

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

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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. monocytogenes* 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

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3 **Antimicrobial activity of gaseous *Citrus limon* var *Pompia* leaf essential oil against**
4 ***Listeria monocytogenes* on ricotta salata cheese**
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34 **Abstract**
35

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

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180 films or nano-carriers able to interact directly with both the atmosphere and the food; iii) vapor
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182 treatment of food with gaseous EOs.
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184 Gaseous EOs (EO gases) are not added directly onto foods, but can be used as primary antimicrobial
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186 agents in food packaging materials and also as sanitizing agents for food surfaces and food-contact
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188 surfaces. The advantage of using EO gases is that they often cause minimal alterations to food aroma
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190 and flavor because there is minimal penetration into the subsurface area (Goñi et al., 2009; Tyagi et
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192 al., 2012). Some EO gases have been found to exert greater antimicrobial activity against foodborne
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194 pathogens and spoilage bacteria when compared with EO in liquid phase (Tyagi and Malik, 2010).
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196 Devices have also been proposed for studying the antimicrobial activity of EO vapor or other volatile
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198 compounds applied directly onto the food matrix, such as compact chambers made from acrylic
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200 materials able to contain the food and produce EO vapor or gas (Venditti et al. 2009; Tyagi and
201
202 Malik 2012; Petretto et al. 2013, Ladu et al. 2015, Lee et al. 2018).
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204

205 Our research group recently studied the chemical composition and the *in vitro* antimicrobial activity
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207 of the EO extracted from the leaves of a particular variety of Sardinian lemon, namely *Citrus limon*
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209 *var. pompia* Camarda (Fancello et al 2016). Our *in vitro* experiments against several strains of *Listeria*
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211 *monocytogenes* showed its EO to exert antilisterial activity.
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214 *L. monocytogenes* can be present in many environmental niches (Vivant et al., 2013) given its
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216 widespread distribution and ability to grow in a broad range of conditions (Ferreira et al., 2014),
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218 including the harsh conditions that may be encountered in food. *L. monocytogenes* has been isolated
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220 from a variety of raw and processed food matrices, including milk and dairy products, meat and egg
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222 products, seafood, vegetables, and other ready-to-eat foods (Ferreira et al., 2014). Dairy products
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224 have been associated with approximately half of all reported outbreaks of listeriosis in Europe and
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226 the United States (CDC, 2016; Filipello et al., 2017).
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228

229 Considering its physico-chemical properties, *ricotta salata* cheese is prone to contamination of
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231 pathogenic and spoilage microorganisms (Casti et al., 2016). According to Spanu et al. (2012), *ricotta*
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233 *salata* cheese can become easily contaminated by *L. monocytogenes* due to its frequent manipulation
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239 during the production process, posing serious challenges to *L. monocytogenes* growth control. A
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241 multi-state outbreak of listeriosis in the United States linked to *ricotta salata* imported from Italy was
242
243 recently recorded. *Ricotta salata* cheese is usually stored at refrigeration temperature and its shelf-
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245 life may vary from three weeks up to several months (Spanu et al., 2015). It is often used in salads as
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247 well as many other dishes and may be sliced, crumbled or grated.

248
249 In the present work, a mild hurdle strategy was used to test the growth of *L. monocytogenes* inoculated
250
251 onto *ricotta salata* cheese using *Pompia* leaf essential oil and citral gases at refrigeration temperature.
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254 255 256 **2. Material and Methods**

257 258 *2.1. Plant material and extraction of EO*

259
260 Three samples of *Pompia* leaf were collected in May-June from the Sardinian town of Siniscola, Italy.
261
262 A sample of leaves weighing 500 g was suspended over 1.000 mL water and subjected to steam
263
264 distillation for 2 h using a Clevenger type apparatus (European Pharmacopoeia 2002). The yield of
265
266 EOs ranged between 0.43 % (v/w) and 0.52 % (v/w), calculated from the dry weight. Extraction was
267
268 carried out in triplicate, and the obtained EOs were collected separately, dried over anhydrous sodium
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270 sulfate (Na_2SO_4), and then stored under a nitrogen atmosphere at 4 °C in amber glass vials until
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272 analyzed.
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275 276 277 *2.2. Gas Chromatography-Mass Spectrometry (GC-MS) analysis*

278
279 The GC-MS analysis was carried out using an Agilent 7890 GC equipped with a Gerstel MPS
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281 autosampler, coupled with an Agilent 7000C MSD detector. The chromatographic separation was
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283 performed on a HP-5 capillary column (30 m × 0.25 mm, film thickness 0.17 µm) (Agilent). The
284
285 following temperature program was used: 60 °C hold for 3 min, then increased to 210 °C at a rate of
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287 4 °C/min, then held at 210 °C for 15 min, then increased to 300 °C at a rate of 10 °C/min, and finally
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289 held at 300 °C for 15 min. Helium was used as the carrier gas at a constant flow of 1 mL/min. The
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291 data were analyzed using a MassHunter Workstation B.06.00 SP1, with identification of the
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298 individual components (Table 1) performed by comparison with the co-injected pure compounds and
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300 by matching the MS fragmentation patterns and retention indices to the built-in libraries, literature
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302 data, or commercial mass spectral libraries (NIST/EPA/NIH 2008; HP1607 purchased from Agilent
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304 Technologies).

305 306 307 308 309 *2.3. Gas Chromatography-Flame Ionization Detector (GC-FID) analysis*

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311 The GC analysis of the EOs was carried out using an Agilent 6890N instrument equipped with an
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313 FID and an HP-5 capillary column (30 m × 0.25 mm, film thickness 0.17 μm). The column
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315 temperature was held at 60 °C for 3 min, then increased to 210°C at a rate of 4 °C/min and held at
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317 210°C for 15 min, then increased to 300 °C at a rate of 10°C/min, and finally held at 300 °C for 15
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319 min. The injector and detector temperatures were both 250 °C. Helium was used as carrier gas at a
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321 flow rate of 1 mL/min. The quantification of EO compounds was carried out using the internal
322
323 standard method and involved injecting a solution of EOs diluted in hexane (dilution ratio 1:200). A
324
325 calibration curve was constructed for each standard compound. When standards were unavailable,
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327 quantification was performed using the calibration curve for a compound of the same class of volatile
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329 (monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenates
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331 sesquiterpenes) present in the EO. The results are expressed as mg per mL of distilled EO (Petretto
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333 et al. 2016).
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339 *2.4. SPME conditions*

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341 The isolation of box headspace **volatile compounds** was carried out using a manual 100 μm
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343 PDMS/DVB/CAR (Polydimethylsiloxane/Divinylbenzene/Carboxen) coated fiber (Supelco, Sigma
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345 Aldrich, St. Louis, Mo., U.S.A.), which was conditioned prior to use according to manufacturer
346
347 instructions. After 80 and 180 min of equilibration time, the conditioned fiber was injected through
348
349 the septum and suspended in the headspace of the box device. The fiber was exposed for 2 min in
350
351 order to extract the volatiles. The fiber was then retracted, removed from the box, and placed
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357 immediately into the injector of the GC. Thermal desorption was performed inside the injector at a
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359 temperature of 250 °C for 5 min in splitless injection mode. Prior to and after each analysis, the fiber
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361 underwent a further bake-out step for 5 min at 250 °C.
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367 2.5. Bacterial strains and culture conditions.

