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# Characterisation of supercritical carbon dioxide extracts from olive leaves: antimicrobial activity, volatile composition and mineral oil contamination

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ABSTRACT

This study investigated the antimicrobial efficacy of olive leaf extract (OLE) obtained *via* supercritical carbon dioxide (SC-$CO_2$) extraction, with additional attention to volatile composition and contamination by mineral oil hydrocarbons (MOH). The water-soluble fraction of OLE inhibited the growth of pathogenic (*Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*) and spoilage (*Pseudomonas fluorescens*, *Leuconostoc mesenteroides*) bacteria, while OLE vapor phase was also able to inhibit fungi such as *Penicillium roqueforti*. These effects were attributed to the combined presence of water soluble polyphenols (139.95 ± 2.50 mg GAE/$g_{DW}$) and bioactive volatiles with antimicrobial properties (*e.g.*, 1-pentanol, nonanal), which may have differentially impacted the tested microorganisms. However, OLE was found to be contaminated with MOH, likely deriving from harvesting or processing operations. Although these findings highlight the strong antimicrobial potential of OLE obtained by SC-$CO_2$ extraction, they also emphasize the need to address contamination risks for its safe use in food and pharmaceutical applications.

## 1. Introduction

Pruning of olive trees (*Olea europea L.*) as well as defoliation of harvested olives generates substantial amounts of discarded leaves (Proietti et al., 2015; Roig et al., 2006; Şahin & Bilgin, 2018). The latter are mainly directed to the production of biogas and cattle feed, or used as soil amendment, operations that lead to their downcycle (Espeso et al., 2021; Michailides et al., 2011; Sansoucy & Alibes, 1985). In recent years, a valuable strategy for the upcycling of olive tree leaves has been investigated, suggesting the possibility of using them as a source for the recovery of bioactive compounds (Lo Giudice et al., 2021). Olive leaves are peculiarly rich in phenolic compounds, including oleuropein, verbascoside, and flavonoids (*e.g.*, luteolin- and apigenin-7-*O*-glucoside) (Palmeri et al., 2022; Talhaoui et al., 2015), which are characterised by well-known antioxidant and antimicrobial activities (Bensehaila et al., 2022; Martín-García et al., 2022; Romani et al., 2019). Such a pool of phenolic compounds is thus extremely interesting for application in both food (*e.g.*, dietary supplements, food preservation) and non-food (*e.g.*, cosmetics, pharmaceutics) industries (Dauber et al., 2023; Martillanes et al., 2017; Ronca et al., 2024; Öztürk et al., 2025). In addition to phenolic compounds, olive leaves also contain other minor components worth mentioning, such as bioactive volatiles, which contribute to the defense of olive tree leaves against microbial pathogens (Brahmi et al., 2012; Campeol et al., 2001; Flamini et al., 2003; Malheiro et al., 2016).

Several studies have aimed to valorize olive leaves by producing olive leaf extract (OLE) for the food sector (Difonzo et al., 2018; Khalifa et al., 2016; Thielmann et al., 2017), by extracting both polar and non-polar leaf compounds. For instance, Ağçam and Ozyılmaz (2022) and Ghomari et al. (2019) demonstrated the antioxidant activity of OLE produced with conventional maceration with water and/or ethanol or Soxhlet extraction with organic solvents. Additionally, several authors showed that OLE presents a broad-spectrum bacteriostatic activity against foodborne pathogens (*e.g.*, *Escherichia coli*, *Staphylococcus aureus*) and spoilage microorganisms such as *Pseudomonas fluorescens*, thereby extending the shelf life of perishable food products (Gökmen et al., 2014; Lahreche et al., 2020; Liu et al., 2017; Moudache et al., 2017). These results pointed out OLE as a promising upcycled natural alternative to synthetic antioxidants and antimicrobials, such as nitrates, nitrites, BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) (Difonzo et al., 2021, 2022), aligning with circular economy

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principles within the food waste management hierarchy (Krzywonos et al., 2025).

Despite these promising results, OLE is often obtained by maceration and Soxhlet extraction of olive leaves at high temperatures for long times, requiring high energy consumption as well as use of toxic solvents. These conditions not only pose environmental concerns but can potentially lead to degradation, oxidation, and hydrolysis of thermolabile components such as phenolic compounds or bioactive volatiles as terpenoids (Blicharski & Oniszczuk, 2017; Cao et al., 2025; López-Bascón & Luque de Castro, 2020). To enhance the sustainability of olive leaf extracts production while preserving their bioactive compounds, various green extraction technologies have been proposed, including ultrasound-assisted extraction, solvent-free microwave-assisted extraction, and supercritical fluid extraction using $CO_2$ (Cruz et al., 2017; Martín-García et al., 2022; Şahin et al., 2017). Among these emerging strategies, supercritical fluid extraction with $CO_2$ combines the diffusivity, viscosity, and surface tension typical of gases with the density and solvating power of liquids (Yıldırım et al., 2024). The use of supercritical $CO_2$ as a solvent has many advantages: i) a critical point of 31.06 °C and 7.386 MPa, allowing for a favorable operating range; ii) abundance, cost-effectiveness, and non-toxicity of $CO_2$; iii) operation in the absence of oxygen, preventing degradation of sensitive compounds; iv) possibility to steer extraction yield and selectivity by addition of polar co-solvents, such as water or ethanol; v) minimal need for extracts refinement or post-treatment (Díaz-Reinoso et al., 2006; Pinto et al., 2021; Pise & Thorat, 2022; Şahin et al., 2011). In this regard, supercritical fluid extraction with carbon dioxide has been applied to olive leaves to obtain eco-friendly and value-added extracts (Cejudo Bastante et al., 2019; Jimenez et al., 2011; Kyriakoudi et al., 2024). Particularly, Cejudo Bastante et al. (2019) applied this technology at 120 bar and 80 °C for 2 h to prepare OLE using ethanol as a co-solvent. The obtained extract presented not only significant antioxidant activity but also antimicrobial efficacy against a few foodborne pathogens. Despite these promising results, questions remain about the components accounting for the observed bioactivity. OLE complex composition includes both a water-soluble fraction, rich in polyphenols, and a variety of volatile compounds, whose potential as food antimicrobials is largely underexplored. Moreover, to the best of our knowledge, no evidence is currently available on the capacity of supercritical $CO_2$-extracted OLE to inhibit not only pathogenic microorganisms but also food spoilage ones.

On the other hand, safety OLE aspects related to the presence of mineral oil hydrocarbons (MOH), posing significant public concern (*e.g.*, saturated and aromatic hydrocarbons, MOSH and MOAH), have never been explored before (Buijtenhuijs & van de Ven, 2019; Jaén et al., 2022). To the best of our knowledge, no data on MOH contamination in olive leaves, or in derived extracts are currently available, even if their presence has been reported in olives and olive oils following post-harvesting and processing operations (Menegoz Ursol et al., 2023). Owing to their lipophilic nature, it cannot be excluded that MOH could accumulate in OLE, being extracted under supercritical $CO_2$ conditions along with antioxidant and antimicrobial compounds. Additionally, contact with plastic materials during OLE processing may lead to contamination with polyolefin saturated hydrocarbons (POSH).

Based on these considerations, this work aimed to characterize OLE obtained by supercritical carbon dioxide extraction, with particular reference to antimicrobial activity, composition of volatile compounds and presence of mineral oil contaminants. The paper was divided in three parts. The first part focused on the antimicrobial effect of water-soluble OLE compounds a activity of water-soluble OLE compounds against foodborne pathogens and spoilage organisms gainst foodborne pathogens and spoilers. In the second part, the antimicrobial activity of OLE volatiles was tested against the same microorganisms by a vapor diffusion assay. To interpret the acquired results, OLE volatiles were analysed by HS-SPME-GC-MS, identifying compounds with potential antimicrobial activity. Finally, in the third part of the paper, the level of caution required during OLE preparation was assessed by quantifying MOSH and MOAH contamination using high-performance liquid chromatography coupled on-line to gas chromatography with flame ionization detection (on-line HPLC-GC-FID). This was followed by confirmatory analysis using comprehensive GC coupled with FID and mass detector (GC × GC-FID/MSD).

## 2. Materials and methods

### 2.1. Materials

The reagents used for this study were sodium carbonate solution ($Na_2CO_3$) and Folin-Ciocalteu reagent (Carlo Erba, Milan, Italy); gallic acid and glycerol (Sigma-Aldrich, Milan, Italy); Brain Heart Infusion (BHI) Broth, De Man Rogosa Sharpe (MRS) broth, Plate Count Agar (PCA), Maximum Recovery Diluent (MRD), Malt Extract Agar (MAE), Malt Extract Broth (MEB) and bacteriological agar (Oxoid, Milan, Italy); hexane, potassium hydroxide (KOH), m-chloroperbenzoic acid (m-CPBA), sodium thiosulfate, absolute ethanol, anhydrous sodium sulphate, activated aluminium oxide 90 (active basic, 0.063–0.200 mm, activity stage I), dichloromethane (Merck, Darmstadt, Germany). Milli-Q water (System advantage A10, Millipore S.A.S., Moldheim, France) was used for all experiments.

