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Antifungal activity of *Lactiplantibacillus plantarum* isolated from fruit and vegetables and detection of novel antifungal VOCs from fungal-LAB co-cultures

Nicola De Simone [a,b], Lucía López [c], Carmen S. Ciudad [c], Angela Scauro [a], Pasquale Russo [d], Jorge Rodríguez [c], Giuseppe Spano [a,*], Beatriz Martínez [c,**]

[a] Department of Agriculture Food Natural Science Engineering (DAFNE), University of Foggia, Foggia, Italy
[b] Institute of Sciences of Food Production, National Research Council (CNR), C/o CS-DAT, Foggia, Italy
[c] Instituto de Productos Lacteos de Asturias, CSIC, Villaviciosa, Asturias, Spain
[d] Department of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy

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ABSTRACT

Undesired microbiota, such as filamentous fungi, negatively impacts the food sector leading to huge losses of food produce. For this reason, preservatives are often used to reduce microbial contamination. In this context, the use of food-grade antagonistic microorganisms has gained international relevance as a promising alternative to synthetic additives. Lactic acid bacteria (LAB) with antifungal activity might have the strain-specific ability to control decay agents and their application as bio-control tools is growing worldwide. In this work, eight *Lactiplantibacillus plantarum* strains were selected and their antifungal activity against *Aspergillus niger*, one of the main fungal contaminants of foods, were deeply investigated. The overlay method was used for an initial fast screening, then the presence of antifungal metabolites was evaluated in the cell-free supernatants (CFSs) by HPLC/DAD and in the volatilome by GC–MS analysis. Contribution of volatile organic compounds (VOCs) to antifungal activity was assessed in a plate-on-plate method without physical contact between moulds and LAB. In order to determine the presence of inducible antifungal VOCs, fungal-LAB co-cultures were carried out in glass tubes for direct GC-MS analyses. Antifungal VOCs, such as acetic acid, 2-Nonanone and 2-Undecanone are produced constitutively by all strains. Whereas fourteen VOCs appeared only in the fungal-LAB co-cultures. Six VOCs out of fourteen identified in co-cultures, and the three VOCs previously mentioned were individually tested for antifungal activity against *A. niger* in airtight contact-less petri dishes assay. Among those, *trans*-2-Octenal and 2-Nonanol had the higher activity against *A. niger*. These results showed that antifungal VOCs produced by *L. plantarum* could effectively inhibit the growth of *A. niger* when tested *in vitro*. In addition, different antifungal VOCs were detected only in fungal-LAB co-cultures, pointing to metabolism modulation mediated by VOCs that results in an enhanced antifungal activity.

1. Introduction

In the agro-food sector, undesired microbiota, including filamentous fungi, are responsible for quality deterioration and decay of food commodities, leading to lower yield, reduction of shelf-life and merchantability (De Simone et al., 2020; 2021a). In addition, fungal contaminations can result in the production of toxic compounds, such as mycotoxins. The use of preventive treatments and hurdle technologies aims to reduce or limit microbial contamination in order to ensure food safety. A common safety measure to prevent fungal deterioration and lengthen the shelf-life of food products is bio-preservation. This method, which is gaining more attention in the last years, is based on the use of protective cultures and/or their metabolites.

Lactic acid bacteria (LAB) are an important group of Gram-positive, usually non-sporulating, facultative anaerobic bacteria associated to soil, plants, several types of food and within the gastro-intestinal tract of

* Corresponding author.
** Corresponding author.
E-mail addresses: nicola.desimone@unifg.it (N. De Simone), llopezfe@ipla.csic.es (L. López), carmen.santos@ipla.csic.es (C.S. Ciudad), angela.scauro@unifg.it (A. Scauro), pasquale.russo1@unimi.it (P. Russo), jorge.rodriguez@ipla.csic.es (J. Rodríguez), giuseppe.spano@unifg.it (G. Spano), bmf1@ipla.csic.es (B. Martínez).

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humans and animals. Alongside to being widely used in food fermentation, where they improve the nutritional and organoleptic qualities, LAB have also been intensively explored for their potential to produce functional foods (Quinto et al., 2014). In fact, they are also associated to beneficial roles for human health, to promote host gut functions and several strains have been claimed as probiotics (Vinderola, Ouwehand, Salminen, & von Wright, 2019). They are authorized for use in food production and as feed additives, and many are included in the QPS and GRAS lists (Koutsoumanis et al., 2022).

Since LAB can be deliberately added to food, in recent years several studies have explored the employment of LAB with antimicrobial activity as bio-preservatives against the growth of spoilage fungi in foods (Ribes, Fuentes, Talens, & Barat, 2018). In fact, antifungal LAB have the strain-specific ability to control decay agents by competing for resources or by producing antifungal metabolites (Siedler, Balti, & Neves, 2019). Metabolites of LAB have been subjected to extensive research in the last years, and many substances have been labeled as antifungal over time (Crowley, Mahony, & van Sinderen, 2013; Honoré et al., 2016; Schwenninger, Meile, & Lacroix, 2011). Organic acids and their derivates, including lactic acid, acetic acid, propionic acid and diacetyl are the main antifungal metabolites produced by LAB, contributing to lowering the pH of the substrate and to create an unsuitable environment for other microorganisms (Singhvi, Zendo, & Sonomoto, 2018). However, these compounds are also flavor volatiles and their flavor threshold concentration is lower than the active concentration against fungi (Siedler et al., 2019), thus their use could impair the sensorial attribute of the foods. Besides organic acids, several other secondary metabolites with antifungal activity, such as peptides (Atanassova et al., 2003; Magnusson & Schnürer, 2001; Ström, Sjögren, Broberg, & Schnürer, 2002), 3-hydroxy fatty acids (Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003), and volatile organic compounds (Kadyan & Pradhan, 2020; Li et al., 2022), have been found in LAB. Antifungal metabolites are of particular interest also because they could be produced in bio-factories and could be used instead of the living microbes thus avoiding the side-effects of LAB catabolism such as acidification, off-flavor, etc. However, although these substances have been confirmed to exhibit activity, the level they produce in biological systems is considerably lower than the minimum inhibitory concentration (MIC). Interestingly, metabolite combinations may boost LAB's antifungal activity, which may be evidence of metabolite synergy (Kadyan & Pradhan, 2020). However, despite research on the antifungal substances produced by LAB has been considerable, little is known about the synergy and mode of action during fermentation (Salas et al., 2017). For these reasons, a focus on the exometabolome, defined as the spectrum of extracellular metabolites, including residual nutrients, may reveal novel findings on the antifungal abilities of LAB and on the synergies between metabolites. In addition, several studies have underlined that metabolism modulation occur when the strains are co-cultured in presence of other microbes (Ström, Schnürer, & Melin, 2005). To this aim, untargeted metabolomics is often used to compare the metabolomes of the test and control groups to find differences in the metabolic profiles. These metabolomic variations might be important for particular biological circumstances.