368
369 *L. monocytogenes* 20600 DSMZ (serovar 1/2a), obtained from DSMZ (*Deutsche Sammlung von*
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371 *Mikroorganismen und Zellkulturen*, i.e., German Collection of Microorganisms and Cell Cultures),
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373 and two wild strains, *L. monocytogenes* B STAA and *L. monocytogenes* E STAA, previously isolated
374
375 from *ricotta salata* cheese were used. The latter two strains belong to serogroup 3 (1/2b, 3b e 7) (data
376
377 not shown). All strains were stored at -80 °C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke,
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379 UK) with glycerol (20 % v/v) and subcultured twice in BHI broth at 37 °C for 18 h to reach the early
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381 stationary phase (~9 log CFU/mL).
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387 2.6. *In vitro* antimicrobial assay

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389 The *in vitro* antilisterial activity of gaseous PLEO and citral was evaluated using the disc
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391 volatilization method (Nedorostova et al., 2009) at five different concentrations (ranging from 0.001
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393 to 0.168 µL/cm³ for PLEO, and from 0.005 to 0.076 µL/cm³ for citral). First, Petri dishes with exactly
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395 20 mL of BHI agar were inoculated with 100 µL suspensions of 10⁶ CFU/mL *L. monocytogenes*.
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397 Sterile paper disks (Whatman No. 1, diameter 55 mm) were then dosed with PLEO (10.4, 5.2, 2.6,
398
399 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.
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401 In accordance with Nedorostova et al., (2009), the two lowest doses of PLEO (1.3, 0.65 µL) were
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403 diluted in diethyl ether 1:10 to facilitate the oil's solubilization. Then, plates were sealed with
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405 parafilm[®] to prevent gas leakages and incubated at 37 °C for 24 h. After incubation, the minimum
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407 inhibitory concentrations (MICs) were recorded. The MICs were expressed as microliters of PLEO
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409 or citral per volume of atmosphere above the inoculated agar surface and defined as the lowest
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414 concentration which clearly produced a visible inhibition zone. Each test was performed in triplicate
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418 and the experiments were repeated twice.
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422 *2.7. Evaluation of the effect of gaseous PLEO treatment and refrigeration temperature*

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424 Twelve BHI agar Petri dishes for each strain were individually inoculated singularly with of one the
425 three strains of *L. monocytogenes* or with the mix of these strains, with a final concentration of $\sim 10^3$
426 cells of *L.monocytogenes* per plate. For the treatment, 6 inoculated Petri dishes were placed inside a
427 20-liter sealed polyethylene box, previously sterilized by an overnight UV treatment, equipped with
428 a circulation fan, to facilitate the circulation of the oil gas inside the box, and a heating system, to
429 obtain the gradual evaporation of PLEO, both of which were connected to a power supply - see
430 paragraph 2.9 - (Venditti et al., 2009; Ladu et al., 2015). The inoculated plates were incubated at 25
431 °C for 3 h in the presence of 0.5 ml of PLEO. The other 6 plates (used as positive control) were
432 incubated at same conditions without the gas treatment. After three hours, treated and positive control
433 plates were incubated at 37 °C and at 5 °C. The growth of *L.monocytogenes* strains were monitored
434 for 2 and 10 days for plates incubated at 37 °C and 5 °C respectively.
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450 *2.8. L. monocytogenes determination on ricotta salata cheese.*

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452 *Ricotta salata* cheeses were purchased in 300-gram portions from a local retail grocer (Sassari, Italy),
453 transported under controlled temperature to the laboratory and analyzed. The presence of *L.*
454 *monocytogenes* strains was ascertained according to standard methods (EN ISO 11290-1:2017).
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461 *2.9. Inoculation of ricotta salata cheese with L. monocytogenes and EO vapor treatments.*

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463 For the artificial inoculation of the *ricotta salata* cheese, two different culture suspensions were
464 prepared using: 1) an 18-hour culture of *L. monocytogenes* 20600 DSMZ; and 2) an 18-hour culture
465 including the three strains used in this work (see above). Each inoculum was serially diluted in 0.1 %
466 Buffered Peptone Water (BPW) to obtain a concentration of approximately 6 log CFU/mL for the
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475 subsequent experiments. *Ricotta salata* cheeses were aseptically cut into 10 g slices, transferred into
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477 sterile Petri dishes, and inoculated with 100 µL of the previously prepared inocula. *L. monocytogenes*-
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479 inoculated *ricotta salata* cheese slices were then placed inside a 20-litres sealed polyethylene box
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481 equipped with a circulation fan and a heating system, both of which were connected to a power supply
482
483 (Figure S1) (Venditti et al. 2009; Ladu et al., 2015). Samples were incubated at room temperature
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485 (20±2 °C) for 3 h in the presence of PLEO or citral gases, used separately, at two different
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487 concentrations (0.5 mL and 1.0 mL). In order to obtain their gradual evaporation, EOs were pipetted
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489 into a heatproof glass vessel, already placed on a hot plate, while the circulation of the oils inside the
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491 box was obtained by the attached fan. After the gaseous treatments, the exact quantity of liquid PLEO
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493 and citral still present in the glass vessel containing the EOs was measured in order to estimate the
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495 concentration of EO evaporated. Specifically, the residual oil was weighed, the weight subtracted
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497 from the initial weight, and the result divided by the volume (cm³) of the polyethylene box. Post-
498
499 treatment, the *ricotta salata* slices were picked up using sterile disposable tweezers and placed in
500
501 sterile Petri dishes that were then sealed with parafilm[®] and incubated for 30 d at 5 °C. *Ricotta salata*
502
503 slices inoculated but not treated and incubated at 5 °C for 30 d were used as positive control (PC).
504
505 Three slices were analyzed for each sampling at each time point: at the beginning of the experiment
506
507 (T0), after 15 d (T15), and after 30 d (T30). All experiments were repeated twice. The enumerations
508
509 of *L. monocytogenes* were conducted according to standard methods EN ISO 11290-2:2017.
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516 2.10. Survival of natural flora in ricotta salata cheese after the gaseous EO treatments

517
518 The effects of PLEO and citral gases and control on the natural mesophilic bacteria population and
519
520 lactic acid bacteria of *ricotta salata* cheese at the end of the experiment were estimated by putting the
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522 cheeses in a sterile stomacher bag (filter 0.5 mm pore size) (VWR, Milan) and homogenized for 1
523
524 min in 90 mL BPW. Total mesophilic bacteria count (TMC) was enumerated in Plate Count Agar
525
526 (PCA, Microbiol, Cagliari, IT) and incubated at 30 °C for 48 h; whereas lactic acid bacteria (LAB)
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534 were enumerated in MRS (De Man, Rogosa, Sharpe) agar (Microbiol, Cagliari, Italy) after incubation
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536 at 30 °C for 48 h.
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539 540 541 *2.11 Scanning and Transmission electron microscopy.*

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543 For the SEM morphologic investigation, samples of *L. monocytogenes* cells treated with 0.5 mL
544
545 gaseous PLEO by the volatilization method and untreated cells were fixed for 3 h with 2.5 %
546
547 glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed in 1.0 % osmium tetroxide
548
549 in 0.1 M sodium cacodylate buffer for 1 h. Samples were then dehydrated in an acetone series
550
551 (ascending acetone concentrations) and critical point dried using a Polaron Jumbo critical point drier
552
553 with carbon dioxide (CO₂) as medium. Dried samples were mounted on aluminum stubs and sputter
554
555 coated with gold/palladium in an Edwards S150A Sputter Coater unit and examined using a Zeiss
556
557 DSM 962 scanning electron microscopy.
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559
560 For TEM investigations, samples of *L. monocytogenes* cells treated with gaseous 0.5 mL PLEO by
561
562 the volatilization method and untreated cells were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate
563
564 buffer at pH 7.2 for 3 h and post-fixed in osmium tetroxide (OsO₄) 1 % for 1 h. Specimens were then
565
566 dehydrated in an acetone series, embedded in Agar 100 epoxy resin, and ultra-thin sections were cut
567
568 with a diamond knife on an RMC MT-7 ultramicrotome and stained with uranyl acetate and lead
569
570 citrate. Samples were examined in a Zeiss EM 109 transmission electron microscope operating at 50
571
572 kV.
573

574 575 576 577 *2.12 Statistical analyses*

578
579 All microbial counts were log transformed to obtain a normal distribution. Data were analyzed by
580
581 analysis of variance (ANOVA). When a significant effect was observed ($P < 0.05$), the differences
582
583 between means were separated using the Tukey–Kramer multiple comparisons test. The mean TMC
584
585 and LAB counts for control and treated samples were compared using Student's t-tests. SPSS
586
587 software, version 19.0, was used to conduct the statistical analyses.
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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

