014. No induction of antimicrobial resistance in *Staphylococcus aureus* and *Listeria monocytogenes* during continuous exposure to eugenol and citral. FEMS Microbiol. Lett. 354, 92-101.

Bennaoum, Z., Benhassaini, H. Falconieri, D., Piras, A., Porcedda, S. 2017. Chemical variability in essential oils from *Ruta* species among seasons, and its taxonomic and ecological significance. Nat. Prod. Res. 19, 2329-2334.

Becerril, R., Gomez-Lus, R., Goni, P., Lopez, P., Nerin, C., 2007. Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon or oregano against *E. coli* and *S. aureus*. Anal. Bioanal. Chem. 388, 1003–1011.

Bicchi, C., Liberto, E., Matteodo, M., Sgorbini, B., Mondello, L., d'Acampora Zellner, B., Costa R. and Rubiolo, P., 2008. Quantitative analysis of essential oils: a complex task. Flav. and Frag. J. 23, 382-391.

Boukhatem, M. N., Ferhat, M. A., Kameli, A., Saidi, F., Mekarnia. M., 2014. Liquid and vapour phase antibacterial activity of *Eucalyptus globulus* essential oil ¼ susceptibility of selected respiratory tract pathogens. Am. J. Infect. Dis. 10, 105–17.

Casti, D., Scarano, C., Pala, C., Cossu, F., Lamon, S., Spanu ,V., Ibba, M., Mocci, A. M., Tedde, F., Nieddu, G., Spanu, C., De Santis, E. P. L., 2016. Evolution of the microbiological profile of vacuum-packed ricotta salata cheese during shelf-life. Ital. J. Food. Sci. 5, 5501.

CDC, 2016. *Listeria* Oubreaks Summary. http://www.cdc.gov/listeria/outbreaks/index. html (accessed 12.4.16).

Desai, M.A., Soni, K.A., Nannapaneni, R., Schilling, M.W., Silva, J.L., 2012. Reduction of *Listeria monocytogenes* in Raw Catfish Fillets by Essential Oils and Phenolic Constituent Carvacrol. J. Food Sci. 77, M516-M522.

European Pharmacopoeia, 6th edn. EDQM (Council of Europe), Strasbourg, France, 2008; 680.

Fancello, F., Petretto, G., Zara, S., Sanna, M.L., Addis, R., Maldini, M., Foddai, M., Rourke, J.P., Chessa, M., Pintore, G., 2016. Chemical characterization, antioxidant capacity and antimicrobial activity against food related microorganisms of *Citrus limon* var. *pompia* leaf essential oil. LWT – Food. Sci. Technol. 69, 579-585.

Fancello, F., Zara, S., Petretto, G.L., Chessa, M., Addis, R., Rourke, J.P., Pintore, G., 2017. Essential oils from three species of Mentha harvested in Sardinia: chemical characterization and evaluation of their biological activity. Int. J. Food Prop. 20, 1751-1761.

Feyaerts, A.F., Mathé, L., Luyten, W., De Graeve, S., Van Dyck, K., Broekx, L., Van Dijck, P., 2018. Essential oils and their components are a class of antifungals with potent vapour-phase-mediated anti-*Candida* activity. Sci Rep. 8, 3958.

Ferreira, V., Wiedmann,M., Teixeira, P., Stasiewicz, M.J., 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J. Food Prot. 77:150–170.

Filipello, V., Gallina, S., Amato, E., Losio, M. N., Pontello, M., Decastelli, L., Lomonaco, S., 2017. Diversity and persistence of *Listeria monocytogenes* within the Gorgonzola PDO production chain and comparison with clinical isolates from the same area, Int. J. Food Microbiol. 245, 73-7.

Flamini, G., Pistelli, L., Nardoni, S., Ebani, V.V., Zinnai, A., Mancianti, F., Ascrizzi, R., Pistelli, L., 2019. Essential oil composition and biological activity of "Pompia", a Sardinian citrus ecotype. Molecules.

Frankova, A., Smid, J., Kloucek, P., Pulkrabek, J., 2014. Enhanced antibacterial effectiveness of essential oils vapors in low pressure environment. Food Control 35, 14-17.

Giaouris, E., Heir, E., Desvaux, M., Hébraud, M., Møretrø, T., Langsrud, S., Doulgeraki, A., Nychas, G. J., Kacániová, M., Czaczyk, K., Ölmez, H., Simões, M., 2015. Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. Front. Microbiol. 6:841. doi: 10.3389/fmicb.2015.00841

Goñi, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., Nerín, C., 2009. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. Food Chem. 116, 982–989.

Guevara, L., Antolinos, V., Palop, A., Periago, P.M., 2015. Impact of moderate heat, carvacrol, and thymol treatments on the viability, injury, and stress response of *Listeria monocytogenes*. Biomed Res. Int. 2015, 10.

Gustafson, J.E., Liew, Y.C., Chew, S., Markham, J., Bell, H.C., Wyllie, S.G., Warmington, J.R., 1998. Effects of tea tree oil on *Escherichia coli*. Lett. Appl. Microbiol. 26, 194-198.

Inouye, S., Takizawa, T., & Yamaguchi, H. 2001. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. J. Antimicrob. Chemother. 47, 565–573.

Inouye, S., Abe, S., Yamaguchi, H., Asakura, M., 2003. Comparative study of antimicrobial and cytotoxic effects of selected essential oils by gaseous and solution contacts. Int. J. Aromather. 13, 33-41.

Jun, H., Kim, J., Bang, J., Kim, H., Beuchat, L.R., Ryu, J.-H., 2013. Combined effects of plant extracts in inhibiting the growth of *Bacillus cereus* in reconstituted infant rice cereal. Int. J. Food Microbiol. 160, 260–266

Kadam, S. R., den Besten, H. M.W., van der Veen, S., Zwietering, M. H., Moezelaar, R., Abee, T., 2013. Diversity assessment of *Listeria monocytogenes* biofilm formation: Impact of growth condition, serotype and strain origin, Int. J. Food Microbiol.165, 259-264.

Koyama, S., Yamaguchi, Y., Tanaka, S., & Motoyashiya, J., 1997. A new substance (yoshixol) with an interesting antibiotic mechanism from wood oil of Japanese traditional tree (kiso-hinoki), *Chamaecyparis obtusa*. Gen. Pharmacol. , 28, 797–804.