### 2.2. OLE preparation

OLE was produced following the protocol and equipment described by Machado et al. (2025). It was produced by Enhanced Solvent Extraction, combining supercritical $CO_2$ and ethanol as co-solvent (1:1) to promote the phenolic compounds' recovery. The extraction conditions were selected based on previous optimization of the processing parameters based on extraction yield (Sánchez-García et al., 2025; Verano-Naranjo et al., 2025). In detail, crushed leaves (180–200 g, ~2–3 mm) were extracted in a high-pressure system model SF1000 from Thar Technologies (Pittsburgh, USA), equipped with a 1000-mL thermostatic vessel, an automatic back pressure regulator and a pressure pump. The leaves were introduced in a paper cartridge and inserted into the vessel. Then, 500 mL of ethanol was added, and the vessel was hermetically closed. The system was pressurized at 10 g/min until it reached the set pressure conditions. The extraction process was performed at 120 bar and 80 °C in static conditions for 24 h. The system was then depressurized at 40 g/min and the extract was recovered and stored at 4 °C in dark. The resulting OLE was previously characterized by Sánchez-García et al. (2025) and Verano-Naranjo et al. (2025) and was reported to contain 12.6 ± 0.5 g/L of oleuropein and 0.6 ± 0.1 g/L of luteolin-7-glucoside, with an extraction yield of 66.2 ± 2.9 g/L.

### 2.3. OLE aqueous extract

An aqueous suspension containing 10 g/100 g of OLE was prepared. An aliquot of 1.5 mL of the suspension was taken under agitation, placed inside a 2 mL Eppendorf® and centrifuged at 20,000×*g* for 15 min at 4 °C using a microcentrifuge (Mikro 20, Hettich Italia srl, Milan, Italy). The supernatant was then collected by percolation and filtered through sterile syringe filters made of polyethersulfone (PES) with a pore size of 0.22 μm (Clear Line, Dominique Dutscher SAS, Brussels, Belgium). The total solids content (w/v) in the filtered aqueous extract of OLE was determined by weight after drying in a thermostatic oven (Vuotomatic 50, Bicasa, Milan, Italy) overnight at 72 °C and a vacuum level of 70 mmHg.

### 2.4. Total phenolic content of OLE aqueous extract

The total phenolic content (TPC) of the OLE aqueous extract was determined using the Folin-Ciocalteu method, as described by Singleton et al. (1999) with slight modifications. Briefly, 250 μL of the OLE aqueous extract, pre-diluted (1 mL/30 mL) in water, was mixed with

12.5 mL of water and 1.25 mL of Folin-Ciocalteu reagent. Subsequently, 5 mL of a 15 g/100 mL $Na_2CO_3$ solution was added, followed by 6 mL of water to reach a final reaction volume of 25 mL. The mixture was allowed to react for 2 h at room temperature in the dark. The absorbance was then measured at 750 nm using a UV-Vis spectrophotometer (UV-2501 PC, Shimadzu, Kyoto, Japan) at 25 °C with a 1 cm path-length cuvette. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/$g_{DW}$), based on a calibration curve obtained using serial dilutions of a gallic acid standard solution ($R^2 = 0.9974$).

### 2.5. Antibacterial activity of OLE aqueous extract

The antimicrobial activity of OLE aqueous extract was tested against foodborne pathogens and spoilers, particularly *Listeria monocytogenes* Scott A, *Salmonella enterica* subsp. *arizonae* DSM 9386, *Staphylococcus aureus* DSA 226, *Escherichia coli* DSA 8048, *Enterococcus faecium* DSM 20477, *Pseudomonas fluorescens* L22, and *Leuconostoc mesenteroides* DSA_O. The strains were stored at - 80 °C in BHI or MRS broth (*Lc. mesenteroides*) added with 30 mL/100 mL glycerol. Before each trial, a loopful of each culture was inoculated into 1 mL of BHI or MRS broth and incubated at 37 °C or 30 °C (*Ps. fluorescens* and *Lc. mesenteroides*) for 18 h. Each culture was centrifuged at 13,000×*g* for 5 min in a Mirko 20 centrifuge (Hettich Italia S.r.l., Milan, Italy), washed three times with MRD, and then diluted in MRD at a ratio of 1:1000. The antibacterial activity was assessed in two biological replicates by turbidimetry in 96-well U-bottom polystyrene microplates (Corning Incorporated, Corning, NY). For each replicate, duplicate wells containing 200 μL of BHI spiked with increasing concentrations of extract (1 mL/10 mL; 1 mL/5 mL; 1 mL/1 mL; sterilized by filtration) were inoculated with 10 μL of microbial culture (final viability about $10^4$ Colony-Forming Units per milliliter, CFU/mL). Control wells containing BHI inoculated with the target microorganisms, and blank wells containing only BHI, were also set up. Only for *Lc. mesenteroides*, trials were carried out in MRS. The microplates were incubated for 48 h at 37 °C or 30 °C (*Ps. fluorescens* and *Lc. mesenteroides*) in a Sunrise microplate reader (Tecan, Cernusco s. N., Milan, Italy) where the Optical Density (OD) at 630 nm ($OD_{630}$) was read at 30-min intervals. $OD_{630}$ readings were used to generate turbidimetric curves. Data were modeled with DMFit online (https://browser.combase.cc/DMFit.aspx) using Baranyi-Roberts' growth model (Baranyi & Roberts, 1994), to estimate values for lag phase duration (λ, expressed in hours), maximum growth rate ($\mu_{max}$, expressed in $OD_{630}$/h) and maximum optical density ($OD_{max}$, expressed in $OD_{630}$).

### 2.6. Antifungal activity of OLE aqueous extract

The antifungal activity against *Penicillium roqueforti* was evaluated using the disk diffusion assay, conducted in two biological replicates (Hudzicki, 2009). The fungal strain was isolated from *Penicillium roqueforti* N (Sacco s.r.l., Cadorago, CO, Italy) and stored at - 80 °C in MEB added with 30 mL/100 mL) glycerol. Before each trial, one loopful of the culture was streaked onto MEA and incubated at 30 °C for 72 h. Spores were then collected using a sterile loop and resuspended in MRD to an optical density at 600 nm ($OD_{600}$) of 0.10, and 100 μL of the suspension was evenly spread onto MEA. Then, a 13-mm diameter filter paper disk (Whatman®, Cytiva, Maidstone, UK) was impregnated with 100 μL of OLE aqueous extract and placed onto the surface of the inoculated agar plates. The plates were incubated at 30 °C for 72 h, and the inhibition zone around the disks was measured after the incubation time.

### 2.7. Antimicrobial activity of OLE volatiles

Overnight-grown washed bacterial cultures, prepared according to the methodology outlined in paragraph 2.5 were serially diluted in MRD at 1:1,000,000. The antibacterial activity was evaluated following the method of López et al. (2005) with specific adjustments. Petri dishes (53 mm in diameter) containing 10 mL of BHI agar (for *L. monocytogenes*, *S. enterica*, *Staph. aureus*, *E. coli*, and *Ent. faecium*), MRS agar (*Lc. mesenteroides*), and PCA (*Ps. fluorescens*) were inoculated by spreading with 35 μL of each microbial suspension (final count about $10^3$ CFU/mL). Under sterile conditions, 0.25 g of OLE was uniformly spread across a 3 × 3 cm square area on the inner part of the Petri dish lids using a CytoOne Cell Scraper (StarLab, Milan, Italy). Each culture was then covered with either an OLE-treated lid or a standard lid (control) and sealed with Parafilm®.

Plates were then placed inside plastic containers made of amorphous polyethylene terephthalate (APET; capacity: 0.7 L; oxygen transmission rate, OTR = 1.81 $cm^3$(STP)/$m^2$ day; water vapor transmission rate, WVTR = 3.19 g/ $m^2$ day; COMPAC s.r.l., Castelnovo di Sotto, Italy) and sealed with a high-oxygen-barrier film composed of polypropylene/polyethylene terephthalate (PP/PET, OTR = 2.13 $cm^3$(STP)/$m^2$ day, WVTR = 5.20 g/ $m^2$ day, COMPAC s.r.l., Castelnovo di Sotto (RE), Italy) to ensure airtight conditions. Each container, holding four Petri dishes, was incubated at 37 °C (for *L. monocytogenes*, *S. enterica*, *Staph. aureus*, *E. coli*, and *Ent. faecium*) or 30 °C (*Ps. fluorescens* and *Lc. mesenteroides*). Images of the plates were taken after 24 h of incubation for all microorganisms, except for *Lc. mesenteroides*, which was imaged after 48 h. Images were acquired using a digital camera (EOS SSOD, Canon, Milan, Italy) positioned 50 cm above the Petri dish, which was placed on a black background within a photo booth (Immagini & Computer, Bareggio, Italy). Illumination was provided by four 100 W photographic reflectors to minimize shading and glare. The camera settings were as follows: exposure time of 1/30 s, F-stop t/10, and focal length of 60 mm. Images were saved in.CR2 format with a resolution of 5184 × 3456 pixels. The diameter of microorganism colonies (Area of Interest, AOI) was estimated through image analysis employing the contour detection method reported by (Yu et al., 2025), implemented in OpenCV *via* the cv2 module, with slight modifications. Images were converted to grayscale, pre-processed applying Gaussian blurring to reduce noise and improve edge detection (Smereka & Dulęba, 2008). Circular contours of AOIs were detected as minimum enclosing circles using the Hough Circle Transform. To identify microorganism colonies or mold mycelia, which have considerably different size, Canny edge detection threshold and predefined minimum and maximum radii were optimized (Bradski, 2000). Diameters of AOI, estimated in pixels, were converted to millimeter using a 0.0028 mm/pixel factor, and reported as mean ± standard deviation (Taniwaki et al., 2006). Similarly, the antifungal activity of OLE volatiles was assessed by adapting the protocol described above. Briefly, a *P. roqueforti* suspension was prepared as outlined in paragraph 2.6 MEA plates were spot-inoculated (without spreading) with 35 μL of the spore suspension and incubated at 30 °C. Mycelial expansion of *P. roqueforti* was then monitored and imaged at 3, 4, and 7 days post-inoculation, and the diameter of the fungal mycelia was quantified using image analysis.