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652 of the compounds released into the headspace was carried out by HS-SPME/GC-MS. The chemical
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654 analyses were carried out at two different times and the results are reported as relative percentages
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656 obtained by peak area normalization (Table 2). In agreement with our previous study (Petretto et al.
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658 2013), the concentration of the more volatile components is greater in the vapor phase with respect
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660 to the pure oil; they include the components that elute in the first part of the chromatogram (i.e., those
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662 with smaller retention times), such as limonene, myrcene, and other monoterpene compounds. By
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664 contrast, the less volatile compounds, such as neral, geranial, linalyl acetate, and the sesquiterpene
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666 fraction were present at lower concentrations in the vapor phase with respect to the pure oil. These
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668 results were also confirmed by the qualitative analysis of the residue (Figure 1) obtained by rinsing
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670 the Petri dishes with hexane once HS-SPME/GC-MS was complete. In addition to some
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672 decomposition derivatives, the residue was characterized by higher amounts of neral, geranial, and
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674 linalyl acetate, as well as lower amounts of limonene, sabinene, and the other more volatile
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676 components of PLEO.
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682 3.3. MIC of PLEO and citral gases

683
684 The MICs of PLEO and citral gases, determined using the disc volatilization method, showed a strain
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686 dependent behavior. While the MIC of PLEO gas ($0.086 \mu\text{L}/\text{cm}^3$) was the same for all three *L.*
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688 *monocytogenes* strains tested, differences were observed in relation to citral gas sensitivity: the MIC
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690 of citral gas on *L. monocytogenes* 20600 DSMZ was $0.014 \mu\text{L}/\text{cm}^3$, whereas it was $0.028 \mu\text{L}/\text{cm}^3$ for
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692 the other two *L. monocytogenes* strains.
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697 3.4. Determination of synergic antimicrobial effect between gaseous PLEO treatment and 698 699 refrigeration temperature.

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701 The treatment at different temperatures showed opposite results. At 37°C no differences were
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703 observed in the growth of *L. monocytogenes* treated with gaseous PLEO respect to the control plate,
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705 except for a slight growth delay on the first day of incubation. On the contrary, when treatment with
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711 gaseous PLEO was followed by incubation at 5 °C, a marked growth delay of *Listeria* was observed,
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713 even after 10 days of incubation. (Figure S2). In particular the growth of the strain *L. monocytogenes*
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715 E STAA was completely inhibited by the treatment and the following incubation at 5 °C, while *L.*
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717 *monocytogenes* B STAA was the less sensitive to the treatment. These results showed that
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719 refrigeration temperature and gaseous PLEO have a synergic effect.
720

721 722 723 724 3.5. Determination of the evaporation of PLEO and citral gases inside of the polyethylene box 725

726 The concentration of PLEO that evaporated inside the box in 3 h at room temperature, from the initial
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728 1 mL and 0.5 mL doses was estimated by subtracting the weight of oil still present in the vessel post
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730 treatment from its initial weight, corresponding to 0.043 $\mu\text{L}/\text{cm}^3$ and 0.021 $\mu\text{L}/\text{cm}^3$ respectively.
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732 These values were thus considered as the concentrations of gaseous PLEO inside the container and
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734 they corresponded to half and a quarter of the MIC, respectively (previously determined by
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736 comparison with the disc volatilization method). Less evaporation occurred from 1 mL and 0.5 mL
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738 of liquid citral, giving final gaseous concentrations of 0.028 $\mu\text{L}/\text{cm}^3$ and 0.014 $\mu\text{L}/\text{cm}^3$ respectively,
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740 also corresponding to half and a quarter of the MIC.
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745 3.6. Antimicrobial activity of gaseous PLEO against *L. monocytogenes* strains 746

747 The effects of two concentrations of gaseous PLEO on *L. monocytogenes* 20600 DSMZ strain
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749 inoculated onto *ricotta salata* cheese were evaluated as described in the paragraph 2.9 of the materials
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751 and methods (Figure 2A). Specifically, the activities of a 0.5 mL (low dose, LD) or 1 mL (high dose,
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753 HD) dose of PLEO were assessed. The positive control for bacterial growth (from here on, PC) was
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755 represented by *L. monocytogenes* 20600 DSMZ inoculated onto ricotta cheese in the absence of
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757 PLEO. Under these PC conditions, *L. monocytogenes* 20600 DSMZ reached a cell density of about
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759 7 \log_{10} CFU/g. The antilisterial effect of PLEO was both time and concentration dependent. Thus, the
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761 *L. monocytogenes* 20600 DSMZ viable count was significantly ($p < 0.001$) reduced in the presence of
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763 a HD of PLEO gases (Figure 2A). The effect was noticeable after 15 days of incubation, with
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770 significant reductions ($p < 0.001$) of about 3 and 4 log units with respect to PC at T0 and at T15,
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772 respectively. At T30, the viable count was 1.69 log₁₀ CFU/g, about 5.5 log₁₀ CFU/g lower with
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774 respect to that of *L. monocytogenes* 20600 DMSZ measured in the PC sample at T30. *L.*
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776 *monocytogenes* 20600 DMSZ showed less susceptibility at the LD of PLEO gas, reaching a
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778 significantly lower value ($p < 0.001$) of 6.08 log₁₀ CFU/g at T30, a log unit lower than the PC count
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780 (7.29 log₁₀ CFU/g) at the same sampling time.

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782 The effect of gaseous PLEO treatment on the growth of the mix of the three strains of *L.*
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784 *monocytogenes* on *ricotta salata* cheese was less pronounced with respect to that observed on *L.*
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786 *monocytogenes* 20600 DSMZ alone (Figure 2B). Even in this experimental trial, the concentration of
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788 gaseous PLEO was the main factor that influenced the bacterial growth. In particular, the HD of
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790 PLEO gas caused a significant decrease in the mixed strain count ($p < 0.001$) after 15 d of incubation
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792 (4.08 log₁₀ CFU/g) compared with the T0 count (5.14 log₁₀ CFU/g). After a further 15 d incubation
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794 (i.e., T30), we observed a significant rise ($p < 0.001$) in the colony count, which reached 5.41 log₁₀
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796 CFU/g, but this count was still significantly lower ($p < 0.001$) than the equivalent PC count (6.07 log₁₀
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798 CFU/g). At the LD, gaseous PLEO had a bacteriostatic effect on the growth of the *L. monocytogenes*
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800 mix of strains in *ricotta salata* cheese. After 15 d of incubation we observed a very slight decrease in
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802 the *L. monocytogenes* mix CFU/g. The CFU/g tended to increase significantly ($p < 0.001$) after 30 days
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804 of incubation, but was still significantly different ($p < 0.001$) to that in the PC.
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811 3.7. Antimicrobial activity of gaseous citral against *L. monocytogenes* strains

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813 The effect of gaseous citral on *L. monocytogenes* 20600 DMSZ in *ricotta salata* cheese was lower
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815 with respect to that exerted by PLEO gases, as showed in Figure 3A. At the HD, the effect on *L.*
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817 *monocytogenes* 20600 DMSZ growth was significant ($p < 0.001$), with a reduction of about 2 log₁₀
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819 following 15 d of incubation (3.42 log₁₀ CFU/g) with respect to T0 (5.29 log₁₀ CFU/g), and dropping
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821 to 2.56 log₁₀ CFU/g at T30. On the contrary, at the LD of citral gas, the viable counts were very
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823 similar to those observed following treatment with the LD of PLEO gas.
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829 The antimicrobial efficacies of HD and LD citral gases against the mix of the three *L. monocytogenes*
830 are presented in Figure 3B. The HD of citral gases significantly reduced ($p < 0.001$) the CFU/g of the
831 *L. monocytogenes* mix at T15, which remained constant until the end of experiment (T30) and was
832 significantly different ($p < 0.001$) from the PC count (4.58 log₁₀ CFU/g vs. 6.31 log₁₀ CFU/g,
833 respectively). Similar results were observed for the LD of citral. Finally, it seems that the mix of three
834 *L. monocytogenes* strains proliferated more slowly on *ricotta salata* cheese (PC) with respect to *L.*
835 *monocytogenes* DSMZ alone, obtaining a CFU concentration just above 6 log₁₀ CFU/g at T30.
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846 3.8. Effect of gaseous PLEO and citral treatments on mesophilic and lactic acid bacteria