Ladu, G., Cubaiu, L., d'Hallewin, G., Pintore, G., Petretto, G.L., Venditti, T., 2015. *Rosmarinus officinalis* L. and *Myrtus communis* L. Essential Oils Treatments by Vapor Contact to Control *Penicillium digitatum*. J. Food Process. Technol. 6, 492.

Lee, G., Kim, Y., Kim, H., Beuchat, L.R., Ryu, J.-H., 2018. Antimicrobial activities of gaseous essential oils against *Listeria monocytogenes* on a laboratory medium and radish sprouts. Int. J. Food Microbiol. 265, 49-54.

Lopez, P., Sanchez, C., Batlle, R., Nerin, C., 2005. Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. J. Agric. Food Chem. 53, 6939-6946.

Lorenzo-Leal, A.C., Palou, E., López-Malo, A., 2019. Evaluation of the efficiency of allspice, thyme and rosemary essential oils on two foodborne pathogens in in-vitro and on alfalfa seeds, and their effect on sensory characteristics of the sprouts. Int. J. Food Microbiol. 295, 19-24.

Melito, S., Petretto, G.L, Podani, J, Foddai, M., Maldini, M., Chessa, M., Pintore, G., 2016. Altitude and climate influence *Helichrysum italicum* subsp. *microphyllum* essential oils composition. Ind. Crops Prod. 80, 242-250.

Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., Pulkrabek, J., 2009. Antimicrobial properties of selected essential oils in vapour phase against food borne bacteria. Food Control 20, 157–160.

Ngang, J.J., Nyegue, M.A., Ndoye, F.C., Tchuenchieu Kamgain, A.D., Sado Kamdem, S.L., Lanciotti, R., Gardini, F., Etoa, F.X., 2014. Characterization of Mexican coriander (*Eryngium foetidum*) essential oil and its inactivation of *Listeria monocytogenes* in vitro and during mild thermal pasteurization of pineapple juice. J. Food Prot. 77, 435-443.

Perricone, M., Arace, E., Corbo, M. R., Sinigaglia, M., Bevilacqua, A., 2015. Bioactivity of essential oils: a Review on their interaction with food components. Front Microbiol. 6, 76, 1-7.

Petretto, G.L., Maldini, M., Addis, R., Chessa, M., Foddai, M., Rourke, J., Pintore, G., 2016. Variability of Chemical Composition and Antioxidant Activity of Essentials Oils Between *Myrtus communis* var. *Leucocarpa* DC and var. *Melanocarpa* DC. Food Chemistry, 197, 124-131.

Petretto, G.L., Foddai, M., Maldini, M., Chessa, M., Venditti, T., D'Hallewin, G., Pintore, G., 2013. A novel Device for the Study of Antimicrobial Activity by Vapor-Contact of Volatile Substances on Food Products. Commun. Agric. Appl. Biol. Sci.78, 65-72.

Petretto, G.L., Fancello, F., Bakhy, K., Faiz, C.A., Sibawayh, Z., Chessa, M., Zara, S., Sanna, M.L., Maldini, M., Rourke, J.P., Pintore, G., 2018. Chemical composition and antimicrobial activity of essential oils from *Cuminum cyminum* L. collected in different areas of Morocco. Food Bioscience 22, 50-58.

Rasooli, I., Rezaei, M. B., Allameh, A., 2006. Ultrastructural studies on antimicrobial efficacy of the thyme essential oils on *Listeria monocytogenes*. Int J Infect Dis.10, 236–241.

Sato, K., Krist, S., Buchbauer, G., 2006. Antimicrobial effect of trans-cinnamaldehyde, (-)-perillaldehyde, (-)-citronellal, citral, eugenol and carvacrol on airborne microbes using an airwasher. Biol. Pharm. Bull. 29, 2292-2294.

Seo, H.S., Beuchat, L. R., Kim, H., Ryu, J.H., 2015. Development of an experimental apparatus and protocol for determining antimicrobial activities of gaseous plant essential oils. Int. J. Food Microbiol. 215, 95-100.

da Silva Luz, I., Neto, N.J.G., Tavares, A.G., Nunes, P.C., Magnani, M., de Souza, E.L., 2012. Evidence for lack of acquisition of tolerance in *Salmonella enterica* serovar *typhimurium* ATCC 14028 after exposure to subinhibitory amounts of *Origanum vulgare* L. Essential oil and carvacrol. Appl. Environ. Microbiol. 78, 5021-5024.

Siroli, L., Patrignani, F., Serrazanetti, D.I., Vannini, L., Salvetti, E., Torriani, S., Gardini, F., Lanciotti, R., 2016. Use of a nisin-producing *Lactococcus lactis* strain, combined with natural antimicrobials, to improve the safety and shelf-life of minimally processed sliced apples. Food Microbiol. 54, 11-19.

Sofos, J.N., Beuchat, L.R., Davidson, P.M., Johnson, E.A., 1998. Naturally occurring antimicrobials in food. Regul. Toxicol. Pharmacol. 28, 71–72.

Spanu, C., Scarano, C., Spanu,V., Pala, C., Di Salvo, R., Piga, C., Buschettu, L., Casti, D., Lamon, S., Cossu, F., Ibba, M., De Santis, E.P.L., 2015. Comparison of post-lethality thermal treatment conditions on the reduction of *Listeria monocytogenes* and sensory properties of vacuum packed ricotta salata cheese. Food Control 740, 747-50.

Spanu, C., Scarano, C., Spanu, V., Penna, C., Virdis, S., De Santis, E.P.L., 2012. *Listeria monocytogenes* growth potential in Ricotta salata cheese. Int. Dairy J. 24, 120-122.

Tyagi, A.K., Malik, A., 2010. Antimicrobial action of essential oil vapours and negative air ions against *Pseudomonas fluorescens*. Int. J. FoodMicrobiol. 143, 205–210.

Tyagi, A.K., Malik, A., 2011. Antimicrobial potential and chemical composition of *Eucalyptus globulus* oil in liquid and vapour phase against food spoilage microorganisms. Food Chem. 126, 228-235.

Tyagi, A.K., Malik, A., 2012. Bactericidal action of lemon grass oil vapors and negative air ions. Innovative Food Sci. Emerg. Technol. 13, 169–177.