### 2.8. OLE volatiles

Volatiles compounds in the headspace of OLE sample were assessed through solid phase micro-extraction technique coupled with gas chromatographic analysis and mass spectrometry (HS-SPME-GC-MS). A sealed vial containing 0.50 g of OLE was conditioned at 40 °C for 30 min in a HTA autosampler (HTA model HT2800T, Brescia (BS), Italy).

For extraction, a 2 cm triphasic fiber (Supelco, Merck KGaA, Darmstadt, Germany) was used with a sampling temperature of 40 °C for 15 (after 15 min of prior thermal equilibration before microextraction). A gas chromatography-mass spectrometry (GC-MS) system (GC 2030 Nexis Shimadzu Italia S.r.l., Milan, Italy) equipped with a DB-WAX-MS capillary column (60 m length, 0.25 mm internal diameter, and 0.25 μm film thickness, Agilent Technologies, CA, USA) was used to separate and detect volatile compounds. Desorption into the GC injection port was at 250 °C with splitless-type injection for 3 min. For separation,

Helium was used as carrier gas (0.9 mL/min flow rate), while interface, source, and quadrupole temperatures were 240, 200, and 150 °C, respectively. The temperature program included two phases: 40 °C for 1 min, followed by the ramp at 250 °C for 30 min, which was reached after heating 4 °C/min. The mass spectrometry (GCMS-QP2020 NX Shimadzu Italia S.r.l., Milan, Italy) worked in scan mode with a mass range from 25 to 400 m/z. Chromatographic profiles were evaluated using the GC-MS solution software (version 4.52, Shimadzu Corporation, Kyoto, Japan) and compounds were identified by spectra comparison using the NIST/EPA/NIH 20 Mass Spectral Library (John Wiley & Sons Inc., Hoboken, NJ, USA) and by comparing their Kovat's retention index with those reported in the literature (http://webbooking.nist.gov/chemestry/).

### 2.9. MOSH and MOAH contamination

To evaluate saturated and aromatic hydrocarbons (MOSH and MOAH) contamination, OLE was reduced to a fine powder and 0.5 g were directly weighed into a Teflon vessel (Green Chemplus CEM Corporation, Matthews, North Carolina, USA), added with 10 μL of internal standard mixture (IS), 10 mL of *n*-hexane and 10 mL of a saturated methanolic KOH solution. The samples underwent microwave-assisted saponification (MAS) in a MARS 5 (CEM Corporation, Matthews, NC, USA) (Moret et al., 2016). After a pre-heating step (5 min), the samples were heated at 120 °C for 20 min under magnetic stirring. Once cooled to ambient temperature, they were added with 40 mL of water and 3 mL of methanol, then placed at −20 °C for 30 min. Subsequently, the separated hexane extracts were withdrawn using a Pasteur pipette, concentrated to 700 μL, and subjected to epoxidation following a slightly modified Nestola and Schmidt (2017) protocol. Briefly, 500 μL of an ethanolic 20 g/100 mL m-CPBA solution were added to the extract and left at room temperature for 15 min under stirring. Then, 2 mL of aqueous sodium thiosulfate solution (10 g/100 mL) and 500 μL of ethanol to stop the reaction were added. The vial was shaken for 3 min, and finally, 500 μL were transferred into an autosampler vial filled with a spatula tip of sodium sulphate. For MOAH analysis, the sample extract obtained was injected into the HPLC-GC-FID apparatus. For the MOSH, 20 μL of saponified and epoxidized sample was loaded into a 6-mL glass cartridge (10 mm i.d.) containing 5 g of activated aluminum oxide 90 active basic, previously conditioned with 10 mL of *n*-hexane. The elution of the MOSH fraction was carried out with further 10 mL of *n*-hexane, which were recovered and reconcentrated before HPLC-GC-FID analysis.

The separation and analytical determination of MOSH and MOAH were conducted using an LC-GC 9000 system from Brechbühler (Zurich, Switzerland). This system included a Phoenix 40 module with three syringe LC pumps, four switching valves, an UV-VIS spectrophotometric detector (UV-2070 Plus, Jasco, Japan), an autosampler PAL LHS2-xt Combi PAL (Zwingen, Switzerland) and a GC Trace 1300 (Thermo Scientific, Milan, Italy) with a double-channel configuration to allow simultaneous analysis of MOSH and MOAH in a single run. Each GC channel was equipped with a 10 m × 0.53 mm id uncoated pre-column (retention gap), followed by a steel T-piece union connected to the solvent vapor exit, and a 10 m × 0.25 mm id PS-255 separation column (0.15 μm film thickness) from Mega, Italy. The LC column used was an Allure Si (25 cm × 2.1 mm i.d., 5 μm) from Restek (Milan, Italy). The transfer of the LC fraction into the GC was carried out through a Y-interface using the retention gap technique. The flame ionization detector (sampling frequency of 50Hz) and solvent vapor exit were heated at 360 and 140 °C, respectively. For analysis, the sample was eluted at 300 μL/min using a gradient starting with hexane and reaching 30 mL/100 mL dichloromethane. MOSH and MOAH fractions were transferred to the GC between 2.1 and 3.6 min, and between 3.8 and 5.3 min, respectively. After transferring the fractions, the LC column was back-flushed with dichloromethane and reconditioned with hexane to prepare the system for the subsequent injection. Data were acquired and processed by Chromeleon software (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

To characterize MOSH and MOAH fractions obtained by liquid chromatography (LC), a comprehensive two-dimensional GC × GC-FID/MS system was employed. This technique offers improved separation power and compound identification, especially in complex matrices such as hydrocarbon fractions. The system consisted of an Agilent 7890 GC equipped with a 7693 autosampler and an on-column injector with track-oven configuration. Detection was achieved simultaneously with a FID and a MSD (5977B, Agilent Technologies, Santa Clara, CA, USA). The column setup included an uncoated retention gap (10 m × 0.53 mm i.d., Mega, Legnano, Italy), followed by a DB-17 column (15 m × 0.25 mm i.d., 0.15 μm film thickness, Agilent Technologies, Santa Clara, CA, USA) used as the first-dimension (1D) separation, and a DB-5ms column (2.5 m × 0.15 mm i.d., 0.05 μm film thickness, Mega, Legnano, Italy) employed as the second-dimension (2D) separation and modulation loop. The effluent from the second dimension was split between the FID and MS *via* two fused silica capillaries (1 m × 0.10 mm i.d. to the MS and 1 m × 0.18 mm i.d. to the FID), achieving an approximate split ratio of 70:30 in favor of the FID. The FID was operated at 340 °C, with hydrogen flow at 30 mL/min, air at 400 mL/min, and nitrogen as make-up gas at 25 mL/min. The sampling frequency was set at 100 Hz. The transfer line and ion source temperatures were both maintained at 340 °C, while the quadrupole was operated at 150 °C. Ionization was carried out in electron ionization (EI) mode at 70 eV, with a spectral acquisition range from m/z 50 to 550, at a rate of 50 spectra per second. The injection volume was 25 μL introduced into the on-column injector at a rate of 150 μL/min. Helium was used as the carrier gas with an initial flow of 4.5 mL/min for 5.7 min, which was then decreased to 1.5 mL/min and held constant for the remainder of the run. The oven temperature program was as follows: 45 °C (1 min), ramped to 65 °C at 30 °C/min (held 4.5 min), then to 340 °C at 4 °C/min (held 5 min). Thermal modulation was performed using a ZX2 modulator (Zoex Corporation, Houston, TX, USA) with closed-cycle refrigeration, controlled *via* Optimode™ v2.0 (SRA Instruments, Cernusco sul Naviglio, Italy). The hot jet pulse was set at 350 ms, with a modulation period of 9 s. The hot jet temperature increased from 250 °C (after 5.1 min) to 400 °C at a ramp of 4 °C/min. Cold jet flow, managed by a mass flow controller (MFC), followed a programmed decrease: starting at 50%, reduced to 40% during the first 700 s, then to 5% after 35 min, and maintained at this level until the end of the analysis. MassHunter software (Agilent Technologies, Santa Clara, CA, USA) was used for data acquisition, while two-dimensional chromatograms were processed with GC Image® GC × GC Edition (version 2020r1, Zoex Corporation, Houston, TX, USA).

### 2.10. Statistical analysis

All determinations were expressed as the mean ± standard deviation (SD) of at least three repeated measurements from two experiment replicates (n = 2). Statistical analysis was performed by using R (The R Foundation for Statistical Computing, Wien, Austria, version 4.4.3) within the RStudio environment (version 2024.12.0, Build 467). Bartlett's test was used to check the homogeneity of variance; one-way ANOVA was carried out and the Tukey test was used to determine statistically significant differences among means ($p < 0.05$). Pearson correlation analysis was performed to assess the overall linear relationships between OLE concentration and the microbial growth parameters ($OD_{max}$, $\mu_{max}$, and $\lambda$). All experimental data from the seven bacterial strains across the four OLE concentrations were pooled for this analysis ($n = 28$). The strength and direction of the relationships were evaluated by calculating correlation coefficients ($p < 0.05$).