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848 The treatment with gaseous PLEO and citral did not affect the TMC (total mesophilic bacteria) (8.02
849 log₁₀ CFU/g Vs 8.03 log₁₀ CFU/g, control and treated respectively) or LAB counts (7.17 log₁₀ CFU/g
850 Vs 7.47 log₁₀ CFU/g) with no significant differences detected at T30 in their viable counts.
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857 3.9. SEM and TEM analyses of PLEO gas-treated *L. monocytogenes* cells

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859 The treatment of *L. monocytogenes* with gaseous PLEO *in vitro* showed a marked effect on cell
860 morphology. SEM analysis showed that whereas untreated cells present intact, turgid, smooth
861 surfaces (Figure 4A) and fibrillar structures (Zaamer et al., 2013), PLEO gas-treated cells (Figure 4B)
862 showed no fibrillar structures. Moreover, PLEO gas-treated cells showed modifications in cell wall
863 morphology and the presence of small bulges of coagulated structures – possibly proteins coming
864 from the cell membrane and cytoplasm, as suggested by Koyama et al. (1997). To explore the ultra-
865 structural modifications occurring in vapor-treated cells further, transmission electron microscopy
866 was used. While *L. monocytogenes* cells showed a regular and delineated cell wall and plasma
867 membrane with some dense structures well distributed through the cytoplasm (Figure 5A-B), most of
868 the treated cells showed a thinner cell wall and white dense areas within the cytoplasm. Other cells
869 showed internal damage and several deformities; in particular, an altered periplasmic space and dense
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888 zones asymmetrically distributed throughout the cytoplasm (Figure 5C). Finally, some cells showed
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890 evident cell wall and plasma membrane ruptures (Figure 5D).
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893 894 **4. Discussion** 895

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897 Essential oils are considered to be powerful antimicrobial agents, with a plethora of potential
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899 applications in the food and food packaging industries, and as sanitizing agents for food surfaces.
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901 Moreover, there is growing evidence that EOs in the vapor phase are more effective antimicrobials
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903 than in the liquid phase (Tyagi and Malik, 2011; Lopez et al., 2005; Inouye et al., 2003). In the present
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905 work, we show that the MIC of PLEO gas was the same for all three tested strains of *L.*
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907 *monocytogenes*, while the MIC of citral gases varied among the strains, with the MIC for *L.*
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909 *monocytogenes* 20600 DSMZ being lower than that for the other two strains tested. These results are
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911 in accordance with those from other authors (Desai et al., 2012; Fancello et al., 2017; Yuan et al.,
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913 2017 Petretto et al., 2018), who have shown that the antimicrobial activity of EOs are species- and
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915 strain- dependent.
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918 Moreover, we found that gaseous PLEO and citral were much more effective than their liquid phases
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920 (as reported by Fancello et al., 2016). We can postulate that gaseous PLEO and citral have different
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922 mechanisms of action in the vapor phase vs. liquid phase. Indeed, it has been previously demonstrated
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924 that essential oils used as vapors have better antimicrobial activities than the essential oils used in the
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926 liquid phase, probably as a result of better penetration and increased contact of the essential oil in the
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928 vapor phase (Tiagy et al., 2010 and 2011). Our results, in accordance with Inouye et al. (2001), also
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930 show that the vapors of aldehyde components, such as citral and cinnamaldehyde, were more active
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932 compared with the terpene ketone and terpene ether components, demonstrating that, in general, the
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934 antimicrobial activity of essential oils may depend to the volatility of the active compounds that
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936 makes the essential oils better antimicrobial agents in the vapor phase (Tyagi and Malik, 2012).
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938 Recently, Feyaerts et al. (2018) showed that the presence of vapor-phase-mediated antimicrobial
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940 activity could be predicted from the chemical class that predominates in the EOs, with aldehyde-,
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947 phenol-, monoterpenol-, ether- and ketone-rich EOs showing the highest inhibitory activity. The
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949 greater antimicrobial activity of the vapor phase could also be explained by the fact that water reduces
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951 volatility, considering that components with hydroxyl groups may be more solvated and remain in
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953 the water phase (Sato et al., 2006). In relation to this, Boukhatem et al. (2014) stated that lipophilic
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955 molecules in the aqueous phase associate to form micelles and thus restrain the attachment of EOs to
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957 microorganisms. Moreover, we found that the strong antilisterial activity was dependent on the
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959 concentration of PLEO and citral used and on the type of inoculum (the 20600 DSMZ strain alone or
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961 mixed strains). Specifically, the antimicrobial activity of PLEO and citral gases was more effective
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963 when *ricotta salata* cheese was inoculated with just a single strain than with a mix of strains, and
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965 gaseous PLEO showed greater antimicrobial activity than pure citral gas, as already observed by other
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967 authors (Tyagi et al., 2012), thus indicating a synergistic or additive role of the EO components,
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969 enhancing the antimicrobial activity of the oil. Finally, we found that, the antilisterial activities of the
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971 gaseous PLEO tended to be more efficient when *L. monocytogenes* 20600 DSMZ cells were
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973 inoculated on *ricotta salata* slices than on BHI agar medium. This result lies in contrast with those of
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975 other studies where the activities of the EOs gases tended to be lower when used on contaminated
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977 food matrices (Lee et al., 2018), although Lorenzo-Leal et al. (2019) observed that the vapor from
978
979 all-spice EO showed greater antimicrobial activity when assessed in alfalfa seeds than on laboratory
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981 media, in line with our findings.

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

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989 For this reason, the mild hurdle approach to fight the growth of *L. monocytogenes* in *ricotta salata*
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991 cheese using gaseous PLEO in combination with refrigeration was first tested *in vitro*, by comparing
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993 the effect at 5 °C with an incubation at 37 °C. The *in vitro* results confirmed the synergic effect of the
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995 gaseous oil combined with the refrigeration temperature. Once applied to the *in vivo* tests, results of
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997 the mild hurdle technology, showed that at 5 °C the antimicrobial activity of gaseous PLEO was very
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1006 strong with a significant reduction in terms of CFU counts of a single strain (or mix of strains) of *L.*
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1008 *monocytogenes* expressed as log units after 15 and/or 30 days of incubation compared with the
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1010 control.

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1012 We might therefore postulate that the use of mild hurdle technology, such as storage at 5 °C post
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1014 treatment, favors bacterial cell damage and importantly prevents the cells from repairing and
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1016 reforming their damaged cellular structures. In the case of our 3-strain mix, the lower anti-bacterial
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1018 efficacy of the PLEO and citral gases on inoculated *ricotta salata* cheese could be due to the cross-
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1020 resistance effect of the different *L. monocytogenes* strains (Giaouris et al., 2015; Kadam et al, 2013).
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1022 The efficacy of the mild hurdle technology used in this work has also been confirmed by other authors
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1024 (Guevara et al., 2015; Ngang et al., 2014). Their studies showed that the combination of moderate
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1026 heat with carvacrol and thymol or curcuma had a synergistic effect, leading to inactivation values that
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1028 were three- or four-fold faster than when heat was used alone. Recent findings have suggested that
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1030 cellular damage can already occur at sub-lethal concentrations of citral (Siroli et al., 2016); indeed,
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1032 the sub-MIC concentration used in our work was capable of causing damage to the bacterial cells,
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1034 and the post-treatment low storage temperature could hinder cellular repair. Interestingly, the
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1036 continuous exposure of *L. monocytogenes* to citral does not induce the development of antimicrobial
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1038 resistance (Apolonio et al., 2014).