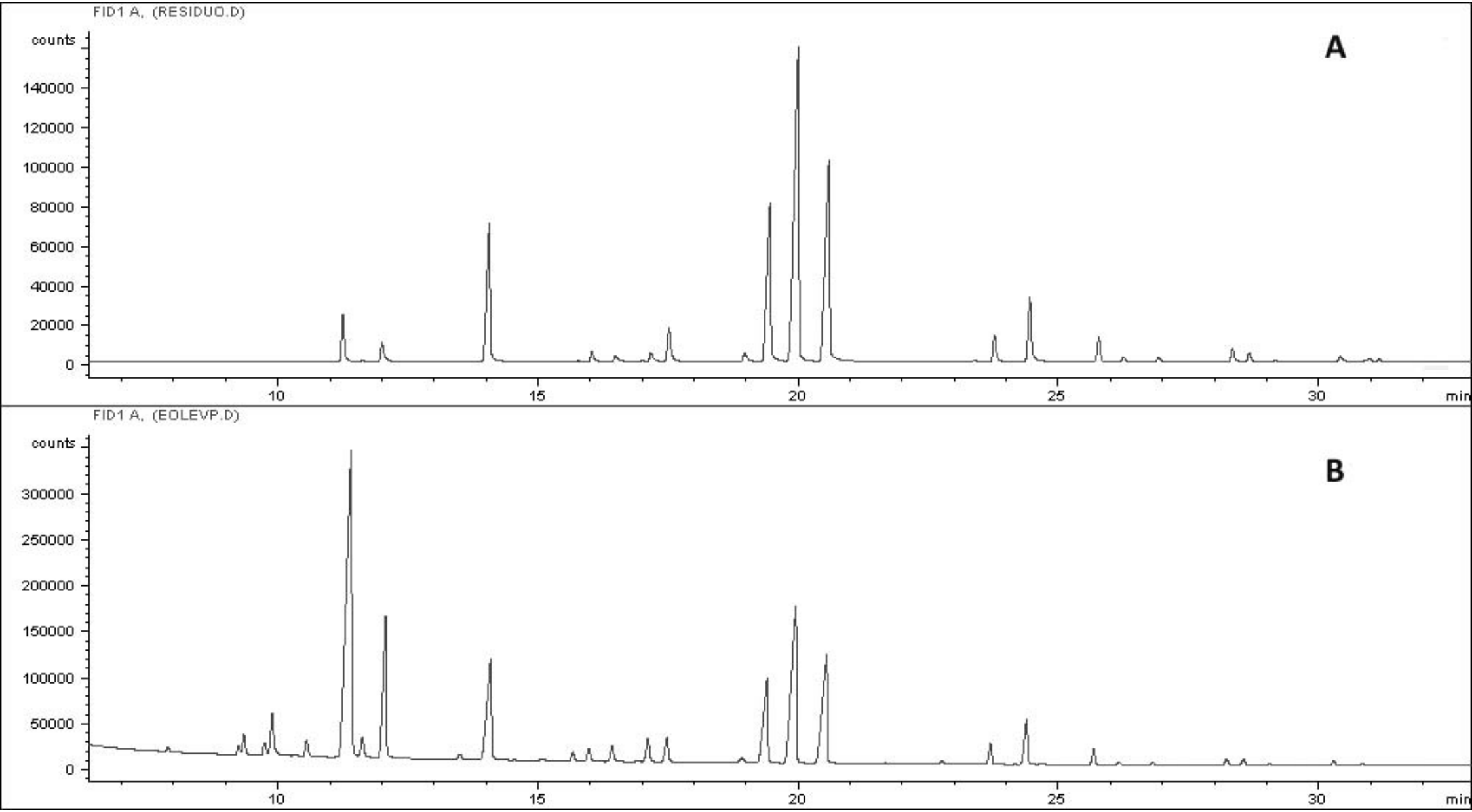
Venditti, T., Dore, A., Molinu, M.G., Agabbio, M., D'hallewin, G., 2009. Combined effect of curing followed by acetic acid vapour treatments improves postharvest control of *Penicillium digitatum* on mandarins. Postharvest Biol. Tec. 54, 111-114.

Vivant, A.L., Garmyn, D., Piveteau, P., 2013. *Listeria monocytogenes*, a down-to-earth pathogen. Front. Cell. Infect. Microbiol. 3. http://dx.doi.org/10.3389/fcimb.2013.00087.

WHO. (2002). Food safety and foodborne illness. Geneva, Switzerland: World Health Organization Fact sheet 237 (revised January 2002).

Yuan, W., Lee, H.W., Yuk, H.-G., 2017. Antimicrobial efficacy of *Cinnamomum javanicum* plant extract against *Listeria monocytogenes* and its application potential with smoked salmon. Int. J. Food Microbiol. 260, 42-50.