The present study aims to investigate the exometabolic profiles of eight *L. plantarum* strains selected for their antifungal activity against *Aspergillus niger*. To this aim, different methods were used to assess the antifungal potential of the strains using living bacteria, cell-free supernatants and volatile compounds. Then, the metabolic profiles of two selected strains were compared when the strains are co-cultured or not with the target fungus, in order to underline metabolism modulation through the production of antifungal volatile compounds.

2. Materials and methods

2.1. Microbial strains and growth conditions

Lactiplantibacillus plantarum strains previously isolated from various plant-based matrices and cryopreserved at the Laboratory of Industrial Microbiology of the University of Foggia, were routinely cultured at 30 °C in MRS broth (Biolife Italiana, Milano, Italy) from cryopreserved stock in MRS supplemented with 25% glycerol (Sigma-Aldrich, St Louis, MO, USA), and plated on MRS agar (1.5% agar). *Aspergillus niger* CECT 2805, purchased from the Spanish Type Culture Collection (CECT, Paterna, Spain), was used as target strain in the antagonism assays. Cryopreserved culture was plated on Potato Dextrose Agar (PDA, Oxoid, Basingstoke, UK), and incubated at 25 °C for 5 days. Fungal spore suspensions were prepared by brushing the plates surface with sterile 0.86% NaCl solution (Sigma-Aldrich) supplemented with 0.01% Tween 80 (Sigma-Aldrich) using a sterile swab, and stored at 4 °C for short-term uses. Fungal spore concentration was determined by plating serial dilutions on PDA plates and adjusted to approximately 1×10^6 spores/mL.

2.2. Antifungal activity against Aspergillus niger CECT 2805

2.2.1. Screening

The overlay method was used to screen the antifungal activity of LAB strains as previously reported (Russo, Arena, et al., 2017). In summary, 5 μL of bacterial cultures in the middle exponential phase were placed in spots on MRS agar plates. After 24 h of incubation at 30 °C, the plates were covered with a second layer of media (15 mL) of Malt Extract soft agar (0.75% agar, Oxoid) supplemented (1/100 *v/v*) with the fungal spore suspension prepared as above. After 2 days of incubation at 25 °C, the strains were discriminated by the occurrence of the inhibition halos surrounding the bacterial colonies, and classified as having no (−), mild (+), or strong (++) inhibition, for zones of inhibition lesser than 1 mm, between 1 and 3 mm or greater than 3 mm, respectively (Russo, Arena, et al., 2017).

2.2.2. Hyphal radial growth inhibition

In order to determine the antifungal activity present in cell-free supernatants (CFSs), *L. plantarum* strains were grown in MRS for 24 and 48 h at 30 °C. After centrifugation and filtration (0.22 μm pore-filter), an aliquot of the CFSs was neutralized (pH 7.0). The antimicrobial activity was assayed by radial growth inhibition, as described by De Simone, Capozzi, de Chiara, et al. (2021). Briefly, CFSs were added to melted PDA at 10% (v/v). After solidification, 10 μL of fungal suspension at 1×10^4 spores/mL were spotted at the center of the plate. After five days at 25 °C, the diameter of the colony was measured. Then, the inhibition percentage was calculated using the following formula:

$$RGI(\%) = \frac{100 \times (RG_c - RG_t)}{RG_c}$$

Where, RGI% = inhibition percentage of fungal radial growth, RG_c = average radial growth in control plates and RG_t = average radial growth in test plates.

2.2.3. Plate on plate assay

Contribution of volatiles to antifungal activity was assessed in a "plate-on-plate" test system without direct contact between moulds and *L. plantarum* strains, as described by Aunsbjerg et al. (2015), with minor modifications. PDA plates were spotted with 10 μL of *A. niger* spore dilution (10^4 spores/mL), whereas 100 μL of overnight cultures of *L. plantarum* diluted 1/10 in saline solution were spread on the surface of MRS agar plates. After drying, the plates were sealed together with Parafilm® (Heathrow Scientific, Vernon Hills, IL, USA). After 5 days of incubation at 25 °C, the inhibitory activity was calculated by comparing the growth of *A. niger* on double plates set uninoculated with

L. plantarum. RGI (%) was calculated as stated above.

2.3. Analysis of carbohydrates and organic acids by HPLC

Cell-free supernatants from LAB cultures grown in MRS for 24 and 48 h at 30 °C were filtered (0.45 μm) and injected (injection volume, 20 μL) through a chromatographic system composed of an Alliance 2795 separations module, a 2996 photodiode array (PDA) detector, a 2414 refractive index (RI) detector and the Empower software (Waters, Milford, MA, USA). The sample separation was carried out in an ion-exclusion column ICSep ION-300, 300 mm × 7.8 mm, (7 μm) with pre-column filled with the same material, ICSep ICE-GC-801, 20 mm × 4 mm (Transgenomic, Omaha, NE, USA), which was maintained at 65 °C with a column heater. The mobile phase was 0.0085 N sulphuric acid in HPLC-grade water at a flow rate of 0.40 mL/min. Identification and quantification of organic acids was performed with PDA chromatograms extracted at 210 nm and the RI detector was used for determination and quantification of carbohydrates. Concentration (mg/L) of each analyte was calculated using linear regression equations (R^2 0.99) from the corresponding standard curves. In Table 1 the source and purity of volatile compounds (VOCs) used as standard is reported.