## 3. Results and discussion

### 3.1. Antimicrobial activity of OLE aqueous extract

The initial phase of this study consisted of assessing the antimicrobial activity of the olive leaf extract (OLE) against a group of seven bacterial

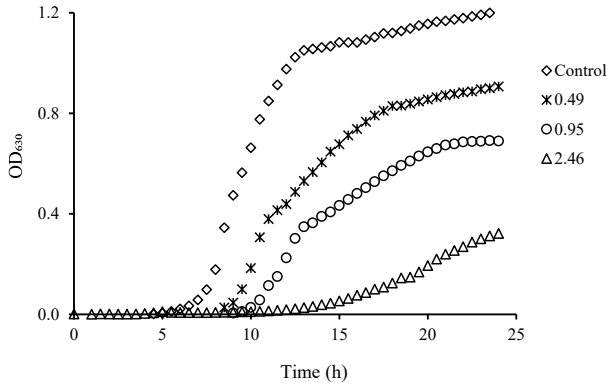


**Fig. 1.** Turbidimetric growth curves of *Listeria monocytogenes* in the presence of increasing concentrations (0.00 (control), 0.49, 0.95 and 2.46 g/100 mL) of OLE aqueous extract in the growth media. The y-axis reports the Optical Density at 630 nm ($OD_{630}$).

**Table 1**
Lag phase (λ), maximum growth rate ($\mu_{max}$), and maximum optical density ($OD_{max}$) estimated from the growth kinetic curves of different pathogenic and spoilage microorganisms in the presence of increasing concentrations of olive leaf extract (OLE) in the growth media.

| Microorganism | OLE (g/100 mL) | λ (h) | $\mu_{max}$ ($OD_{630}$/h) | $OD_{max}$ ($OD_{630}$) |
|---|---|---|---|---|
| *L. monocytogenes* | 0.00 | $6.98^{c} \pm 0.07$ | $0.21^{a} \pm 0.00$ | $1.13^{a} \pm 0.01$ |
| | 0.49 | $7.84^{bc} \pm 0.45$ | $0.11^{b} \pm 0.01$ | $0.86^{ab} \pm 0.13$ |
| | 0.98 | $8.99^{b} \pm 0.61$ | $0.07^{b} \pm 0.01$ | $0.67^{bc} \pm 0.04$ |
| | 2.46 | $14.13^{a} \pm 0.07$ | $0.03^{c} \pm 0.00$ | $0.40^{c} \pm 0.06$ |
| *E. coli* | 0.00 | $3.87^{d} \pm 0.07$ | $0.48^{ab} \pm 0.03$ | $1.31^{a} \pm 0.02$ |
| | 0.49 | $4.28^{c} \pm 0.11$ | $0.51^{a} \pm 0.02$ | $1.28^{ab} \pm 0.04$ |
| | 0.98 | $5.10^{b} \pm 0.09$ | $0.41^{b} \pm 0.02$ | $1.11^{b} \pm 0.00$ |
| | 2.46 | $6.93^{a} \pm 0.01$ | $0.16^{c} \pm 0.01$ | $0.76^{c} \pm 0.07$ |
| *Staph. aureus* | 0.00t | $4.03^{c} \pm 0.06$ | $0.52^{ab} \pm 0.01$ | $1.36^{a} \pm 0.01$ |
| | 0.49 | $4.69^{bc} \pm 0.13$ | $0.55^{a} \pm 0.02$ | $1.29^{ab} \pm 0.10$ |
| | 0.98 | $5.39^{b} \pm 0.04$ | $0.40^{b} \pm 0.07$ | $1.06^{b} \pm 0.02$ |
| | 2.46 | $7.33^{a} \pm 0.33$ | $0.18^{c} \pm 0.01$ | $0.74^{c} \pm 0.11$ |
| *En. faecium* | 0.00 | $3.96^{c} \pm 0.14$ | $0.43^{b} \pm 0.06$ | $1.46^{a} \pm 0.00$ |
| | 0.49 | $4.83^{bc} \pm 0.06$ | $0.64^{a} \pm 0.03$ | $1.27^{b} \pm 0.03$ |
| | 0.98 | $5.65^{b} \pm 0.30$ | $0.62^{a} \pm 0.17$ | $1.08^{c} \pm 0.01$ |
| | 2.46 | $7.59^{a} \pm 0.71$ | $0.18^{c} \pm 0.01$ | $0.56^{d} \pm 0.02$ |
| *S. enterica* | 0.00 | $4.20^{c} \pm 0.08$ | $0.60^{a} \pm 0.07$ | $1.46^{a} \pm 0.02$ |
| | 0.49 | $4.66^{c} \pm 0.22$ | $0.59^{a} \pm 0.05$ | $1.33^{a} \pm 0.15$ |
| | 0.98 | $5.33^{b} \pm 0.19$ | $0.30^{b} \pm 0.01$ | $0.89^{b} \pm 0.00$ |
| | 2.46 | $7.66^{a} \pm 0.03$ | $0.17^{b} \pm 0.01$ | $0.61^{b} \pm 0.01$ |
| *Lc. mesenteroides* | 0.00 | $7.94^{b} \pm 0.30$ | $0.17^{a} \pm 0.00$ | $1.52^{a} \pm 0.00$ |
| | 0.49 | $9.35^{b} \pm 0.02$ | $0.17^{a} \pm 0.00$ | $1.52^{a} \pm 0.01$ |
| | 0.98 | $13.23^{b} \pm 2.50$ | $0.13^{b} \pm 0.00$ | $1.45^{a} \pm 0.00$ |
| | 2.46 | $30.59^{a} \pm 4.85$ | $0.03^{c} \pm 0.01$ | $0.01^{b} \pm 0.01$ |
| *Ps. fluorescens* | 0.00 | $10.26^{a} \pm 0.11$ | $0.12^{a} \pm 0.01$ | $0.75^{a} \pm 0.00$ |
| | 0.49 | $9.09^{b} \pm 0.21$ | $0.08^{b} \pm 0.01$ | $0.63^{a} \pm 0.08$ |
| | 0.98 | $9.27^{b} \pm 0.02$ | $0.06^{c} \pm 0.00$ | $0.57^{a} \pm 0.00$ |
| | 2.46 | $10.42^{a} \pm 0.08$ | $0.05^{c} \pm 0.00$ | $0.60^{a} \pm 0.25$ |

[a-d] For the same microorganism, means within the same column indicated by different letters are significantly different ($p < 0.05$).

species of food interest (*Listeria monocytogenes*, *Escherichia coli, Staphylococcus aureus*, *Enterococcus faecium*, *Salmonella enterica*, *Leuconostoc mesenteroides*, and *Pseudomonas fluorescens)* and the filamentous fungus *Penicillium roqueforti*. Since OLE obtained by supercritical carbon dioxide (SC-$CO_2$) extraction (Cejudo Bastante et al., 2019) contains both lipid- and water-soluble compounds (Manjare & Dhingra, 2019; Markom et al., 2020; Wrona et al., 2017), there could be the risk of non-homogeneous distribution of the OLE in the microbial liquid cultures. To avoid this criticism, an OLE aqueous extract, having a concentration corresponding to the maximum OLE solubility in water (4.92 ± 0.03 g/100 mL), was prepared and tested for antimicrobial activity through addition in the growth media.

Fig. 1 shows the turbidimetric growth kinetics of *L. monocytogenes* in the presence of increasing concentrations of OLE aqueous extract in the growth media. Overall, for *L. monocytogenes*, a clear concentration-dependent inhibitory pattern was observed (Fig. 1). In particular, increasing OLE levels resulted in a progressive lag extension, along with a marked reduction in both maximum growth rate and stationery-phase yield, supporting a dose-effect relationship for the aqueous fraction under the tested conditions.

Similar growth curves in the presence of increasing OLE concentration were also obtained for the other microorganisms considered in the study (data not shown). Growth curves for all microorganisms were modeled to estimate lag phase length (λ), maximum growth rate ($\mu_{max}$), and maximum optical density ($OD_{max}$) (Table 1).

Compared to the control (*i.e.*, 0.00 g OLE/100 mL), increasing concentrations of OLE were particularly effective in inhibiting the growth of bacterial pathogens, as indicated by significant lag phase extension coupled with maximum growth rate and maximum optical density reduction. At the highest OLE concentration, pathogens generally showed an approximately 1.8–2.0-fold increase in λ, accompanied by marked reductions in $\mu_{max}$ (≈58–86%) and $OD_{max}$ (≈42–65%) relative to controls (Table 1). For instance, for *L. monocytogenes*, at 2.46 g/100 mL of OLE, λ increased from 6.98 to 14.13 h, while $\mu_{max}$ decreased from 0.21 to 0.03 $OD_{630}$/h and $OD_{max}$ from 1.13 to 0.40 (Table 1).