Zameer, F., Gopal, S., Krohne, G., Kreft, J., 2010. Development of a biofilm model for *Listeria monocytogenes* EGD-e. World J. Microbiol. Biotechnol. 26, 1143-1147.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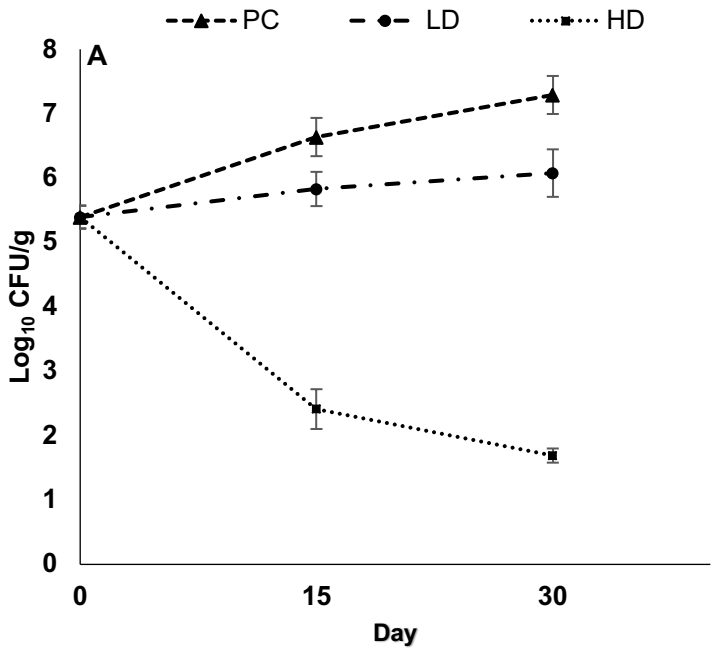
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4
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Log10 CFU/g
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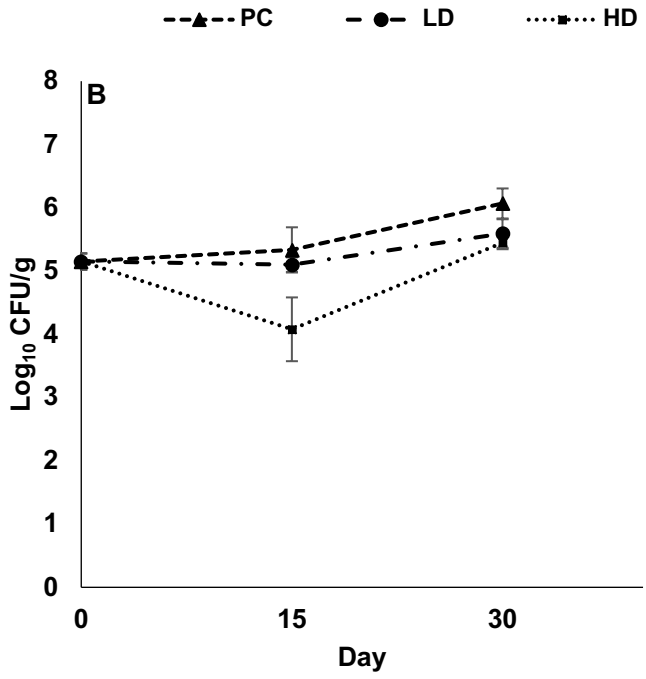
Day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Log10 CFU/g
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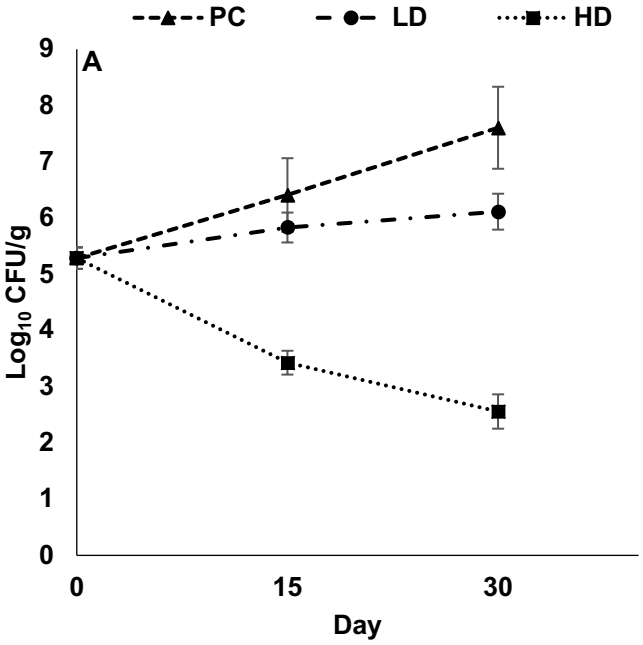
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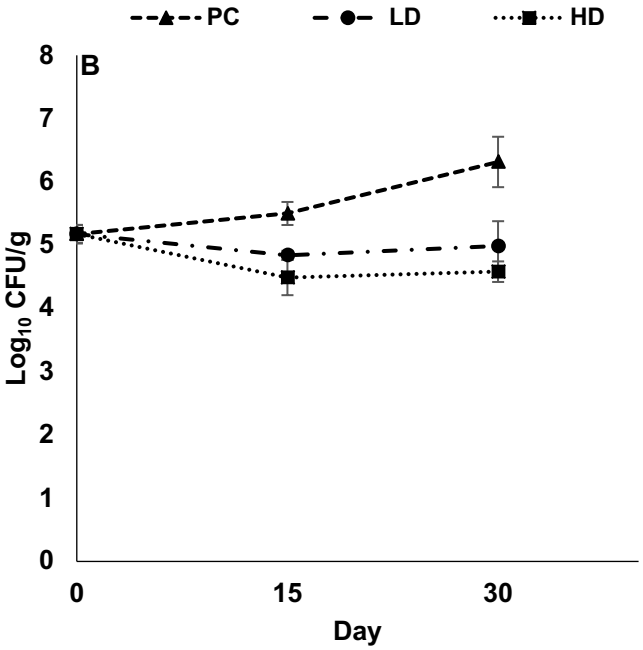
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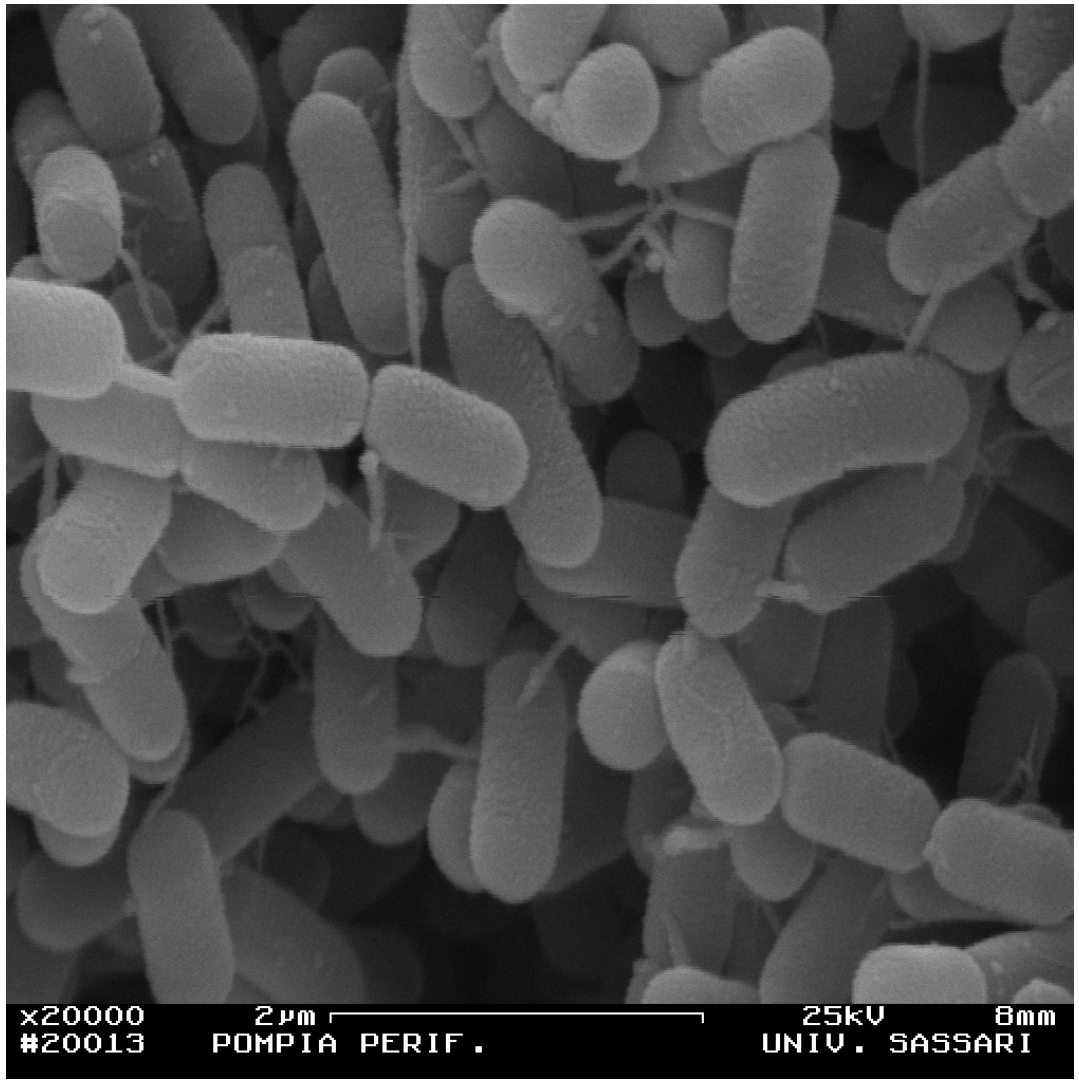
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#20013 POMPIA PERIF. UNIV. SASSARI