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1041 The SEM images of the untreated samples revealed the presence of fibrillar structures, probably
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1043 caused by the shrinkage of the exopolymeric matrix. These fibrillar structures were not, on the other
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1045 hand, observed in PLEO-treated samples, perhaps due to the activity of the EO vapor against the
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1047 matrix structure. Moreover, small bulges of coagulated structures were visible in the PLEO-treated
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1049 cells, which were probably proteins extruded from the cell membrane and cytoplasm, (Koyama et al.,
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1051 1997). These putative proteins may have been pushed out through holes produced in the cell wall by
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1053 the oil vapors. A recent interesting work by Tyagi and Malyk (2012) showed a similar modification
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1055 in cell morphology resulting from the use of gaseous *lemon* grass oil.
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1065 The data obtained by SEM and TEM reinforce the idea that the PLEO could target the cell wall and
1066 the plasma membrane, which could explain the bulges of coagulated structures. Indeed, similar results
1067 were found by: Gustafson et al. (1998), who treated *E. coli* with tea tree oil; Rasooli et al. (2006),
1068 who treated *L. monocytogenes* with thymus; and Becerril et al. (2007), who treated *E. coli* with
1069 cinnamon and oregano and with lemon grass (Tyagi and Malyk, 2012).
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1072 The chemical analysis of the EO employed in this study highlighted some differences compared with
1073 results reported in literature. With the exception of linalyl acetate, the differences regard minor
1074 compounds. The variability in chemical composition of the EO could be related to climatic conditions
1075 (Melito et al. 2016), geographical position (Petretto et al. 2018), and the harvesting period (Bennaoum
1076 et al. 2017).
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1087 1088 **5. Conclusions**