2.4. VOCs analysis from L. plantarum cultures

L. plantarum strains were cultured for 48 h at 30 °C in MRS (5 mL) directly in 20 mL screw capped SPME vials (Agilent Technologies, Santa Clara, CA, USA). The vials were sealed with a magnetic screw capped PTFE/silicone liner septum (Agilent Technologies) and equilibrated to 40 °C for 10 min with pulsed agitation of 5 s at 500 rpm using a PAL RSI 120 (CTC Analytics AG, Zwingen, Switzerland). A Carbon WR/PDMS MS fiber (CTC Technologies) was used for VOCs adsorption. The fiber was exposed to the headspace above the samples for 20 min at depth 40 mm at 40 °C and retracted prior to injection into the GC inlet and desorbed for 1 min at 250 °C using a Agilent 8890 (Agilent Technologies) with a HP-INNOWax (30 m × 250 μm x 0.25 μm) column (Agilent Technologies) using split/splitless injector in splitless mode. The temperature of the column oven was set at 35 °C, held for 0.5 min, increased at 6.5 °C/min to 165 °C then increased at 20 °C/min to 260 °C, yielding total GC run time of 26.5 min. Helium was used as carrier gas, at constant flow rate of 1.4 mL/min. The detector was an Agilent MSD 5977B mass spectrometer detector (Agilent Technologies), ran in single quadrupole mode. The ion source temperature was 230 °C and the interface temperature was set at 280 °C. The MS mode was electronic ionization (70 eV) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library. An auto-tune of the GCMS (ETUNE-F1) was carried out prior to the analysis to ensure optimal GCMS performance.

2.5. Detection of antifungal VOCs in fungal-LAB co-cultures

In order to estimate if there is metabolome modulation when *L. plantarum* strains are co-cultured with *A. niger*, a new experiment was set up in which the strains were co-cultured directly in the GC vials and by maintaining the same conditions of the plate-on-plate method. Specifically, 2 mL of melted PDA was deposited at the bottom of sterile GC glass vial kept horizontally. After solidification, 2 mL of melted MRS was pipetted on the opposite side of the vials. Then, the vials were inoculated by depositing 10 μL of spore suspension onto the PDA layer, whereas *L. plantarum* strains were inoculated by streaking 10 μL of overnight cultures onto MRS layer. Then, GC vials were incubated for 24 h at 30 °C and for 5 days at 25 °C. After incubations, GC vials were analyzed following the same method as above. The nature of VOCs was determined by search in ChemFOnt database (Wishart et al., 2023).

2.6. Antifungal activity of selected pure VOCs

Six VOCs emitted from LAB and identified only in co-cultures, and three VOCs with reported antifungal activity emitted from LAB and identified in co-cultures were selected for further analysis. The synthetic VOCs were individually tested for inhibition of mycelial growth of *A. niger* on PDA in closed petri dishes (9 cm in diameter) assay. Briefly, PDA plates were inoculated at the center of the plate with a single spot of 10 μL of a spore suspension of *A. niger* (10^4 spores/mL). Meanwhile, 50 μL of increasing concentrations of synthetic VOCs (0, 0.1%, 0.5%, 1%, 5%, 10%, 50%, diluted in Ethanol absolute, Sigma-Aldrich) were pipetted onto a piece of filter paper (3.5 cm^2) placed at the center of the cover of the petri dish. Then, plates were sealed with Parafilm® and incubated upside down at 25 °C. After 5 days of incubation the diameter of fungal growth was recorded. Experiment was done in triplicate. The percentage of inhibition of mycelial growth of *A. niger* by a given VOC at each dilution point was calculated on the basis of the difference of the colony diameter without VOC treatment. The concentration value for 50% inhibition of mycelial growth (IC50) expressed as microliters per liter of airspace was inferred from the inhibition percentages and the VOC doses, as previously described (Huang et al., 2011).

2.7. Statistical analysis

Antifungal activity, residual sugars and organic acids were discriminated with One-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test, with $p < 0.05$ as the minimal level of significance. Experimental data were analyzed using SAS Studio Version 9.4 (SAS Institute, Cary, NC, USA). Z-scores were calculated using SPSS Version 25 software (IBM, Armonk, NY, USA). Heatmap of metabolites content was generated using Heatmapper (http://www.heatmapper.ca). Principle component analysis (PCA) was carried out using SPSS Version 25 software.

3. Results and discussion

3.1. Inhibitory activity of L. plantarum strains against A. niger

The antifungal activity of eight *L. plantarum* strains (Table 2) against *A. niger* CECT 2805 was evaluated. This fungus was chosen as representative of its genera, which is responsible for postharvest alteration in food commodities and also for production of toxic metabolites (e.g.

Table 1
Source and purity of volatile compounds (VOCs) used in this study.

VOC(s)	CAS	Source(s)	Purity (%)
Acetic acid	20105.292	VWR (Radnor, PA, USA)	>99
1-Hexanol	52830	Honeywell (Charlotte, NC, USA)	>99
trans-2-Octenal	269956	Sigma-Aldrich	94
1-Octen-3-ol	O-528-4	Sigma-Aldrich	98
1-Octen-3-one	W351504	Sigma-Aldrich	50 solubilized in 1-octen-3-ol,
3-Octanone	203-837-1	Sigma-Aldrich	98
2-Nonanol	6540H	Chem Service Inc. (West Chester, PA, USA)	99
2-Nonanone	10,873-1	Sigma-Aldrich	>99
2-Undecanone	U130-3	Sigma-Aldrich	99

Table 2
Antifungal activity against *Aspergillus niger* CECT 2805 of LAB strains used in this work. Values are means and standard deviation of three biological replicates. Values with different letters are significantly different according to Fisher's Least Significant Difference (LSD) test ($p < 0.05$).