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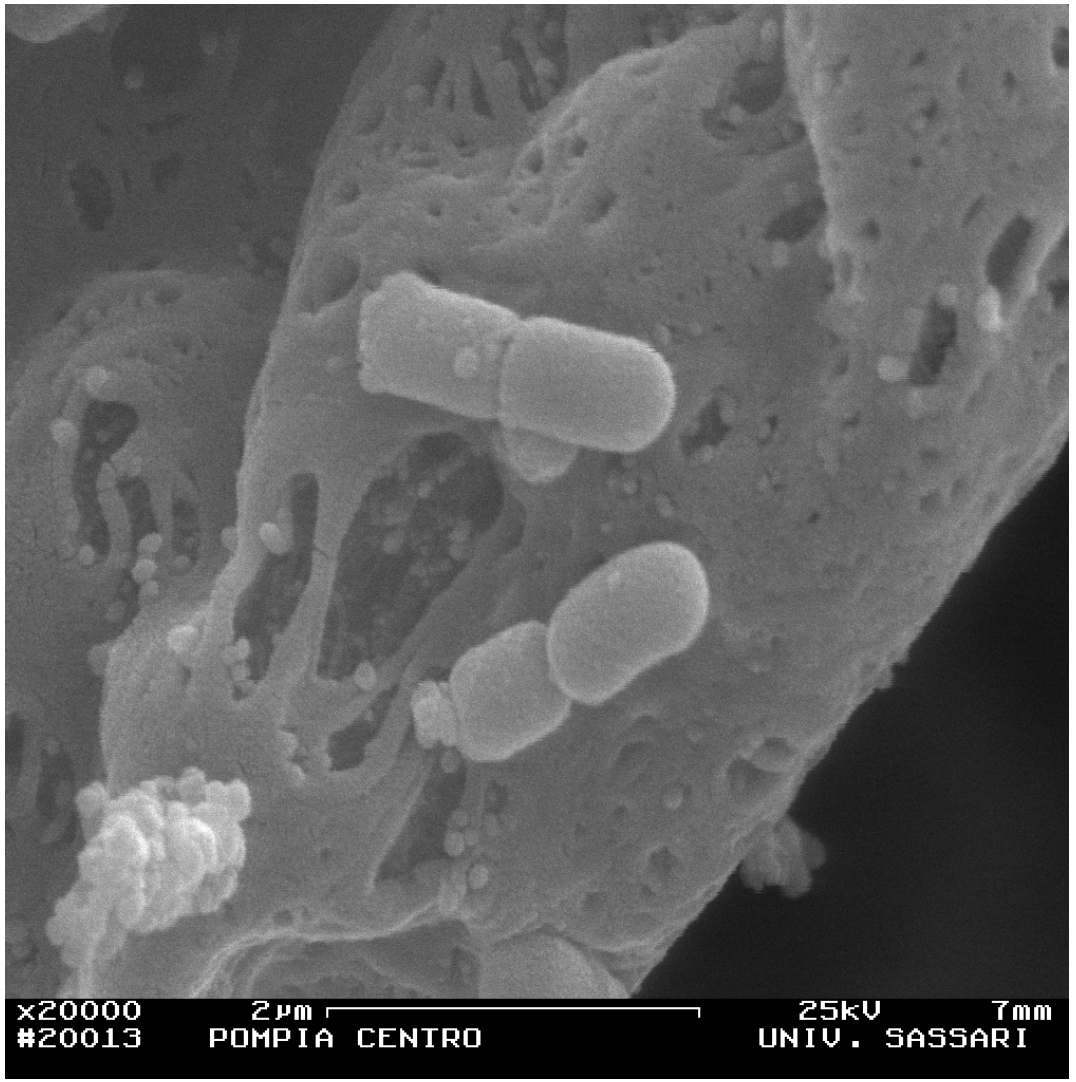
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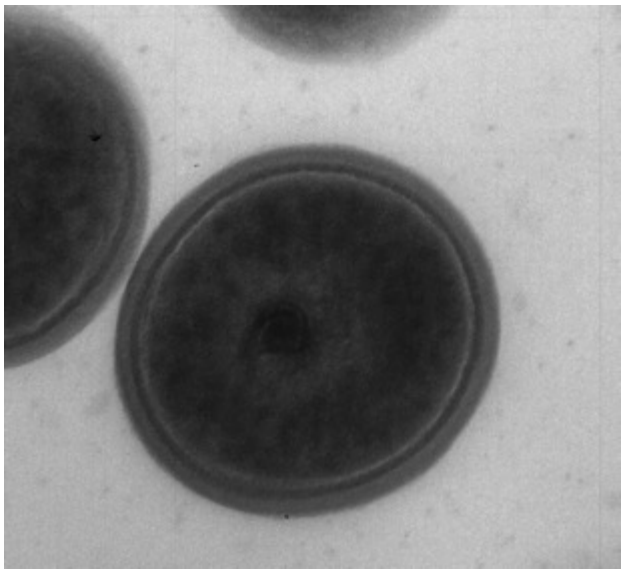