1089 In this work, we showed that different strains of *L. monocytogenes* can be inactivated by PLEO when
1090 used as a gas in artificially contaminated *ricotta salata* cheese. Moreover, we proved that the
1091 antimicrobial activity can be enhanced by mild hurdle technology, in the form of refrigeration. This
1092 combination of methods allowed us to significantly reduce the presence of *L. monocytogenes* in the
1093 *ricotta salata* cheese when the 20600 DSMZ strain was used alone, even at half MIC, and it resulted
1094 in bacteriostatic activity when all three *Listeria* spp. strains were used together. In the latter case, a
1095 possible cross-protection effect between the strains could have caused the reduced antimicrobial
1096 activity of the EO. Electron microscopy analyses implicated the putative targets of the EO to be the
1097 cell wall and the plasma membrane. Finally, it is important to underline that treatment with the
1098 gaseous EO vapor had no significant effect on the *ricotta salata* cheese's resident lactic acid bacteria.
1099 In summary, these results highlight the importance of investigating the effects of PLEO gas on
1100 different pathogen strains, used singularly or mixed, especially because it has been shown that more
1101 than one strain can reside in a specific ecological niche. Our results also highlight the potential
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1124 application of gaseous PLEO as an effective natural alternative to synthetic substances to enhance the
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1126 microbial safety of *ricotta salata* cheese and other foodstuffs, while maintaining high cheese quality.
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1138 sectors.
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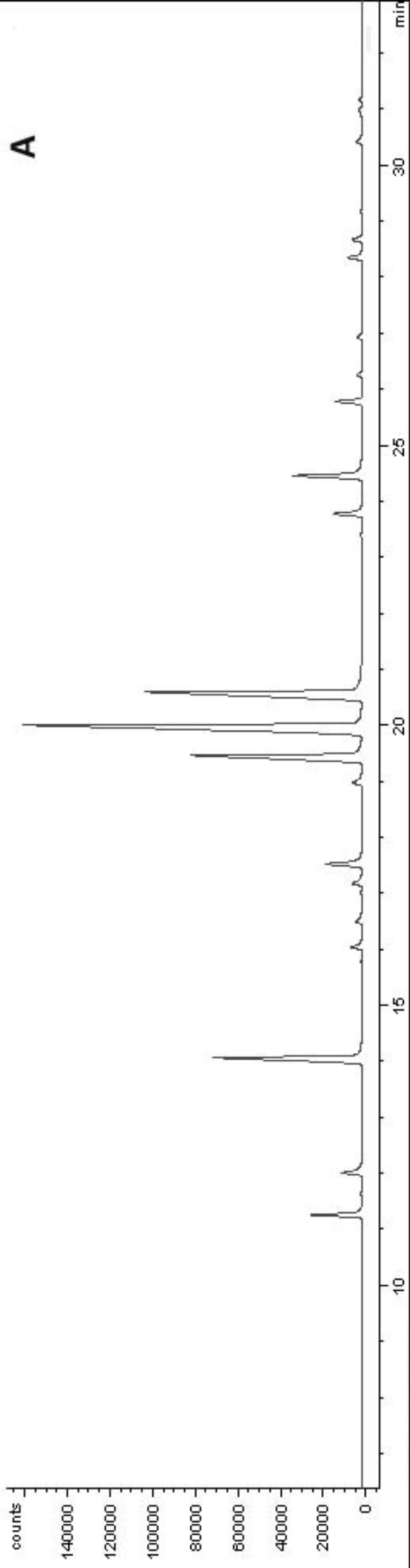
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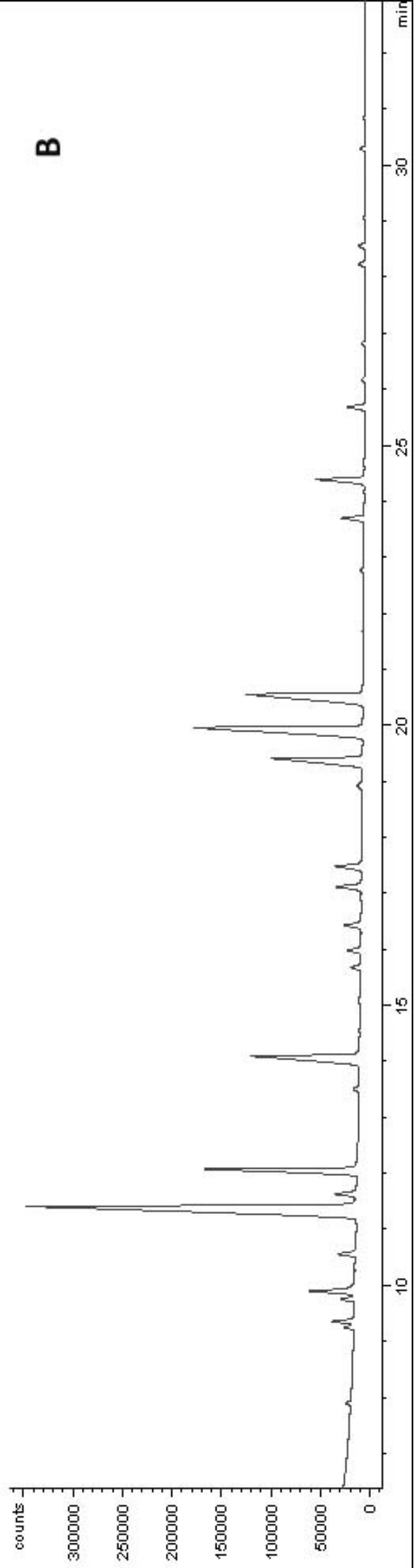
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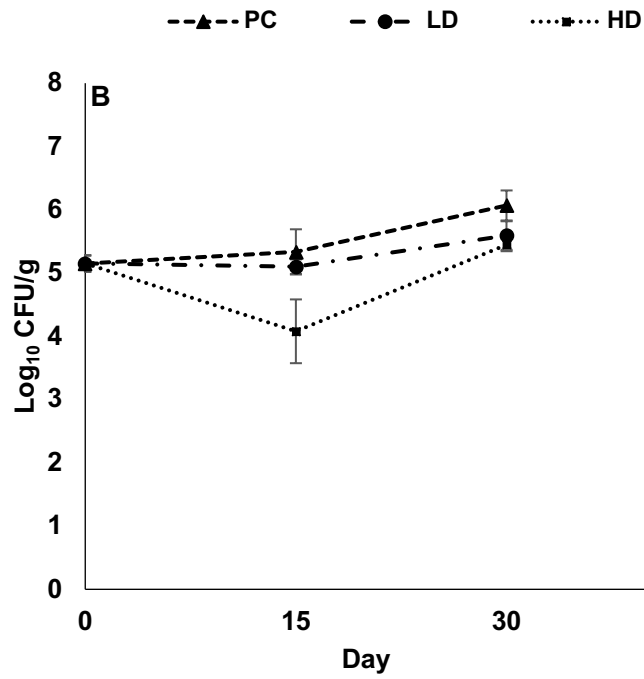
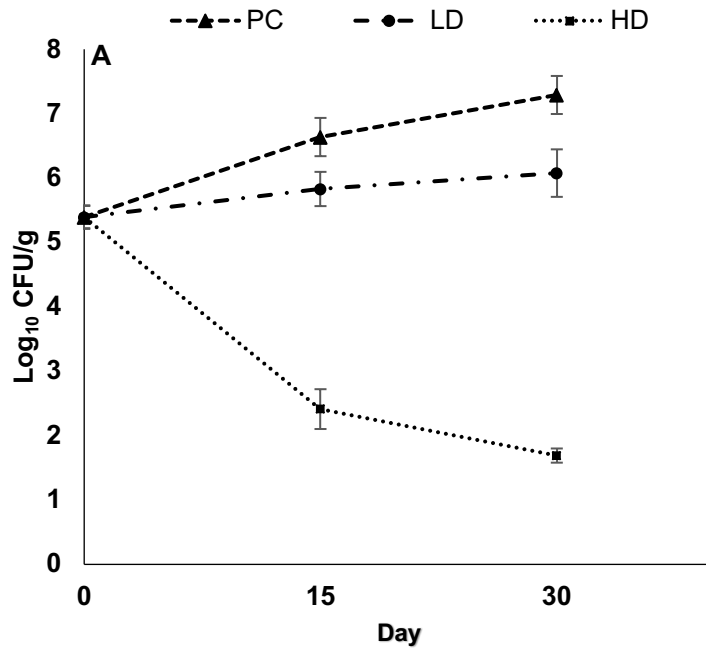
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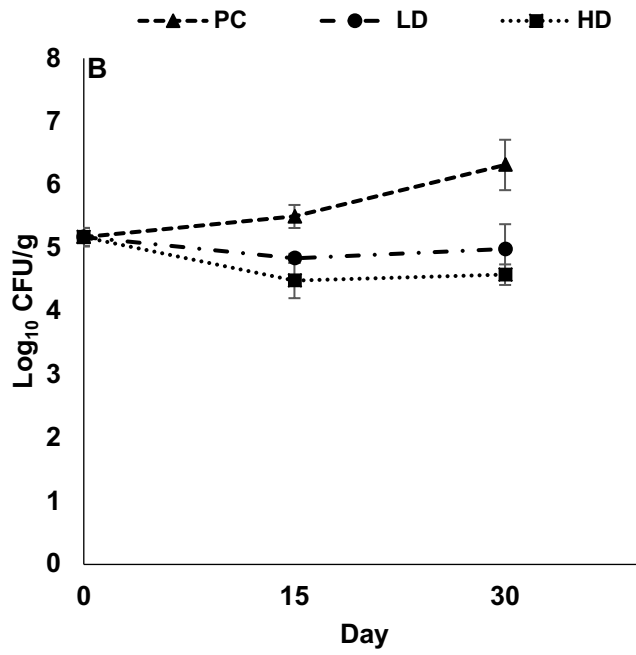
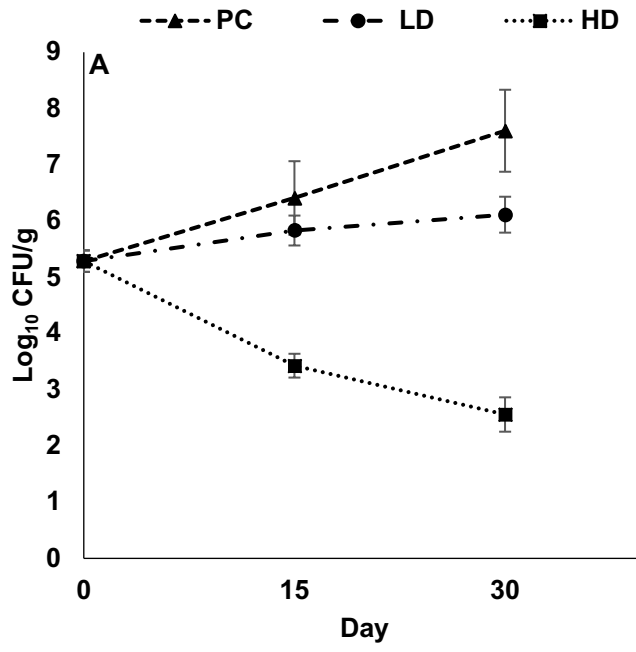
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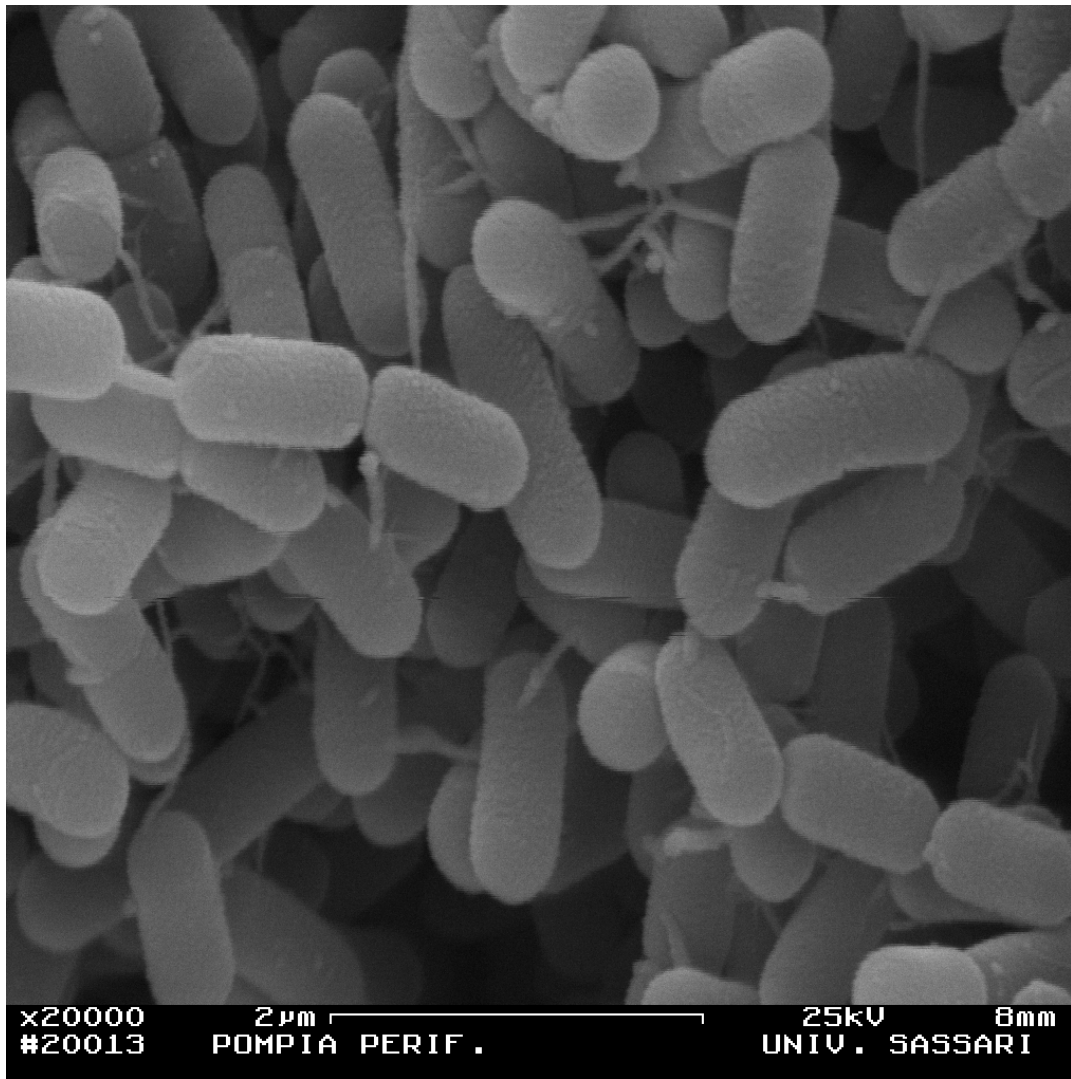