Strain	Screening[a]	Radial growth inhibition percentage (RGI%)			
		24h-CFS[b]	48h-CFS[b]	3d-VOCs[c]	5d-VOCs[c]
L. plantarum 10A	+	11.02 ± 1.20cd	13.56 ± 1.20c	18.49 ± 0.97de	16.46 ± 0.48d
L. plantarum 11A	+	9.32 ± 1.20d	11.02 ± 0.60cd	17.81 ± 1.94e	17.09 ± 0.90d
L. plantarum CB56	++	10.17 ± 2.40d	12.71 ± 1.20cd	19.86 ± 0.97cd	18.35 ± 0.90cd
L. plantarum CZ97	+	9.32 ± 1.20d	10.17 ± 2.40d	14.73 ± 0.48f	12.66 ± 0.97e
L. plantarum CZ103	+	8.47 ± 2.40d	11.86 ± 1.20cd	21.23 ± 0.97c	17.72 ± 1.94d
L. plantarum UFG 121	++	15.25 ± 0.60 ab	18.64 ± 0.60b	24.32 ± 0.48b	20.25 ± 0.97bc
L. plantarum NC8	++	13.56 ± 1.20bc	16.95 ± 1.20b	25.34 ± 0.97b	21.52 ± 0.97b
L. plantarum WCFS1	++	16.95 ± 0.60a	22.03 ± 2.40a	30.48 ± 1.45a	29.11 ± 1.94a

[a] Measured as radium (in mm) of inhibition halo, where + and ++ stand for mild (1–3 mm) and strong (>3 mm) inhibition.
[b] Measured as RGI% after 5 days of mould growth in plates containing 10% of LAB-CFS.
[c] Measured as RGI% after 3 and 5 days of mould growth in presence of VOCs emitted by LAB strains.

mycotoxins), which often contaminates fruits derived products, such as purees, juice and cider (Perrone & Gallo, 2017). Six *L. plantarum* strains were selected because they were previously applied for the enhancement of shelf life in different foods (De Simone, Capozzi, de Chiara, et al., 2021; Russo, Arena, et al., 2017; Russo, Fares, Longo, Spano, & Capozzi, 2017). These strains had been also analyzed for their tolerance in *in vitro* models that simulate oro-gastrointestinal transit, as well as for their immunomodulatory properties *in vitro* (Rocchetti et al., 2023). Moreover, the same strains have been evaluated to increase shelf life and safety as well the functional properties of strawberry in post-harvest (De Simone et al., unpublished results). In addition, two bacteriocin-producing strains, *L. plantarum* NC8 and WCFS1 whose genome is available, were chosen because they are often used as model strains in several studies of international relevance dealing with antimicrobial activity (Jeong et al., 2021; Maldonado, Ruiz-Barba, & Jiménez-Díaz, 2004; Maldonado et al., 2004, 2004; Maldonado-Barragán, Caballero-Guerrero, Lucena-Padrós, & Ruiz-Barba, 2013).

The overlay method was used for a fast initial screening according to Russo, Arena, et al. (2017)). Table 2 shows the strains used in this work, the corresponding source of isolation and the results of the antagonism tests. All the strains had antifungal activity against the model organism, and the degree of inhibition was recorded between mild (1–3 mm) and strong (>3 mm).

Subsequently, to check if the antifungal activity was due to the production and secretion of metabolites in the culture media, the cell-free supernatants (CFSs) of the selected strains were obtained from late exponential phase cultures (24 h of growth) and from early stationary phase (48 h of growth) by centrifugation and filtration. Aliquots of CFSs were neutralized at pH 7.0, aiming to discriminate the antifungal activity due to the secretion of organic acids. Then, the antifungal activity was evaluated against *A. niger* with the radial growth method of the hyphae in PDA plates supplemented with 10% of CFSs. The antagonistic activity of CFSs was expressed as percentage of radial growth inhibition with respect to the control, after 5 days of mould growth at 25 °C. As shown in Table 2, different values of inhibition and different levels of significance were obtained with the addition of 10% of acidic CFSs. In all the experiments *L. plantarum* WCFS1 showed the highest level of antifungal activity, with 16.95% ± 0.60% and 22.03% ± 2.40% of growth inhibition for 24h-CFS and 48h-CFS, respectively, followed by UFG 121 and NC8, which had a slightly lower degree of inhibition, with 15.25% ± 0.60% and 13.56% ± 1.20% for 24h-CFS, and 18.64% ± 0.60% and 16.95% ± 1.20% for 48h-CFS, respectively. The other strains had lower levels of inhibition, with *L. plantarum* 10A showing appreciable values only with 48h-CFS. However, the antifungal activity of the CFSs from all the strains drastically decreases with the neutralization of pH, and fungal growth was comparable ($p > 0.05$) to those of the control without CFS (data not shown). For this reason, it was hypothesized that acidic metabolites could be the main elements responsible for the detected antifungal activity. This result is in line with current literature on this topic. In fact, other authors reported the same behavior for LAB-CFSs (i.e. lack of activity after neutralization) and against several fungal targets. For instance, the CFS from *L. plantarum* UM55 throw off its inhibitory properties against *Aspergillus flavus* MUM 17.14 (Guimarães, Santiago, Teixeira, Venâncio, & Abrunhosa, 2018) and *Penicillium nordicum* MUM 08.16 (Guimarães, Venancio, & Abrunhosa, 2018), after neutralization. In the same conditions, 24h-CFS from *Lactiplantibacillus paraplantarum* CRL 1905 lost the ability to inhibit conidial germination of *Penicillium digitatum* and *Penicillium italicum* (Volentini et al., 2023).

3.2. Analysis of metabolites from antifungal L. plantarum CFSs

Organic acids are mainly generated by the catabolism of carbohydrates and act by diffusing through the membrane of target organisms in the undissociated state, reducing the intracellular pH, thus heading metabolic activities to a halt (Brul & Coote, 1999). Among those, lactic and acetic acids are the most commonly reported organic acids in *L. plantarum* supernatants since they are the main products of carbohydrate catabolism (Guimarães, Santiago, et al., 2018). Whereas, 3-phenyllactic acid (PLA) was described in the last decades as an efficient antifungal produced by *L. plantarum*, with varying synthesis levels depending on the isolate and a strain-dependent production (Zhang, Zhang, Shi, Shen, & Wang, 2014). In order to explain the different antifungal activity within our *L. plantarum* strains, the organic acids mainly responsible for antifungal activity, such as lactic and 3-phenyllactic acid in 24h-CFSs and 48h-CFSs were quantified by HPLC/DAD along with residual sugars (Table 3).