As regards food spoilers, the OLE aqueous extract exhibited different effects depending on the considered microorganism. In particular, limited inhibitory effects were observed against *Ps. fluorescens*. Indeed, increasing OLE concentrations primarily resulted in a progressive decrease in $\mu_{max}$, whereas λ and $OD_{max}$ remained comparatively stable, suggesting an overall modest susceptibility of this Gram-negative spoilage organism to the OLE aqueous fraction. Such resistance to OLE may agree with the well-known ability of *Pseudomonas* spp. to adapt to the presence of different antimicrobial compounds (Silverio et al., 2022), due to their intrinsic tolerance traits. The latter include an outer membrane that restricts the penetration of many xenobiotics and the activity of multidrug efflux systems, which together can reduce intracellular accumulation of antimicrobial phytochemicals (Nikaido, 2003). In addition, the pronounced stress-response plasticity of *Pseudomonas* spp. and, in some contexts, biofilm-associated tolerance may further contribute to the observed results (Liu et al., 2018; Maifreni et al., 2023). By contrast, *Lc. mesenteroides* exhibited the strongest dose-effect relationship among the tested microorganisms, with a pronounced lag phase extension at the highest OLE concentration, accompanied by a marked reduction in $\mu_{max}$ and an almost complete collapse of $OD_{max}$ (Table 1). This marked sensitivity is consistent with the generally higher susceptibility of Gram-positive bacteria to antimicrobial compounds compared to Gram-negative ones, due to the lack of an outer membrane (Daglia, 2012). These findings are in contrast with those reported by Korukluoglu et al. (2010), in which OLE extracted using water as solvent showed no antimicrobial activity against *Lc. mesenteroides* nor towards the other tested microorganisms (*e.g., E. coli, Staph. aureus,* and *S. enterica*). These discrepancies are likely attributable to the fact that SC-$CO_2$ extraction might favor the extraction of bioactive compounds other than water-soluble ones, which could also contribute to the OLE antimicrobial effect. The observed differences may also be partly related to the strain and type of test used to assess antimicrobial activity.

To assess the strength of the dose-dependent antimicrobial effect of OLE, a Pearson correlation analysis was performed on the pooled dataset reported in Table 1, and the results are shown in Supplementary Table S1. Results revealed linear relationships between OLE concentration and microbial growth parameters. Specifically, OLE concentration was strongly negatively correlated with the $OD_{max}$ ($r = -0.74$). This strong inverse relationship underlines that increasing OLE concentration progressively limited the maximum attainable population size. Similarly, a significant negative correlation was observed for $\mu_{max}$ ($r = -0.49$) and a positive one for λ ($r = 0.47$), suggesting a dose-dependent

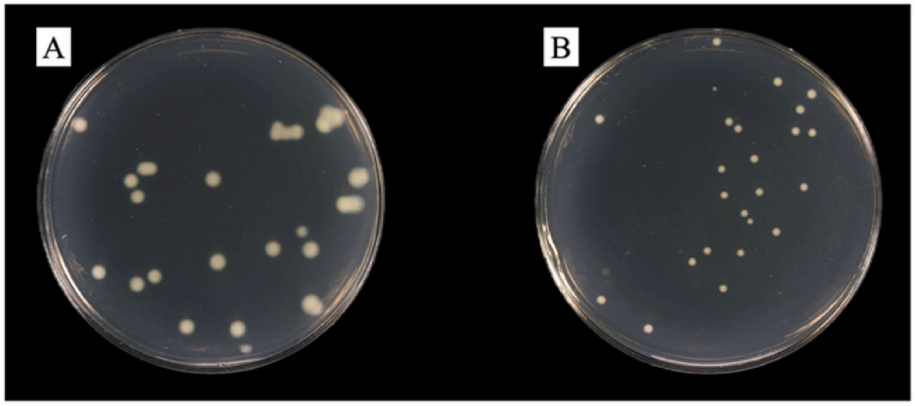


**Fig. 2.** *Pseudomonas fluorescens* colonies in the absence (A) and in the presence (B) of olive leaf extract in the vapor diffusion assay.

**Table 2**
Colony diameter (mm) in the absence (Control) and presence (OLE) of olive leaf extract in the vapor diffusion assay.

| Microorganism | Colony diameter (mm) | |
|---|---|---|
| | Control | OLE |
| *L. monocytogenes* | $1.48^{a} \pm 0.14$ | $1.18^{b} \pm 0.14$ |
| *E. coli* | $2.31^{a} \pm 0.19$ | $1.75^{b} \pm 0.19$ |
| *Staph. aureus* | $2.42^{a} \pm 0.08$ | $1.80^{b} \pm 0.09$ |
| *En. faecium* | $2.54^{a} \pm 0.21$ | $1.50^{b} \pm 0.16$ |
| *S. enterica* | $3.56^{a} \pm 0.18$ | $2.88^{b} \pm 0.07$ |
| *Lc. mesenteroides* | $1.36^{a} \pm 0.14$ | $1.14^{b} \pm 0.10$ |
| *Ps. fluorescens* | $1.78^{a} \pm 0.19$ | $1.33^{b} \pm 0.24$ |

[a-b] For the same microorganism, means within the same line followed by different letters are significantly different ($p < 0.05$).

inhibitory effect of OLE.

OLE aqueous extract was also evaluated for its antifungal activity against *Penicillium roqueforti*. To this aim, since molds do not grow uniformly in liquid media, the disk diffusion assay was used. After the incubation period, no inhibition zones were detected around the OLE-loaded disk, indicating an absence of antifungal activity. This result contrasts with the findings of Korukluoglu et al. (2008), who reported significant efficacy of OLE against several *Penicillium* species, including *P. roqueforti*, using the same method. This discrepancy may arise from differences in OLE extraction methodologies. Those authors, indeed, utilized Soxhlet extraction with organic solvents (*i.e.*, methanol, acetone, or diethyl ether), dissolving the crude extract in methanol for testing, whereas in the present study, an aqueous OLE extract was used. These different approaches probably led to OLEs with different compositions and concentrations of bioactive compounds. In addition, the lack of inhibition observed for *P. roqueforti* in the disk diffusion assay also suggests that the local OLE concentration along the diffusion gradient remained below the inhibitory threshold for this mold. This tolerance may be attributed to fungal-specific cell wall and membrane features (β-glucan/chitin-based wall architecture and associated remodeling), together with inducible tolerance mechanisms (cell-wall integrity signaling, stress-response pathways and efflux transporters) (Brown et al., 2014; Coleman & Mylonakis, 2009).

### 3.2. Antimicrobial activity of OLE volatiles

In the second part of the study, the antimicrobial activity of OLE volatiles was explored. To this aim, a vapor diffusion assay was used: OLE was spread on the inner surface of the lid used to cover the Petri

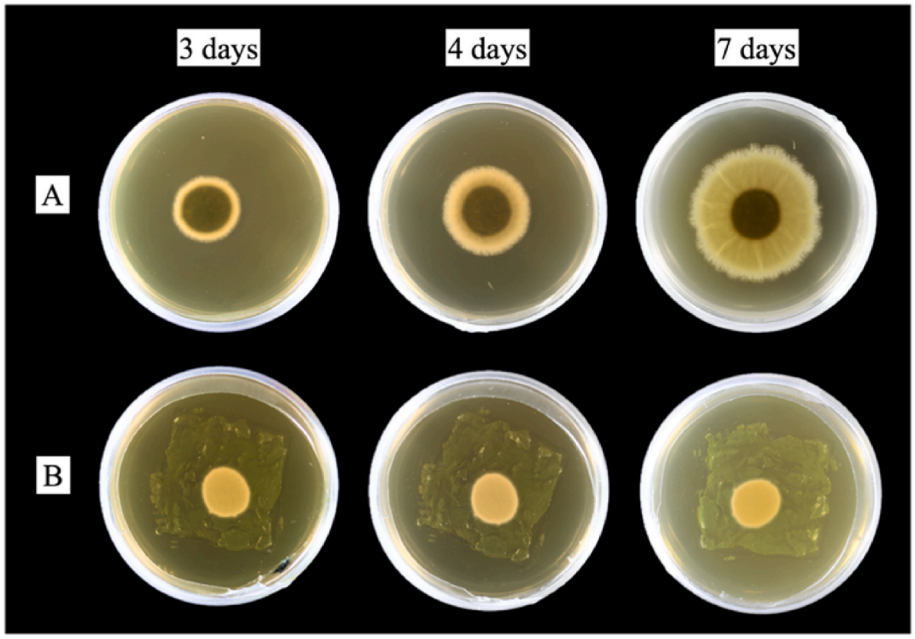


**Fig. 3.** Mycelial expansion of *Penicillium roqueforti* over time in the absence (A) and in the presence (B) of olive leaf extract in the vapor diffusion assay.

dishes holding the inoculated growth media. This assay was used against bacterial pathogens and spoilers, as well as *P. roqueforti*. Fig. 2 shows an example of the antibacterial effect of OLE evaluated with the vapor diffusion assay against *Ps. fluorescens*.

As shown in Fig. 2A, colonies grown in the absence of OLE appeared larger than those exposed to OLE (Fig. 2B). Similar results were also observed for all the other tested microorganisms, indicating that the OLE vapor phase exerted antibacterial activity. Notably, different from what was observed by the addition of OLE aqueous extract into the growth media (Table 1), a more pronounced growth inhibition also against *Ps. fluorescens* was detected in the vapor diffusion assay (Fig. 2). To quantify the differences between treated and untreated colonies, diameters were estimated for each tested microorganism using image analysis, and the results are shown in Table 2.