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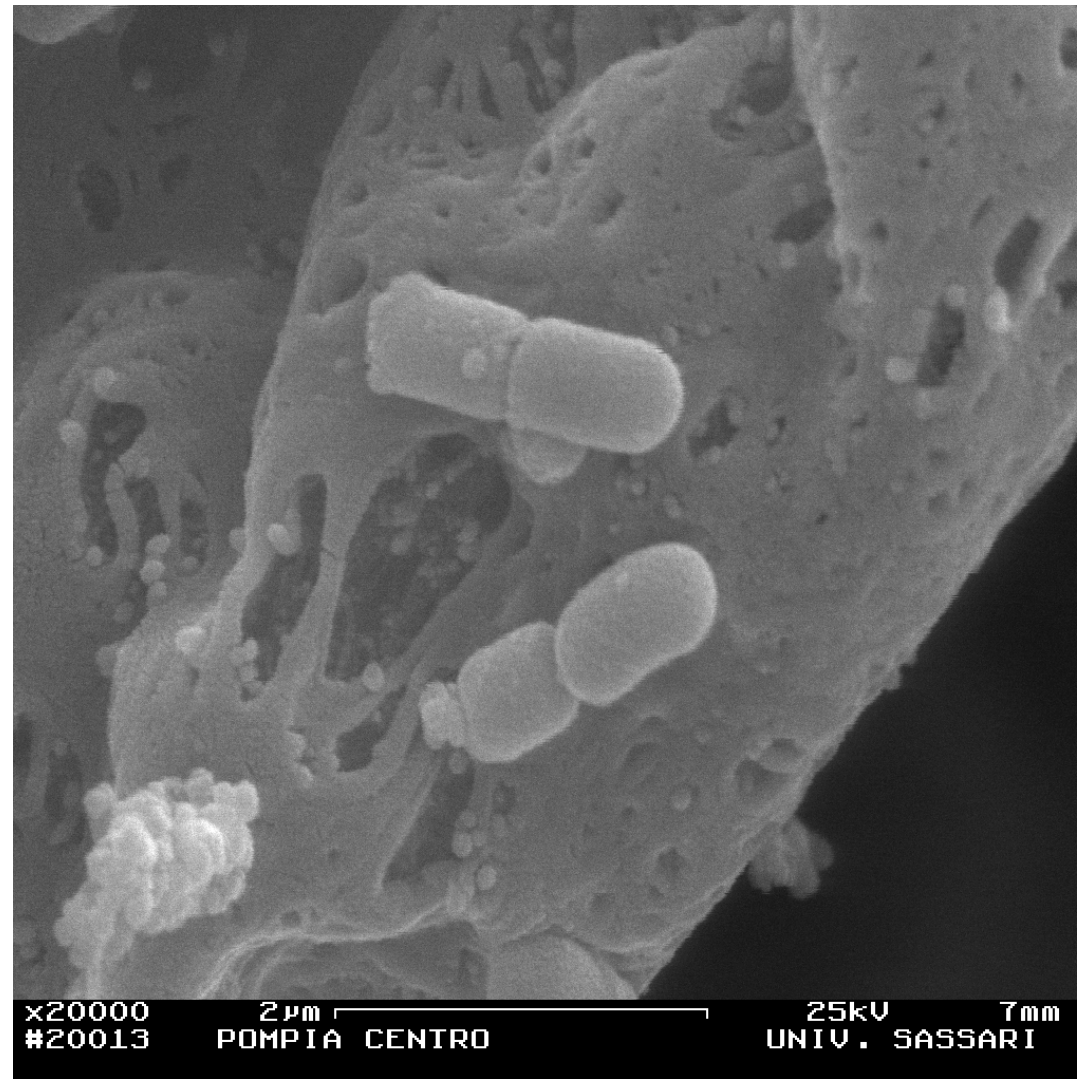