Three carbohydrates were found in the culture medium, i.e. maltose, glucose and fructose. Glucose was the most abundant carbohydrate, with 16.8 ± 0.23 g/L, followed by fructose and maltose with 1.81 ± 0.34 and 1.27 ± 0.17 g/L, respectively. All the strains were able to consume fructose within the 24 h of incubation. Two strains, *L. plantarum* UFG 121 and WCFS1, consumed entirely the maltose, which was not detectable in the 24h-CFS. At the same time, higher concentrations of residual glucose were found in their 24h-CFS that could be explained by the activity of α-amylases, as already described for WCF1 strain (Plaza-Vinuesa, Hernandez-Hernandez, Moreno, de las Rivas, & Muñoz, 2019). In contrast, *L. plantarum* 11A, CB56, CZ97 and CZ103 were not able to consume maltose within the 24 h, and only a small amount was consumed after 48 h. These strains consumed almost all the glucose after 24 h, whose amount underwent a further slight decrease at 48 h. *L. plantarum* 10A and NC8 metabolize maltose and glucose simultaneously with *L. plantarum* 10A consuming maltose slower than *L. plantarum* NC8, since the last strain showed no traces of residual maltose at 24 h. These results support the metabolic diversity already reported in this species (Siezen & van Hylckama Vlieg, 2011). Of note, maltose metabolizing strains are among those with high antifungal activity. Maltose has been recently shown to improve bacteriocin production in *L. plantarum* (Zhao et al., 2021). Whether maltose fermentation also enhances antifungal activity or not deserves further investigation. Significant differences in the production of lactic acid were found among the strains at both incubation times. *L. plantarum* NC8 had the highest production of lactic acid in 24h-CFS, with 16.90 ± 0.42

Table 3
Quantification of organic acids and residual sugars in 24h-CFSs and 48h-CFSs from *L. plantarum* strains. Values are means and standard deviation of three biological replicates. Values with different letters are significantly different according to Fisher's Least Significant Difference (LSD) test ($p < 0.05$). nd: not detected. *: not significant.

	24h-CFSs				48h-CFSs			
	Glucose (mg/L)	Maltose (mg/L)	Lactic acid (g/L)	PLA (mg/L)	Glucose (mg/L)	Maltose (mg/L)	Lactic acid (g/L)	PLA (mg/L)*
10A	22.75 ± 1.14d	356.58 ± 15.03d	16.47 ± 0.13 ab	85.05 ± 3.27cd	18.76 ± 0.05cd	nd	16.96 ± 0.03a	91.49 ± 1.09
11A	25.50 ± 1.37d	1299.53 ± 2.28b	15.64 ± 0.24c	89.44 ± 2.25bc	18.96 ± 2.79cd	1211.32 ± 87.94a	16.12 ± 0.56b	98.02 ± 5.42
CB56	53.76 ± 8.50cd	1303.67 ± 19.62 ab	15.48 ± 0.19c	86.86 ± 0.85cd	16.84 ± 1.58d	1160.69 ± 61.46a	15.56 ± 0.36b	93.81 ± 4.91
CZ97	58.47 ± 19.76c	1319.10 ± 3.53a	15.29 ± 0.18c	82.46 ± 2.16d	17.44 ± 0.65cd	1195.15 ± 101.53a	15.82 ± 0.17b	93.08 ± 2.23
CZ103	51.31 ± 34.34cd	1280.19 ± 9.32c	15.56 ± 0.01c	85.94 ± 1.45cd	15.86 ± 0.16d	938.87 ± 232.15b	15.91 ± 0.20b	91.32 ± 0.57
UFG 121	608.43 ± 0.98b	nd	16.25 ± 0.11b	93.80 ± 0.58 ab	39.79 ± 3.33b	nd	16.99 ± 0.14a	98.44 ± 1.36
NC8	23.19 ± 3.47d	nd	16.90 ± 0.42a	88.45 ± 1.29bc	20.84 ± 0.94c	nd	17.06 ± 0.15a	91.43 ± 1.12
WCFS1	742.10 ± 34.17a	nd	16.60 ± 0.12 ab	95.76 ± 4.14a	43.96 ± 3.48a	nd	17.10 ± 0.22a	98.73 ± 0.14

g/L, in agreement with its high metabolic activity, followed by *L. plantarum* WCFS1, 10A, and UFG 121, with 16.60 ± 0.12, 16.47 ± 0.13, and 16.25 ± 0.11 g/L, respectively. Nevertheless, no significant difference was found in their 48h-CFSs. The other four strains 11A, CB56, CZ97 and CZ103 showed slightly lower levels of lactic acid. The concentration of PLA was similar among strains and, despite the 24h-CFSs showed significant differences, with WCFS1 and UFG 121 as the best producers with 95.76 ± 4.14 and 93.80 ± 0.58 mg/L, respectively, no differences were found in 48h-CFSs. These data are consistent with those previously reported for other LAB strains with antifungal activity. The observed levels of PLA are within the average range of those for medium-high producers reported in the literature (Zhang et al., 2014), ranging 46–99.6 mg/L down to 4.3 mg/L in low producers (Guimarães, Santiago, et al., 2018; Guimarães, Venancio, & Abrunhosa, 2018; Quattrini et al., 2018; Volentini et al., 2023).

Taken as a whole, our *L plantarum* strains can be typed in two groups based on their metabolic activity: *L. plantarum* 10A, UFG 121, NC8 and WCFS1 able to metabolize maltose and to produce higher levels of lactic acid, whereas the other four strains were unable to metabolize maltose and produced lactic acid at a lesser extent. Nevertheless, the levels of both antifungal compounds analyzed here, although significantly different in the case of lactic acid, are unlikely able to explain the degree of fungal inhibition observed with CFSs (see Table 2). In fact, by analyzing individually the differences among strains, e.g. between *L. plantarum* 10A and UFG 121, despite the quantity of lactic acid and PLA detected in 48h-CFSs was similar, there is a significant difference in the antifungal activity of these two strains. Consequently, it was hypothesized that these organic acids alone cannot fully explain the detected activity, and a synergistic effect with other metabolites may be present.