A significant reduction in colony size was observed for each bacterial target, highlighting the broad-spectrum antibacterial efficacy of the OLE volatile fraction. Strikingly, the OLE volatiles were also found to greatly reduce the mycelial expansion of *Penicillium roqueforti* (Fig. 3). Indeed, in the absence of OLE (Fig. 3A), the fungus grew over time, reaching a diameter of $1.95 \pm 0.52$ cm, $2.16 \pm 0.80$ cm, and $3.75 \pm 1.28$ cm at 3, 4, 7 days of incubation, respectively. In contrast, the OLE-exposed fungus (Fig. 3B) demonstrated a substantially diminished growth pattern, exhibiting a surface area that remained virtually constant over time, ranging from $1.40 \pm 0.62$ cm to $1.45 \pm 0.54$ cm at 3 and 7 days of incubation. This result is particularly noteworthy compared with the results obtained from the disk diffusion assay, in which no inhibition zone was observed for the OLE aqueous extract against *Penicillium roqueforti*.

### 3.3. On the broad-spectrum antimicrobial effect of OLE

The results discussed in paragraphs 3.1 and 3.2 indicate a broad-spectrum antimicrobial activity of OLE, mediated by both water-soluble and volatile compounds. In this regard, OLE is a chemically complex mixture of several bioactive constituents; therefore, the observed inhibitory patterns should be interpreted as the combined outcome of the presence of multiple compounds rather than being attributable to a single molecule.

With reference to the OLE aqueous fraction, the observed antibacterial activity against both Gram-positive and Gram-negative bacteria indicates a potential broad-spectrum efficacy (Table 1). These results are highly promising, as the species tested in this study are among the primary sources of foodborne diseases both in Europe and globally (EFSA, 2024; Mather et al., 2024). Our data are consistent with the findings of Markín et al. (2003) and Pereira et al. (2007), who attributed OLE wide-range antibacterial activity to its abundance in polyphenolic compounds (*e.g.*, oleuropein, flavonoids). The latter are well known to exhibit an intense effect in inhibiting microbial growth, by multiple mechanisms, including disassembling of cell membranes, inhibition of key microbial enzymes, and interfering with biofilm formation and expression of specific virulence factors (Di Pasqua et al., 2006; Gonelimali et al., 2018; Lee & Lee, 2010; Rajendran et al., 2015; Wu et al., 2013). In this regard, the OLE aqueous extract used in this study presented a total phenolic content of $139.95 \pm 2.50$ mg GAE/$g_{DW}$, a value substantially higher than those reported in the literature, which typically range from 20 to 70 mg GAE/$g_{DW}$ (Debib & Boukhatem, 2017; Giacometti et al., 2021). Such a difference is likely attributable to the higher extraction efficiency of SC-$CO_2$ over conventional methods (Bimakr & Ganjloo, 2016).

Moreover, SC-$CO_2$ extraction may yield additional non-phenolic constituents that may produce additive or synergistic inhibitory effects with polyphenols (Hyldgaard et al., 2012; Zhang & Wu, 2025). For instance, SC-$CO_2$ extraction has been shown to efficiently isolate diverse phytochemical classes from plant matrices (*e.g.*, alcohols, aldehydes, ketones, esters, and organic acids), many of which are volatile compounds with antimicrobial activity (Moreira et al., 2023). They readily partition into the headspace, reaching microbial surfaces under vapor

**Table 3**
List of detected volatile compounds characterizing the headspace in the presence of olive leaf extract by means of HS-SPME-GC-MS. Literature references are provided for compounds with reported antimicrobial activity.

| Retention time (min) | Compound | Class | Reference |
|---|---|---|---|
| 7.206 | Hexanal ◆ | Aldehyde | Lanciotti et al. (2003) |
| 9.258 | Pentanoic acid, ethyl ester | Ester | |
| 10.356 | 1-Penten-3-ol | Alcohol | |
| 10.967 | Heptanal | Aldehyde | |
| 11.403 | 4-Heptanone, 2,6-dimethyl- | Ketone | |
| 12.972 | Hexanoic acid, ethyl ester ◆ | Ester | Mayser (2015) |
| 13.104 | Hexane, 1,1-diethoxy- | Ether | |
| 13.360 | Octane, 1-chloro- | Alkane | |
| 13.751 | 1-Pentanol ◆ | Alcohol | Li et al. (2022) |
| 14.074 | Propanoic acid, 2-oxo-, ethyl ester | Ester | |
| 14.530 | Hex-5-enoic acid, ethyl ester | Ester | |
| 14.827 | Octanal ◆ | Aldehyde | Liu et al. (2012) |
| 15.375 | 3-Hexenoic acid, ethyl ester | Ester | |
| 15.948 | 2-Heptenal ◆ | Aldehyde | Kubo et al. (1995) |
| 16.208 | 2-Penten-1-ol | Alcohol | |
| 16.574 | Heptanoic acid, ethyl ester | Ester | |
| 18.509 | Nonanal ◆ | Aldehyde | Bisignano et al. (2001) |
| 18.930 | 3-Octen-2-one | Ketone | |
| 20.059 | Octanoic acid, ethyl ester ◆ | Ester | Mayser (2015) |
| 20.256 | Acetic acid | Carboxylic acid | |
| 20.573 | Furfural◆ | Furan derivative (Aldehyde) | Chai et al. (2013) |
| 21.656 | 7-Octenoic acid, ethyl ester | Ester | |
| 21.904 | Formic acid ◆ | Carboxylic acid | Ricke et al. (2020) |
| 22.360 | Benzaldehyde ◆ | Aromatic Aldehyde | Ramos-Nino et al. (1998) |
| 23.190 | Propanoic acid | Carboxylic acid | |
| 23.313 | Nonanoic acid, ethyl ester | Ester | |
| 24.102 | 1-Octanol | Alcohol | |
| 24.482 | Diethyl malonate | Ester | |
| 24.645 | 2,3-Butanediol ◆ | Alcohol | Antoce et al. (1998) |
| 24.772 | 6-Methyl-3,5-heptadiene-2-one | Ketone (Diene) | |
| 25.350 | Pentanoic acid, 4-oxo-, ethyl ester | Ester | |
| 25.510 | Butyrolactone | Lactone | |
| 25.953 | Butanoic acid ◆ | Carboxylic acid | Kennedy et al. (2019) |
| 26.408 | Decanoic acid, ethyl ester ◆ | Ester | Mayser (2015) |
| 26.850 | Benzoic acid, ethyl ester | Ester | |
| 27.207 | Butanoic acid, 2-methyl- | Carboxylic acid | |
| 27.398 | Butanedioic acid, diethyl ester | Ester | |
| 28.491 | 2(3H)-Furanone, 5-ethyldihydro- | Furanone | |
| 29.124 | Pentanoic acid | Carboxylic acid | |
| 32.074 | Hexanoic acid | Carboxylic acid | Mayser (2015) |
| 32.318 | 5,9-Undecadien-2-one, 6,10-dimethyl- | Ketone (Diene) | |
| 32.759 | Benzyl alcohol ◆ | Alcohol | Lucchini et al. (1990) |
| 33.675 | Phenylethyl Alcohol ◆ | Alcohol | Fraud et al. (2003) |
| 35.168 | 2-Hexenoic acid | Carboxylic acid | |
| 37.532 | Octanoic acid ◆ | Carboxylic acid | Mayser (2015) |
| 40.078 | Nonanoic acid | Carboxylic acid | |
| 42.212 | Hexadecanoic acid, ethyl ester | Ester | |
| 42.500 | n-Decanoic acid | Carboxylic acid | |

(*continued on next page*)

**Table 3** (*continued*)

| Retention time (min) | Compound | Class | Reference |
|---|---|---|---|
| 43.630 | 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl | Furanone derivative | |
| 44.971 | Diethyl Phthalate | Ester (Phthalate) | |

The ◆ adjacent to the compound name denotes compounds with validated antimicrobial activity.

conditions and generally exert their antimicrobial effect through interactions with cellular membranes and associated stress responses.

To the best of our knowledge, no data is currently available on the composition of the volatile fraction of OLE obtained through SC-$CO_2$ extraction. Therefore, the OLE volatile profile was analysed using HS-SPME-GC-MS, and the results are listed in Table 3.

Results clearly indicated that OLE was rich in volatile compounds belonging to different classes. In particular, 50 compounds were identified, of which esters (16 compounds) were the most abundant, followed by carboxylic acids (11 compounds), alcohols (7 compounds), aldehydes (7 compounds), ketones (4 compounds), and other minor components. This abundance can be attributed to the efficiency of SC-$CO_2$ in extracting plant constituents, while minimizing their degradation, as the process operates at relatively low temperatures (*e.g.*, 80 °C) using a solvent with amphiphilic properties (Arumugham et al., 2021; Pourmortazavi et al., 2019). The high number of volatiles reported in Table 3 stands in stark contrast to the findings reported by Keskin et al. (2012). These authors identified only 15 components in OLE extracted *via* Soxhlet using water as solvent. The Soxhlet extraction method involves prolonged exposure to high temperatures (*i.e.*, 4 h) with polar solvents (*i.e.*, water), which likely degrade thermolabile compounds and reduce extraction efficiency. Many of the volatiles reported in Table 3 have been demonstrated to exhibit significant antimicrobial activity. Many of these volatiles are indeed known to exert an inhibitory effect since they interfere with membrane integrity and cellular homeostasis (Dorman & Deans, 2000). Several alcohols, including 1-pentanol, 1-octanol, and benzyl alcohol, have been described as having moderate to strong antibacterial and antifungal activity by inducing membrane fluidization, perturbation or damage, as well as protein denaturation (Qin et al., 2022; Yano et al., 2016). Aldehydes, such as hexanal and hexenal, can exert significant activity against microorganisms by disassembling the cell membrane and denaturing proteins and enzymes. Moreover, they can induce oxidative stress, which leads to cellular damage and death (Duan et al., 2023; Patrignani et al., 2008). Similar effects have been also attributed to carboxylic acids and esters (Habib & Karim, 2009; Ng et al., 2023).