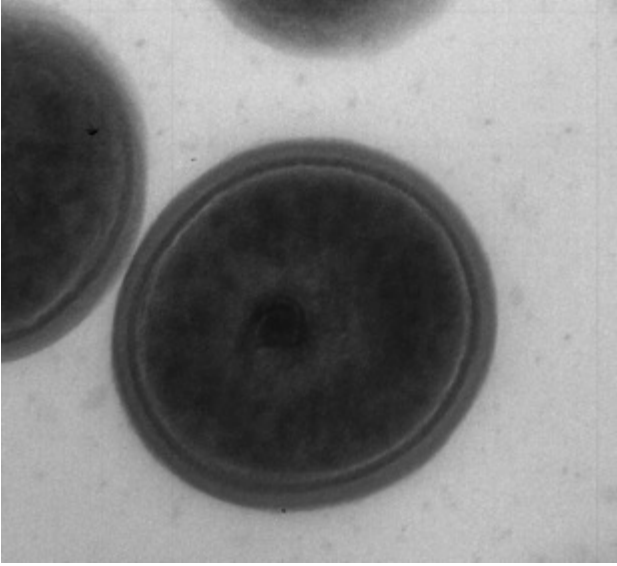




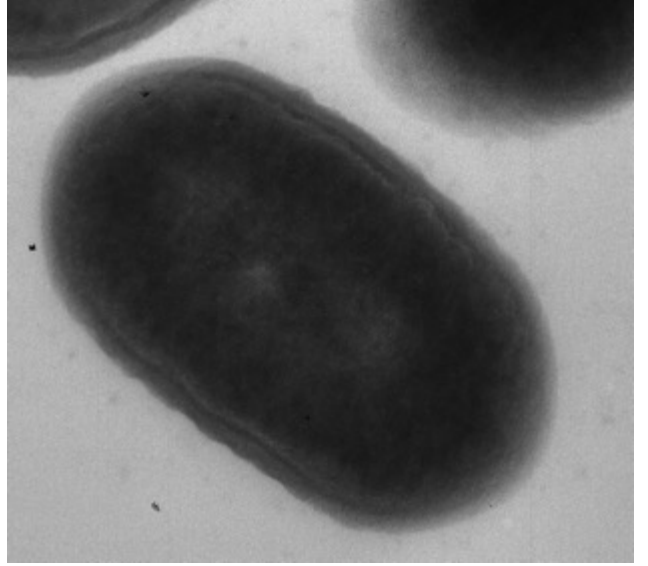
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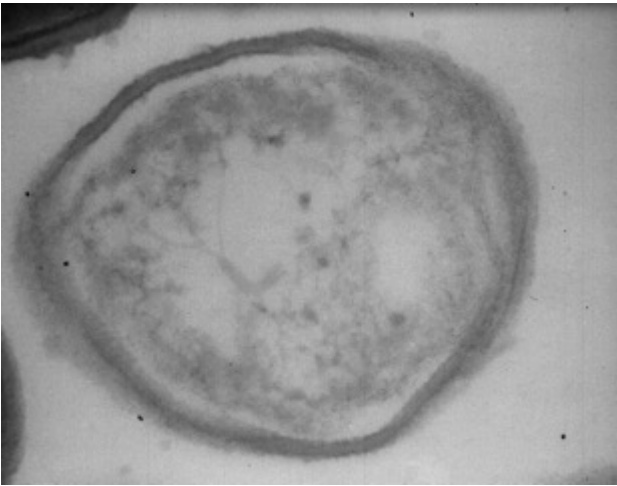
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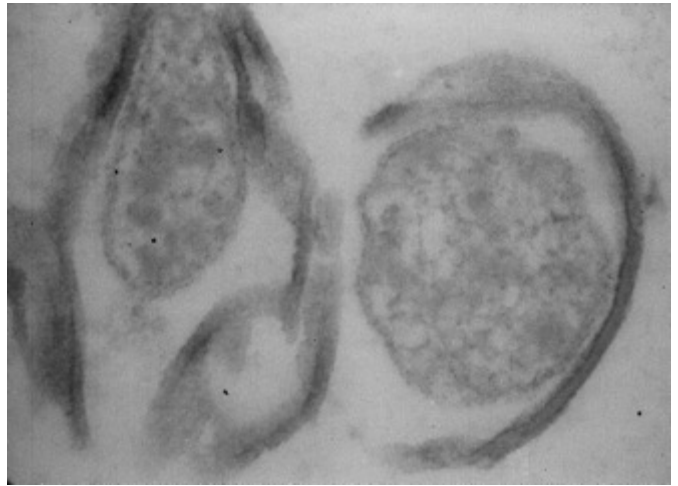
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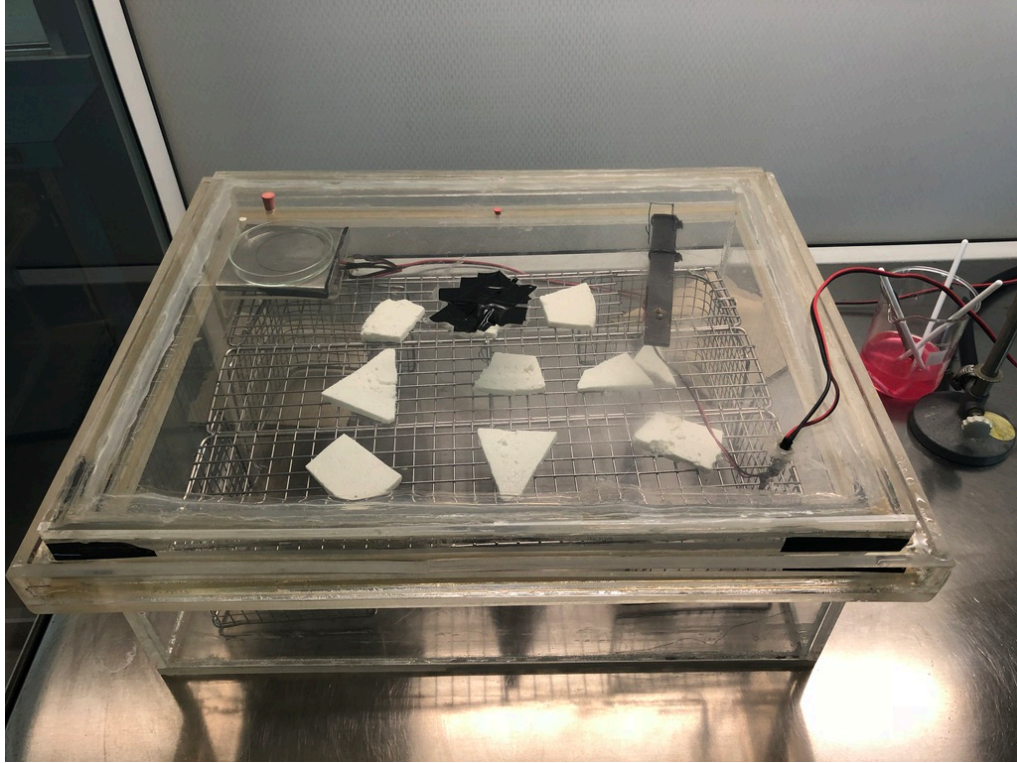
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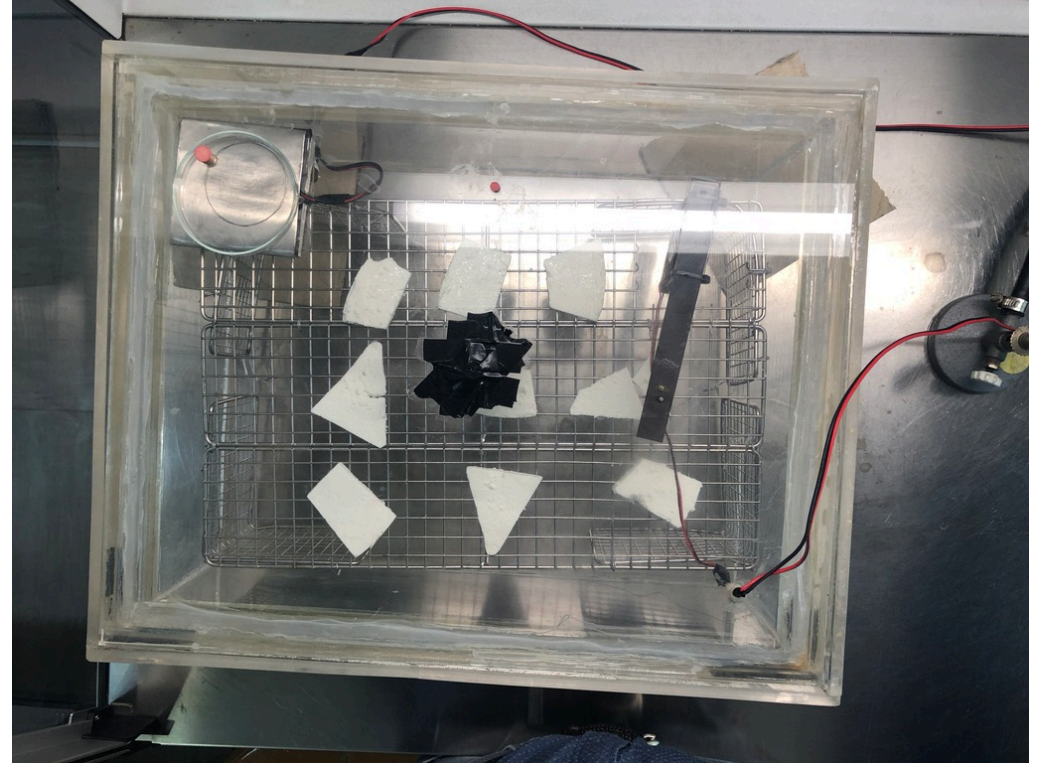
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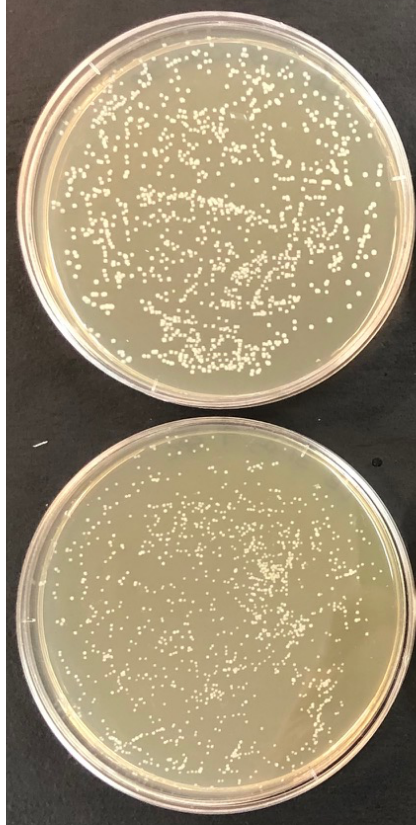
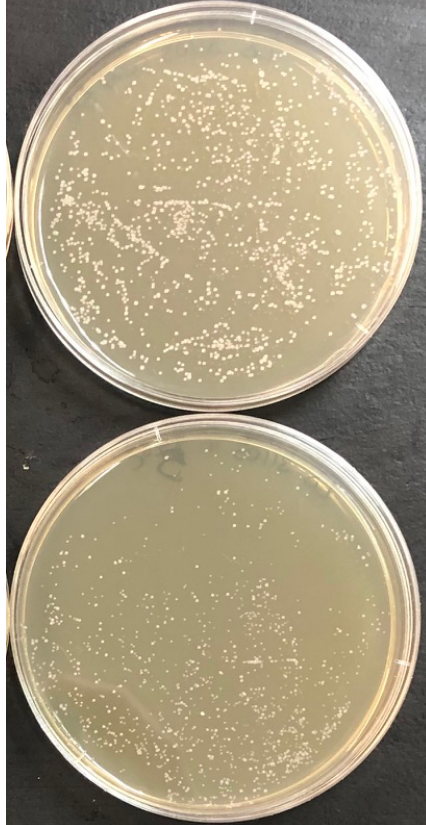
(B)

L. monocytogenes 20600 DSMZ

L. monocytogenes B STAA

L. monocytogenes E STAA

L. monocytogenes MIX



CONTROL

TREATED

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 $\sim 10^5$ 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 $\sim 10^5$ 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 $\sim 10^5$ 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 $\sim 10^5$ 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



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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.

We understand that the Corresponding Author (Severino Zara) is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

Sincerely,

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