Many studies report on the inhibition of fungal species by volatile organic compounds (VOCs) produced by microorganisms (Li et al., 2022). The volatile metabolites extracted from the *L. plantarum* 48h-cultures in MRS were investigated by GC-MS analysis. By excluding the compounds due to the culture media, a total of 27 VOCs were identified on the basis of mass spectral properties (Table S1). These compounds fell into classes of acids (9), ketones (7), alcohols (7), ethers (2) and esters (2). The most abundant compound in the VOC profiles of LAB strains was acetic acid, which accounted between 12.15% and 17.13% of the relative peak area (RPA), with *L. plantarum* UFG 121 as the major producer of this compound. Afterwards, two volatile ketones, 2-Undecanone and 2-Nonanone, had RPA% values of 5.82%–7.74% for 2-Undecanone, and 4.53–5.45% for 2-Nonanone. In all the strains evaluated, the concentration of volatile compounds generally decreased in the following order: acetic acid > 2-Undecanone > 2-Nonanone. This trend confirms the antifungal aptitude of the strains, because these two ketones have been previously reported as antifungal VOCs against *Botrytis cinerea* and *Monilinia* spp. (Calvo et al., 2020). Other six VOCs, such as acetoin, 2-Heptanone, 2-Undecanol, 2-Tridecanone, 2-Tridecanol and 3-Methyl-1-butanol (isoamyl alcohol), were moderately abundant in all the strains investigated, with values about 1%–2%. The other 18 VOCs were the least abundant, with RPA values < 1%.

Then, in order to underline metabolic differences between the strains, a heatmap reflecting the metabolites found in HPLC and in GC-MS was generated (Fig. 1A). In addition, to correlate the metabolites produced by the different strains at the end of the incubation time (48 h) and the antifungal activity, principal component analysis (PCA) was performed (Fig. 1B and C).

With the heatmap it was also possible to discern which metabolites could be correlated with the antifungal activity of the strains. As stated before, high 48h-CFSs-antifungal activity (Fig. 1A row 36) is associated with high lactic acid (Fig. 1A row 34) and low maltose (Fig. 1A row 28). Other metabolites mainly followed the same trend. High level of 2-Nonanone, acetic acid and 3-Methyl-butanoic acid (Fig. 1A row 5, 6 and 13, respectively) were correlated to high antifungal activity. Otherwise, low levels of several compound, such as 2-Undecanol, methyl nonyl ether, heptadecane-2,4-dione, hexanoic acid, benzyl alcohol, 2-Tridecanol, (Z)-4-Decen-1-ol, methyl ether and Octanoic acid (Fig. 1A row 14, 15, 16, 20, 21, 22, 23 and 24, respectively), showed also a negative correlation with antifungal activity.

The Principal Component Analysis (PCA) was also applied to visually highlight the differences among the strains. At 48 h of incubation, the Factors 1, 2 and 3 explained 32.21%, 29.65% and 17.70% of the total variance, respectively, and taken together accounted for 79.57% of the variance in the whole data set. The positive side of Factor 1 was mainly correlated to (Z)-4-Decen-1-ol, methyl ether, 2,3-Butanedione (Diacetyl) and 2-Undecanol. The positive side of Factor 2 mainly correlated to (Z)-Dec-4-enyl isobutylcarbonate and 10-Octadecynoic acid, methyl ester. The positive side of Factor 3 mainly correlated to 2-Heptanone and 2-Nonanone. On the negative side of Factor 1, 2 and 3, the assay mainly correlated to Acetic acid, Citric acid and Nonanoic acid, respectively. The two parameters for antifungal activity (48h-CFSs and 5d-VOCs) were used to cluster and to label the strains in Fig. 1C. The two groups appeared clearly separated in the score plot. Following the previously delineated groups, the strains 10A, 11A, CB56, CZ97 and CZ103, which showed intermediate values of antifungal activity, were homogeneously grouped in the upper right quadrant. Whereas, the strains NC8, UFG 121 and WCFS1, showing higher antifungal activity were grouped in the lower quadrant. The displacement of NC8 in the lower left quadrant of Fig. 1C could be attributable to its higher production of citric acid.

3.3. Determination of presumptive antifungal VOCs in fungal-LAB co-cultures

The presence of antifungal metabolites investigated in 48h-old single LAB cultures in MRS and in their respective CFS may underestimate plausible metabolism modulation when the strains are cultured together. Therefore, a second set of experiments was planned to evaluate if co-culturing selected *L. plantarum* strains and the fungal target without physical contact could modulate some metabolic pathway resulting in the synthesis of unique metabolites. Based on what previously observed, two strains, showing different antifungal activity were selected in order