It must also be considered that interactions between OLE water-soluble polyphenols and volatile compounds may influence the overall antimicrobial response. On the one hand, antagonistic effects cannot be excluded in such a complex mixture, as interactions between water-soluble and volatile compounds may influence partitioning phenomena, possibly reducing the freely bioavailable fraction of individual constituents. On the other, the overall antimicrobial outcome may reflect additive or synergistic effects between phenolics and volatiles, depending on the microorganism and exposure layout (Li et al., 2021; Ye et al., 2013). Based on these considerations, further targeted investigations are needed to assign the observed effects to specific molecules or compound classes.

### 3.4. Mineral oil contamination of OLE

#### 3.4.1. Analytical challenges and method adaptation

Saturated and aromatic hydrocarbons (MOSH and MOAH) analysis consisted of OLE enrichment by microwave-assisted saponification (MAS), purification, and analytical determination *via* on-line HPLC-GC-FID. Sample purification included epoxidation for the MOAH fraction and *n*-alkane removal for the MOSH fraction.

At first instance, the protocol adopted by Menegoz Ursol et al. (2025) for high-sensitivity mineral oil hydrocarbons (MOH) determination in olive oil was applied. According to this protocol, 150 μL of the saponified/epoxidized OLE was loaded (in a total volume of 500 μL of

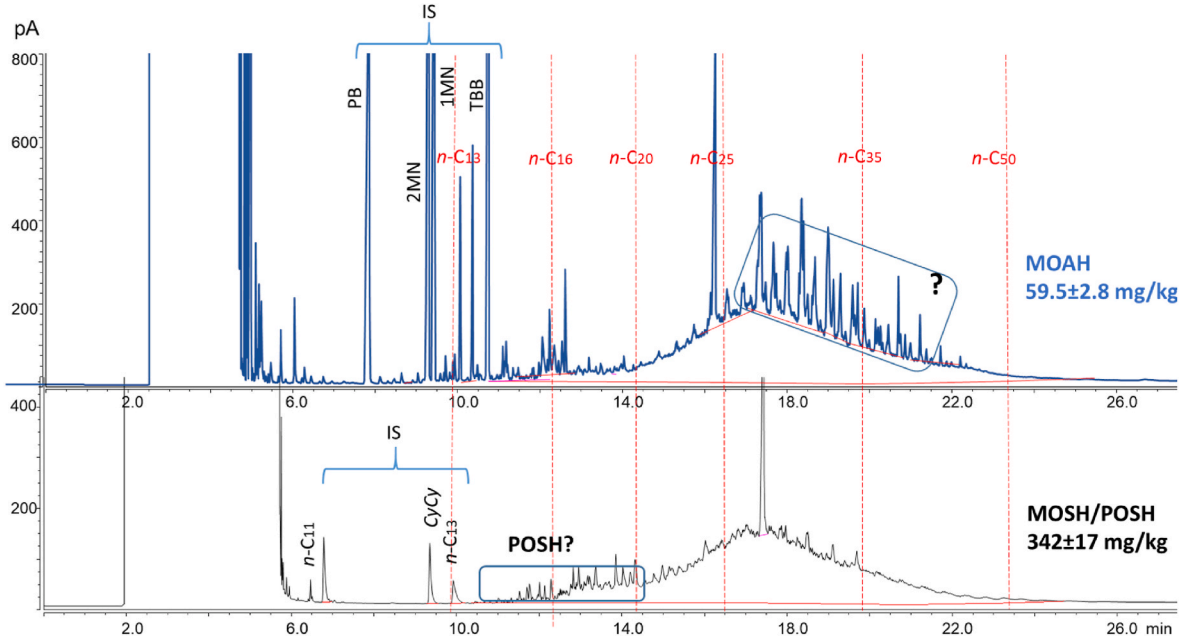


**Fig. 4.** HPLC-GC-FID chromatograms of the MOSH/POSH (bottom) and MOAH (top) fractions of OLE. Retention times of *n*-alkanes from *n*-$C_{13}$ to *n*-$C_{50}$ are indicated to define the molecular weight distribution of the unresolved hydrocarbon humps. The MOSH chromatogram includes contributions from polyolefin oligomeric hydrocarbons (POSH). The MOAH chromatogram displays a clearly detectable aromatic fraction (approximately 15% of the MOSH level), characterized by isolated peaks superimposed on the unresolved hump. Internal standards (IS) were added for quantitative purposes and to verify correct fractionation between MOSH and MOAH. PB, pentyl benzene; 2 MN, 2-methyl naphthalene; 1 MN, 1-methyl naphthalene; TBB, 1,3,5-tri-tert-butyl benzene; CyCy, cyclohexyl cyclohexane.

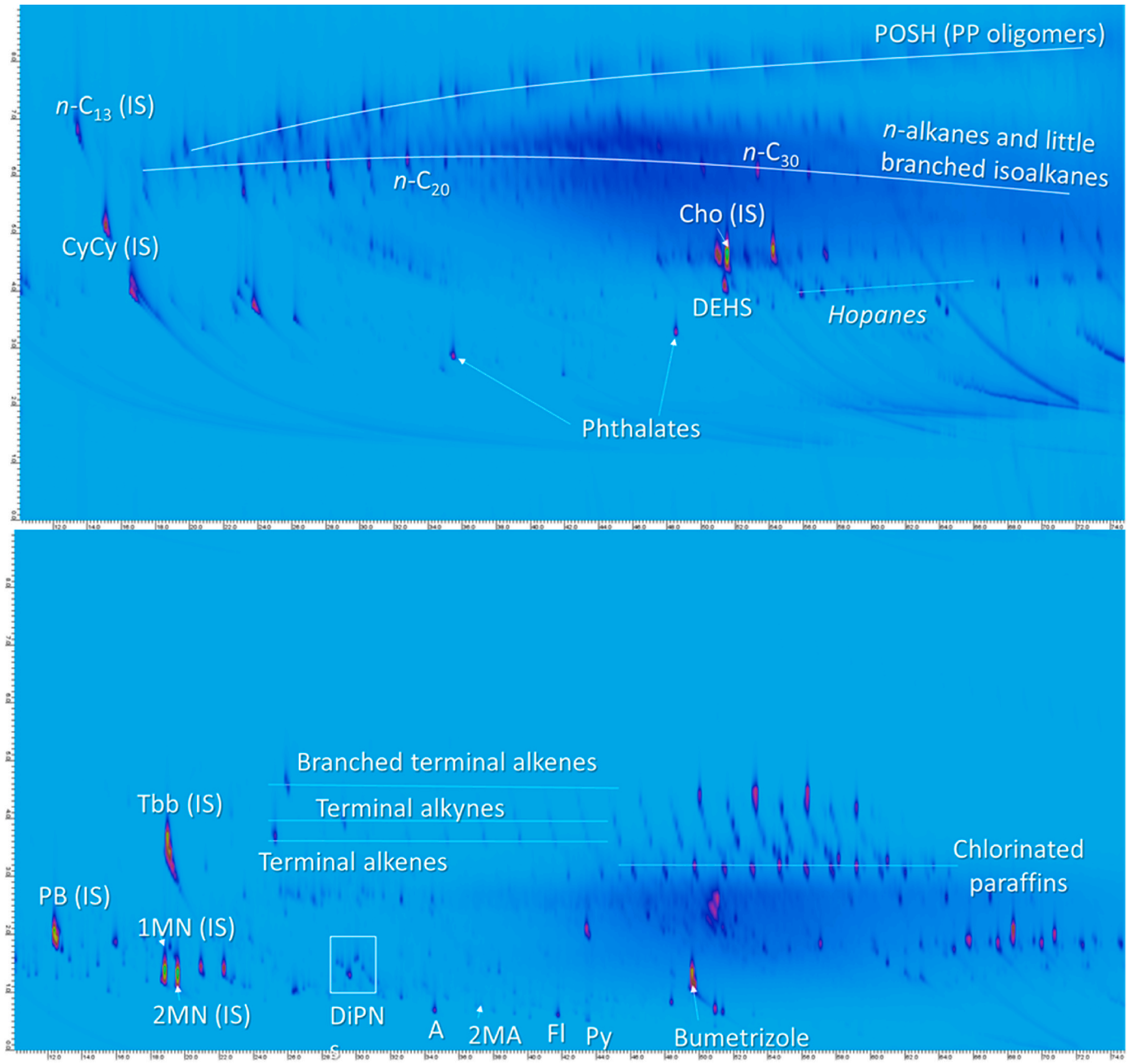


**Fig. 5.** GC × GC-FID plots of the MOSH (Top) and MOAH (Bottom) fractions obtained after HPLC pre-separation of the OLE extract; the MOAH fraction was analysed after epoxidation to remove olefins. In the MOSH plot (top), the presence of polyolefin oligomeric hydrocarbons (POSH), indicative of synthetic hydrocarbon contamination (*e.g.*, polypropylene), is clearly visible, together with typical *n*-alkanes and little branched iso-alkanes. Plasticizers, including decanedioic acid bis(2-ethylhexyl) ester (DEHS), are also indicated. The MOAH plot (bottom) highlights diisopropylnaphthalenes (DiPNs), chlorinated paraffins, and polycyclic aromatic hydrocarbons such as anthracene (A), 2-methyl anthracene (2 MA), fluoranthene (Fl), and pyrene (Py). Internal standards (IS) were added before analysis for quantitative purposes and to verify the correct fractionation between MOSH and MOAH. CyCy, cyclohexyl cyclohexane; Cho, cholestane; PB, pentyl benzene; TBB, 1,3,5-tri-tert-butylbenzene; 1 MN, 1-methyl naphthalene; 2 MN, 2-methyl naphthalene.