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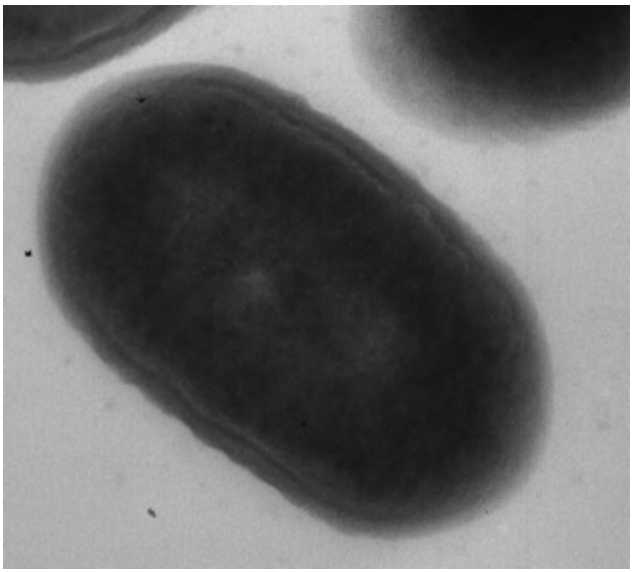

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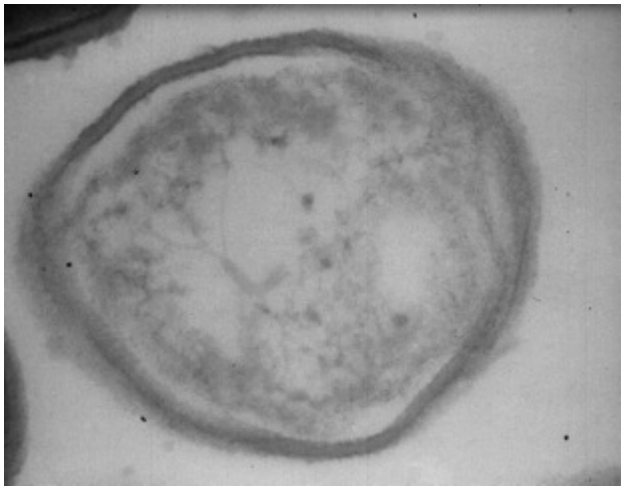

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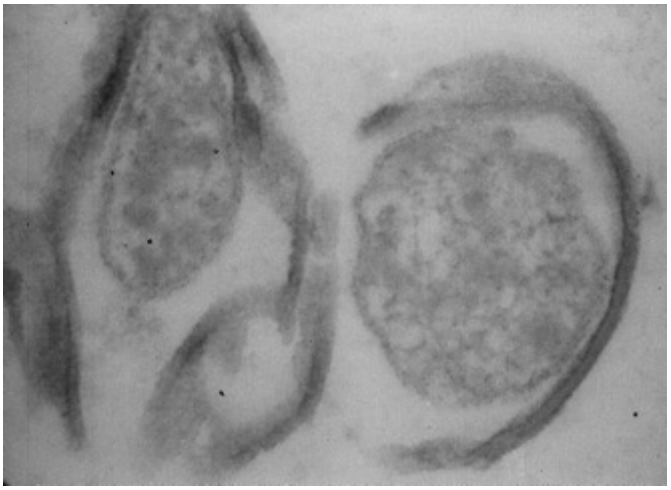

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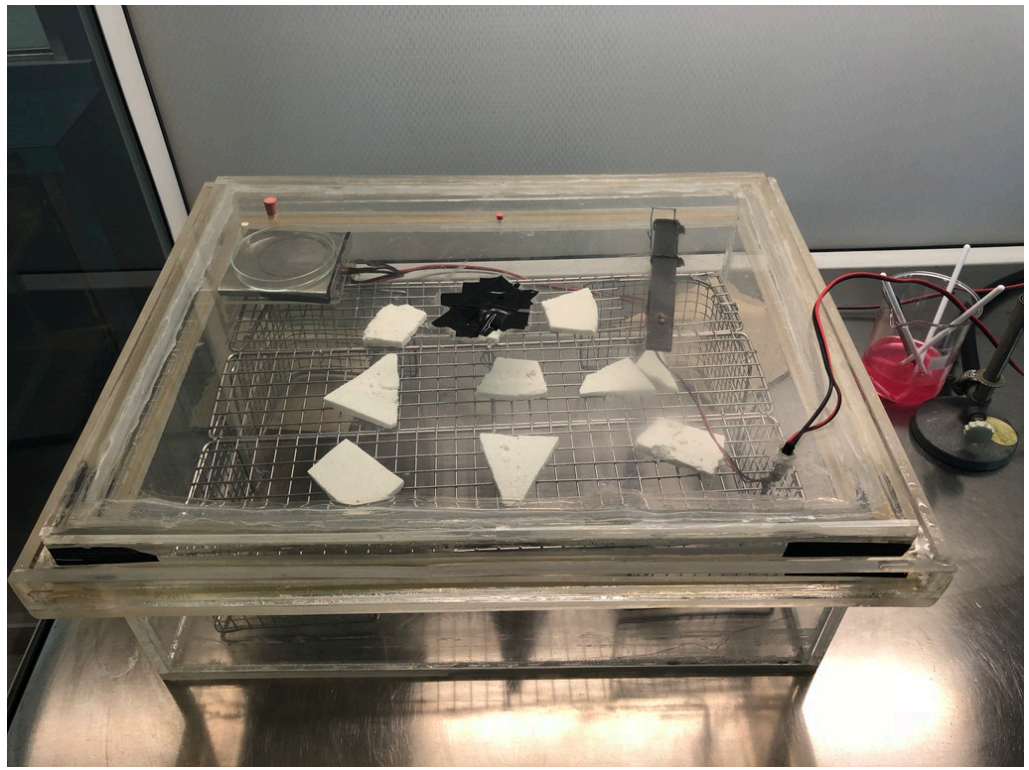