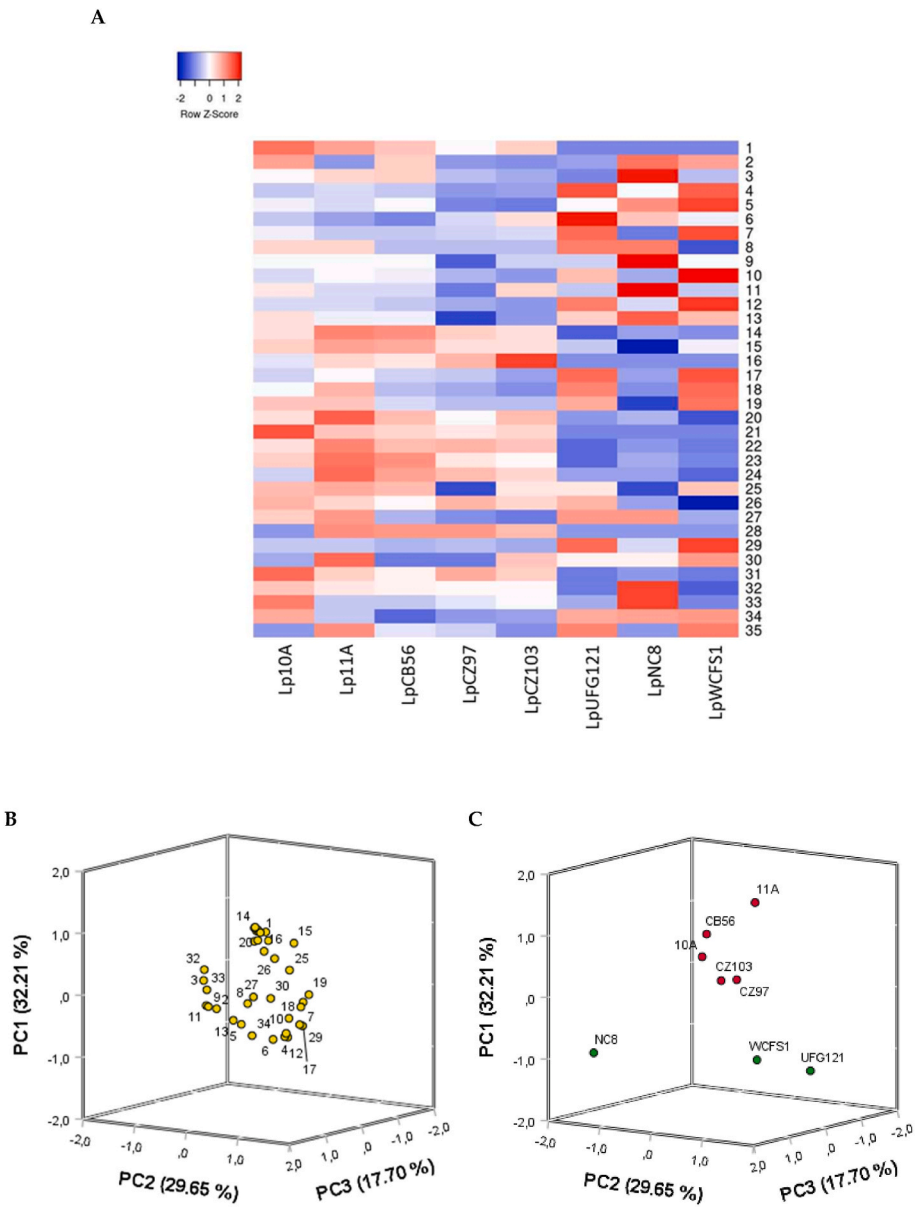

Fig. 1. Metabolic profiling of LAB strains. Heatmap of metabolites content of 48h-CFSs of *L. plantarum* strains (A). Columns represent different *L. plantarum* strains, and rows represent the different metabolites identified. Red color indicates high concentration and blue color indicates low concentration. Principal Component Analysis (PCA), scores plot (B) and loading plot (C) indicating variances in the metabolites profiles across studied strains. Color labeling (red and green dots) of the strains in C refer to antifungal activity (medium and higher, respectively). 1. 2,3-Butanedione; 2. 2-Heptanone; 3. 3-Methyl-1-butanol; 4. Acetoin; 5. 2-Nonanone; 6. Acetic acid; 7. 2,6-Dimethyl-4-heptanol; 8. Propanoic acid; 9. 2-Methyl-propanoic acid; 10. 2-Undecanone; 11. Butanoic acid; 12. (Z)-Undec-6-en-2-one; 13. 3-Methyl-butanoic acid; 14. 2-Undecanol; 15. Methyl nonyl ether; 16. Heptadecane-2,4-dione; 17. 2-Tridecanone; 18. (Z)-Dec-4-enyl isobutylcarbonate; 19. 10-Octadecynoic acid, methyl ester; 20. Hexanoic acid; 21. Benzyl alcohol; 22. 2-Tridecanol; 23. (Z)-4-Decen-1-ol, methyl ether; 24. Octanoic acid; 25. (Z)-6-Pentadecen-1-ol; 26. Nonanoic acid; 27. n-Decanoic acid; 28. Maltose; 29. Glucose; 30. Oxalic acid; 31. Orotic acid; 32. Citric acid; 33. Piruvic acid; 34. Lactic acid; 35. 3-Phenyllactic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to investigate if this trait could be linked to the biosynthesis of different VOCs in co-culture assay. In particular, UFG 121 was the strains with highest antifungal activity, while strain 10A was selected within the group with intermediate antifungal activity because, as the strain UFG 121, it showed a distinct ability to metabolize maltose.

The strains were co-cultured with *A. niger* directly in the glass vials for VOCs analysis for 5 days at 30 °C. After incubation, VOCs generated and accumulated in the vials were analyzed in GC-MS. Then, the VOCs generated in fungal-LAB co-cultures were compared to those produced by LAB and fungal single cultures in order to determine the presence of unique volatiles in the co-cultures. A total of 51 different compounds were identified in the different conditions on the basis of mass spectral properties. This time, compounds fell into classes of ketones (15), alcohols (14), acids (9), aldehydes (4), esters (4), ethers (1), alkenes and alkanes (4) (Table S2). The presence of shared or unique VOCs among single cultures and fungal-LAB co-cultures, and among the two LAB strains used was displayed by using Venn diagrams (Fig. 2). In these analyses, the compounds detected in all samples (e.g. fungal single culture, LAB single cultures and fungal-LAB co-cultures), such as 3-Metil-1-butanol, acetic acid and phenylethyl alcohol were not counted and removed from the analysis due to the interference with the generation of Venn diagrams.

Fig. 2A shows the comparison between the VOC profile of LAB culture, fungal culture and fungal-LAB co-culture, for each *L. plantarum*