*n*-hexane) on a 6-mL solid phase extraction cartridge (10 mm i.d.) filled with 2.5 g of aluminum oxide activated at 500 °C overnight and topped by 0.5 g of sodium sulphate, previously conditioned with 5 mL of *n*-hexane. The MOSH fraction was then eluted with 5 mL of *n*-hexane and concentrated to the original volume, before injection (100 μL) into the LC-GC-FID apparatus. Due to the large amounts of endogenous *n*-alkanes, overloading the MOSH fraction still occurred under these conditions. To address this issue, it was necessary to double the amount of aluminum oxide (as well as conditioning and elution solvent volumes) and reduce the volume of the loaded sample extract to 20 μL.

### 3.4.2. *Quantification of MOSH and MOAH*

Fig. 4 shows the LC-GC-FID chromatograms of the MOSH and MOAH fractions, reporting average values from three replicate analyses and the corresponding standard deviations. Since the presence of polyolefin oligomeric hydrocarbons (POSH) was suspected, the data of the MOSH fraction were expressed as MOSH/POSH.

OLE showed very high MOSH *n*-$C_{10\text{-}50}$ (342 ± 17 mg/kg), accompanied by 15% of MOAH (59.5 ± 2.8 mg/kg) in the same molecular weight interval. Since no data on MOH contamination in olive leaf extracts are currently available, the extent of contamination in OLE was

evaluated by comparison with levels typically reported for vegetable products. The MOSH and MOAH concentrations detected in OLE were markedly higher than those reported for vegetable products included in the European Food Safety Authority (EFSA) exposure assessment, where MOSH concentrations generally ranged between 0.71 and 1.21 mg/kg, and MOAH were not detected or were below the limit of quantification (EFSA, 2023).

The MOSH profile after treatment with aluminum oxide (to eliminate interference by endogenous alkanes) showed a large hump covering the molecular weight range from *n*-$C_{14}$ to beyond *n*-$C_{50}$ (centered on *n*-$C_{28}$). The MOAH profile largely mirrored the MOSH distribution but was characterized by the atypical presence of clusters of isolated peaks in the *n*-$C_{28\text{-}50}$ retention time interval, which were excluded from MOAH quantification. The presence of a measurable MOAH fraction is of particular concern, as this fraction may include genotoxic and carcinogenic compounds (EFSA, 2023).

### 3.4.3. Characterization of contaminants

As shown in Fig. 5 (top), the GC × GC-FID analysis of the MOSH fraction obtained after HPLC pre-separation clearly confirmed the presence of POSH.

Due to co-elution and signal overlap with MOSH, POSH are difficult to identify in one-dimensional GC traces. However, the enhanced separation power of comprehensive two-dimensional gas chromatography enables their clear recognition and characterization (Biedermann & Grob, 2015). Below the POSH region, typical *n*-alkanes and little branched iso-alkanes were observed. The presence of hopanes, triterpene biomarkers of petroleum origin, was confirmed by the characteristic ions at *m/z* 191 and 205, further supporting MOSH. Additionally, various plasticizers, including phthalates and decanedioic acid bis (2-ethylhexyl) ester (DEHS), were detected.

The GC × GC-FID plot of the MOAH fraction (Fig. 5, bottom) was obtained after HPLC pre-separation of the epoxidized extract to remove olefins. This analysis highlighted the presence of mono-aromatic hydrocarbons (characteristic ions *m/z* 91, 105, 119), as well as long-chain chlorinated paraffins (CPs) containing more than 24 carbon atoms, eluting in the same retention time interval as the atypical, non-identified peaks observed in the MOAH fraction of Fig. 4.

Low-chain and medium-chain CPs (up to *n*-$C_{17}$) have been reported in human tissues, biological fluids, and breast milk (Chen et al., 2022), while long-chain CPs have been detected in vegetable oils (Biedermann et al., 2020). These compounds are widely used as plasticizers, flame retardants, and additives in industrial applications, and are environmentally persistent, bioaccumulative, and toxic to animals and humans.

In addition, extracted-ion chromatograms (*m/z* 212, 197, and 155) revealed the presence of diisopropylnaphthalenes (DiPNs), which are typical markers of recycled cardboard contamination. The UV stabilizer bumetrizole, commonly used in plastics and coatings, was also detected.

In the central region of the GC × GC plot, zones corresponding to terminal alkenes were observed, followed by terminal alkynes and branched terminal alkenes. These compounds appear particularly resistant to epoxidation, which may explain their persistence in the MOAH fraction. In the lower region of the plot, several individual polycyclic aromatic hydrocarbons (PAH), including anthracene, 2-methyl anthracene, fluoranthene, and pyrene, were detected. The occurrence of PAH with three or more aromatic rings and a low degree of alkylation is of toxicological concern, as these compounds are considered among the most hazardous components of the MOAH fraction due to their genotoxic and carcinogenic potential (EFSA, 2023).

### 3.4.4. Potential sources of contamination

MOSH and MOAH contamination of OLE may have occurred at different stages of the production chain, including harvesting, storage, and processing of the leaves. Although a contribution from the SC-$CO_2$ extraction process itself cannot be entirely excluded, the analytical evidence presented in this study suggests that contamination is more likely associated with upstream and downstream steps of the production chain rather than with the extraction technology alone. These steps include harvesting operations (*e.g.*, contact with lubricants), storage, and processing (*e.g.*, contact with plastic materials). Similar sources of MOH contamination have previously been reported for olives and other plant-derived matrices, regardless of the extraction method applied. A systematic comparison between SC-$CO_2$ extracts and extracts obtained using alternative extraction technologies would be valuable to better understand the role of the extraction process in MOH contamination.

Contact with lubricants during harvesting operations represents a plausible source of petrogenic hydrocarbons, as previously reported for olives (Menegoz Ursol et al., 2023). In addition, the presence of POSH, plasticizers, CPs, DiPNs, and UV stabilizers suggests further contributions from synthetic materials, likely arising from contact with plastic packaging, storage containers, or processing equipment.

Although it was not possible to monitor contamination at individual processing steps, the combined HPLC–GC–FID and GC × GC–FID/MS analyses provide evidence for the presence of multiple critical points along the OLE processing chain. These findings indicate that targeted mitigation strategies are required to prevent or minimize contamination with mineral oils and other hydrocarbons of synthetic origin, particularly the MOAH fraction, before considering OLE for food-related applications.

## 4. Conclusions

This study demonstrates that olive leaf extract (OLE) obtained *via* supercritical $CO_2$ extraction exhibits strong broad-spectrum antimicrobial activity against both bacteria and fungi, with efficacy observed under both contact and vapor-phase conditions. This efficacy likely results from the combined presence of water-soluble polyphenols and bioactive volatile compounds. In the context of food applications, OLE can be thus regarded as a plant-based antimicrobial ingredient, alternative to synthetic additives, with potential of integration into innovative active packaging solutions.

To enhance comparability across studies and support practical applications, further investigations should include standardized determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values to complement kinetic analyses, facilitating dose selection for challenge tests in real food matrices. Moreover, further targeted investigations are needed to assign the observed antimicrobial effects to specific molecules or compound classes.

Additionally, this work provides the first report of mineral oil hydrocarbons (MOH) in OLE, highlighting a previously overlooked safety concern in olive leaf valorization. Such contamination underscores the need for improvements in OLE production, aiming at prioritizing contamination prevention. This can be obtained by minimizing contact with lubricants during harvesting and avoiding plastic materials during OLE handling and storage. An optimization of the extraction process is also recommended to maximize bioactivity while minimizing undesired residues. Application of OLE as an antimicrobial ingredient in food systems will therefore depend on the ability to control sources of contamination throughout the chain, all the way from cultivation interventions to leaf management to extract production.

Finally, since OLE possesses distinct color and strong flavor, particular attention should be paid to its impact on sensory properties and consumer acceptability of the systems in which it is incorporated.

## CRediT authorship contribution statement

**Alberto Saitta:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Marco Lopriore:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Marilena Marino:** Writing – review & editing, Methodology. **Laura Barp:** Writing – review & editing, Investigation,

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Data curation. **Chiara Conchione:** Writing – review & editing, Formal analysis. **Sabrina Moret:** Writing – review & editing, Writing – original draft, Methodology. **Stella Plazzotta:** Writing – review & editing, Writing – original draft. **Cristina Cejudo Bastante:** Writing – review & editing, Investigation. **Lara Manzocco:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Funding acquisition, Conceptualization.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2026.119306.

## Data availability

Data will be made available on request.

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