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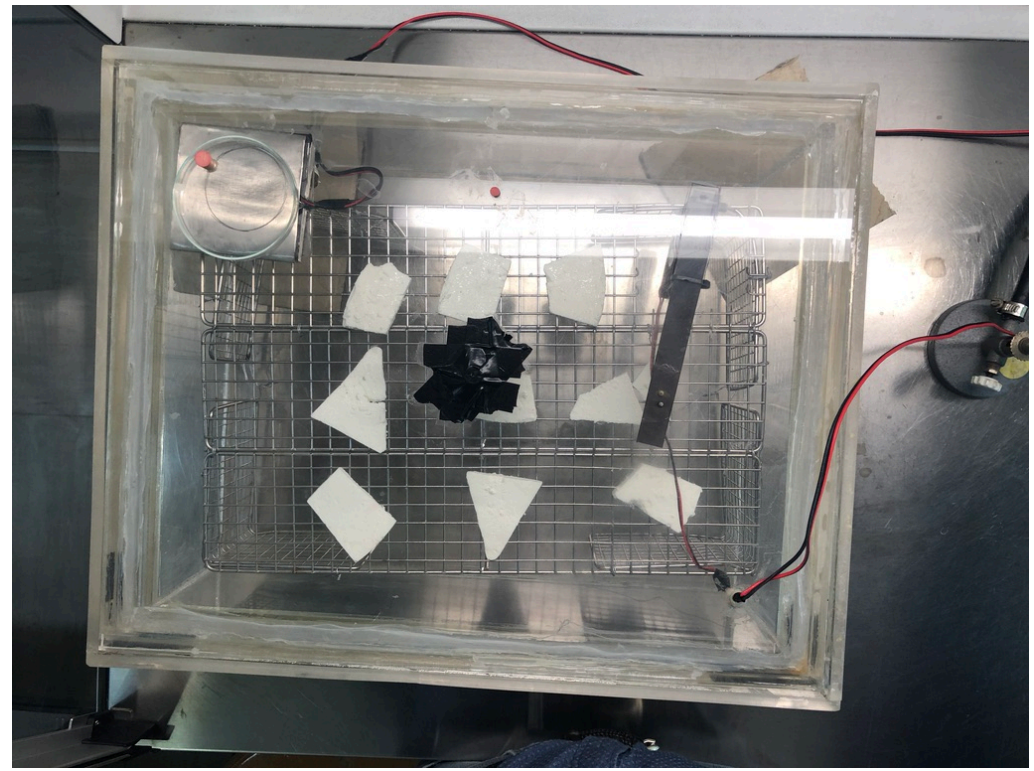
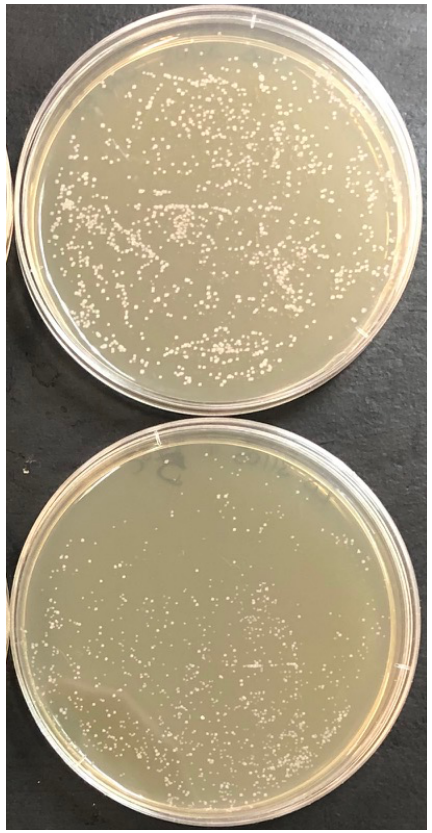
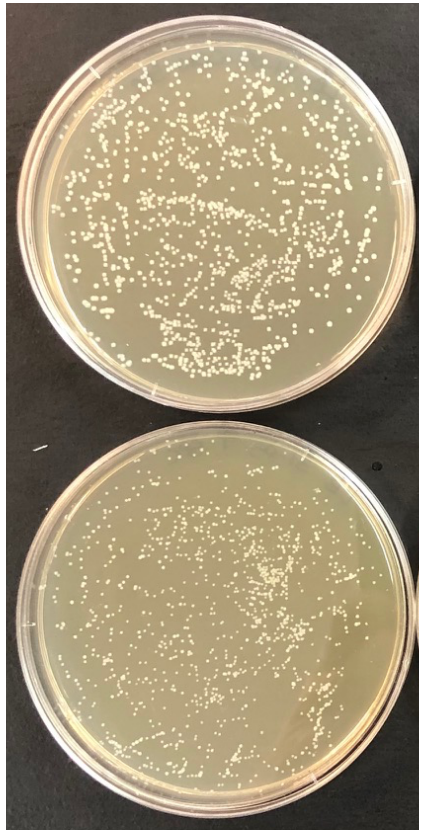


(B)

L. monocytogenes 20600 DSMZ

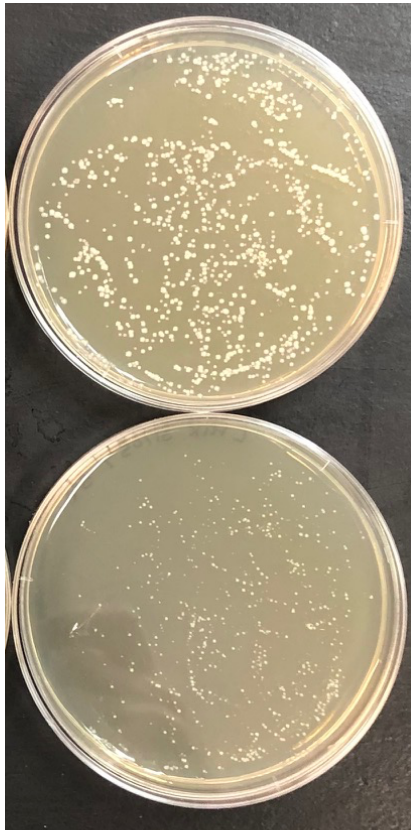


L. monocytogenes B STAA



L. monocytogenes E STAA

L. monocytogenes MIX



CONTROL

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Figure 1: Chromatograms of the residue in the heatproof glass vessel after fumigation experiment (**A**) and of raw EO from Pompia leaves (**B**).

Figure 2. Antimicrobial activity of a 3-hour exposure to a high (HD) or low dose (LD) of pompia leaf essential oil vapor (1mL or 0.5 mL, respectively), performed at room temperature, in samples of *ricotta salata* cheese experimentally contaminated with ~10^5 CFU/g of: **(A)** *L. monocytogenes* DSMZ 20600; or **(B)** *L. monocytogenes* mixed strains (strains DSMZ 20600, strain B STAA and strain E STAA), following 15 and 30 d incubation at 5 °C. Error bars represent standard deviations of the means. Ricotta salata cheese experimentally contaminated with ~10^5 CFU/g of *L. monocytogenes* DSMZ, but not treated provided the control samples.

▲, PC (Positive Control); ●, LD (Low concentration); ■ , HD (High concentration);

Figure 3. Antimicrobial activity of a 3-hour exposure to a high dose (HD) or low dose (LD) of citral vapor (1 mL or 0.5 mL, respectively), performed at room temperature, in samples of *ricotta salata* cheese experimentally contaminated with ~10^5 CFU/g of: **(A)** *L. monocytogenes* DSMZ 20600; or **(B)** *L. monocytogenes* mixed strains (strains DSMZ 20600, Strain B STAA and strain E STAA), after 15 and 30 d of incubation at 5°C. Error bars represent standard deviations of the means. *Ricotta salata* cheese experimentally contaminated with ~10^5 CFU/g of *L. monocytogenes* DSMZ, but not treated provided the control samples.

▲, PC (Positive Control); ● , LD (Low concentration); ■, HD (High concentration);

Figure 4. SEM micrographs of untreated and treated *L. monocytogenes* cells: **(A)** untreated cells (20.00 K); **(B)** PLEO gas treated sample showing deformed cells (20.00 K). In panel **A**, fimbriae-like structures are evident.

Figure 5. TEM micrographs of untreated *L. monocytogenes* cells **(A, B)** and PLEO gas treated cells **(C, D)**. In panel **D**, the PLEO-induced effects on the cell wall and plasma membrane are evident.

Figure S1. 20-litres polyethylene box used for the *in vivo* tests on *ricotta salata* cheese. Inoculated sliced ricotta cheeses are placed on the top of a sterile stainless-steel grid. The heatproof glass vessel containing the PLEO (in the left corner of the box) is collocated on top of the heating system in order to favor evaporation. Pictures of the box are taken from aside (A) and from the top (B). For details about the box, see Ladu et al. (2015).

Figure S2. *In vitro* antimicrobial activity of a 3-hour exposure to a 0.5 mL of gaseous PLEO in plates inoculated with 10^3 cell/mL and incubated at 5 °C for 10 d. In the figure, control plates are showed on the top and treated plates on the bottom. Strains of *L. monocytogenes* were inoculated singularly (20600 DSMZ, B STAA, and E STAA) and mixed as indicated.

Table 1: Main compounds detected in the essential oil extracted by leaves of *Citrus limon* var. *pompia*. Results are expressed as mg/mL of essential oil. tr: trace. RI: experimental retention indexes calculated on a 60 m VF-WAX capillary column.

Compounds	mg/mL	RI
pinene alpha	tr	931
sabinene	tr	971
beta-pinene	tr	975
hepten-2-one (6-methyl)	tr	986
myrcene	10,47	991
alpha-phellandrene	7,07	1004
gamma-terpinene	7,08	1009
limonene	256,87	1032
ocimene z beta	tr	1038
ocimene e beta	52,77	1049
terpinolene	14,47	1088
linalool	84,13	1103
citronellal	13,85	1153
terpinen-4-ol	tr	1178
chrysanthenol cis	tr	1183
alpha-terpineol	41,18	1193
nerol	5,47	1232
neral	**86,81**	1245
linalyl acetate/geraniol	298,65	1260
geranial	**98,39**	1276
neryl acetate	13,56	1365
geranyl acetate	tr	1385
caryophyllene	tr	1423
bergamotene (alpha cis)	tr	1438
humulene alpha	tr	1458
bicyclogermacene	tr	1500
bisabolene	tr	1511

Table 2: Main compounds detected in the headspace of the box by solid phase micro-extraction coupled with GC-MS after 80 min and 180 min from the beginning of evaporation experiments. Results are expressed as relative percent area obtained by internal normalization of GC-MS chromatogram.

	80min	SD	180min	SD	RI
pinene alpha	0.1	0.03	0.1	0.01	931
sabinene	0.2	0.07	0.2	0.03	971
pinene beta	0.5	0.19	0.4	0.07	975
hepten-2-one (6-methyl)	0.3	0.25	0.3	0.14	986
myrcene	5.2	1.79	5.5	0.31	991
phellandrene alpha	0.3	0.13	0.3	0.02	1004
gamma terpinene	0.6	0.19	0.5	0.08	1009
limonene	18.0	6.39	15.4	3.43	1032
ocimene Z beta	1.8	0.43	1.8	0.08	1038
ocimene E beta	7.9	0.80	7.1	1.29	1049
terpinolene	0.6	0.19	0.6	0.01	1088
linalool	7.5	0.68	7.6	0.50	1103
citronellal	0.8	0.03	0.8	0.05	1153
terpinen-4-ol	0.7	0.05	0.7	0.10	1180
chrysanthenol cis	1.3	0.18	1.3	0.11	1183
terpineol alpha	1.9	0.16	2.1	0.11	1193
nerol	0.4	0.18	0.8	0.12	1232
neral	10.7	1.40	10.1	0.61	1245
linalyl acetate	18.4	2.14	17.1	2.30	1260
geranial	14.2	2.97	15.3	1.04	1276
neryl acetate	2.0	0.79	2.5	0.21	1365
geranyl acetate	4.0	1.93	5.4	0.42	1385
caryophyllene e	1.3	0.37	1.5	0.27	1423
bergamotene (alpha cis)	0.3	0.08	0.4	0.10	1438
humulene alpha	0.2	0.11	0.5	0.14	1458
bicyclogermacene	0.3	0.10	0.6	0.16	1500
bisabolene	0.3	0.11	0.6	0.12	1511

Università degli Studi di Sassari
DIPARTIMENTO DI AGRARIA
Sezione Scienze e Tecnologie Ambientali e Alimentari

Viale Italia 39, 07100 Sassari
Tel. +39. 079 229386/17 Fax. +39. 079 229370
Partita IVA 00196350904

Sassari, 13 June 2019

Editorial Office

Food Microbiology

Dear Editor,

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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