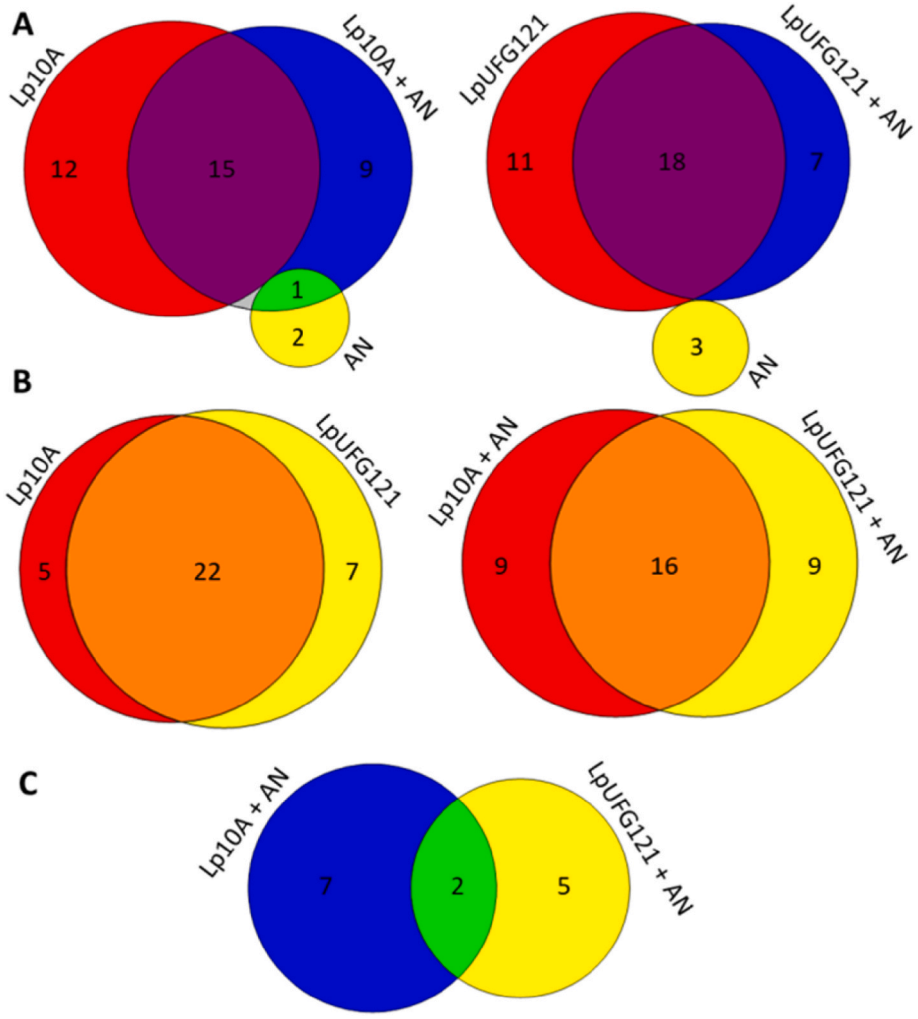

Fig. 2. Venn diagrams of shared and unique VOCs in LAB, fungal culture and LAB-fungal co-cultures. **A.** Unique VOCs in LAB cultures (red), fungal culture (yellow) and LAB/fungal co-cultures (blue). Shared VOCs between LAB culture and LAB/fungal co-culture (purple) and fungal culture and LAB/fungal co-culture (green). **B.** Unique VOCs in *L. plantarum* 10A (red) and UFG 121 (yellow) cultures and fungal co-cultures. Shared VOCs are in orange. **C.** Unique VOCs present only in fungal co-culture of *L. plantarum* 10A (blue) and UFG 121 (yellow). Shared VOCs are in green. AN: *Aspergillus niger*. VOCs generated by the culture media and unique VOCs present in all samples were not counted. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

strains utilized. Three compounds have been detected in fungal single culture and among those only one was also found in fungal-Lp10A co-culture, whereas none of them was found in the fungal-LAB co-culture with the strain UFG 121. For the first condition, 27 compounds were produced by 10A in single culture, and among those only 15 were found also in the respective co-culture. For the second condition, 29 compounds were produced by UFG 121 in single culture, and among those only 18 were found also in the respective co-culture. From the two co-cultures, nine and seven compounds were unique in 10A and UFG 121, respectively. With regard to the LAB single cultures, the 27 compounds produced by Lp10A and the 29 by UFG 121 were compared by overlapping the two VOCs profiles, and the majority of them, 22 compounds, were produced by both LAB, as expected (Fig. 2B). However, when in co-cultures out of the 25 compounds produced by each fungal-LAB co-culture, only 16 compounds were shared and 9 unique VOCs were present in each condition (Fig. 2B). Then, the unique VOCs present only in fungal-LAB co-cultures, i.e. the 9 compounds from 10A and the 7 compounds from UFG 121 were compared, showing that only 2 of them were present in both conditions (Fig. 2C). Finally, a total of 14 compounds were found only in the co-culture of *A. niger* with at least one *L. plantarum* strain. Table 4 reports the distribution of the 14 compounds detected in the co-cultures, their molecular formula, and nature. It should be noted that at this stage is not possible to know which microorganism is actually producing these molecules. Following the previous results, the 14 compounds detected only in co-cultures were investigated about their presence in literature, metabolism, and antifungal activity.

Four eight-carbon volatiles out of fourteen were detected in the co-cultures. These compounds probably belong to the same biosynthetic pathway, also because they have a very similar molecular formula. In other microorganisms 1-Octen-3-one is converted in 3-Octanone in a pH-dependent manner or via enzymatic reaction (Combet, Henderson, Eastwood, & Burton, 2009). However, it is not clear which pathways contribute to the eight-carbon volatiles, in either *L. plantarum* or *A. niger*. This class of compounds were widely studied in literature as antifungal. For example, 1-Octen-3-ol was previously reported as a volatile self-inhibitor produced by *Penicillium paneum* (Chitarra, Abee, Rombouts, Posthumus, & Dijksterhuis, 2004). In another work, 1-Octen-3-ol completely inhibited the growth of *Fusarium tricinctum* and *Fusarium oxysporum* at the concentration of 4.0 mg/mL (Xiong et al., 2017). In the same work, 1-Octen-3-one had no effect on the growth rate of tested fungi at the same concentration (Xiong et al., 2017). Whereas, 3-Octanone exerted antimicrobial activity on a wide range of bacterial and fungal plant pathogens (Muto, Fukushima-Sakuno, Ishihara, & Osaki-Oka, 2023). In a recent article, (E)-2-Octenal, together with other (E)-2-Alkenals, were tested as antifungal against *A. flavus* NRRL 3357,

Table 4
Unique VOCs detected only in fungal-LAB co-cultures and their distribution and Relative Peak Area percentage (RPA%) among the fungal-LAB co-cultures.

Compound(s)/Syn	Molecular formula	Type of compound	RPA%	
			10A	UFG 121
Propanoic acid	$C_3H_6O_2$	Organic acids and derivatives	–	0.076 ± 0.021
1-Hexanol	$C_6H_{14}O$	Fatty acyls/Lipids and lipid-like molecules	–	0.066 ± 0.011
Acetic acid, pentyl ester (Amyl acetate)	$C_7H_{14}O_2$	Organic acids and derivatives	0.227 ± 0.062	–
1-Octen-3-one	$C_8H_{14}O$	Fatty acyls/Organic oxygen compounds	0.205 ± 0.076	–
(E)-2-Octenal (*Trans*-2-Octenal)	$C_8H_{14}O$	Fatty acyls/Organic oxygen compounds	0.120 ± 0.006	–
3-Octanone (Ethyl pentyl ketone)	$C_8H_{16}O$	Fatty acyls/Organic oxygen compounds	2.443 ± 0.268	0.188 ± 0.031
(Z)-2-Octen-1-ol (*cis*-2-Octenol)	$C_8H_{16}O$	Fatty acyls/Lipids and lipid-like molecules	0.229 ± 0.022	–
7-Methyl-1-octene	C_9H_{18}	–	0.099 ± 0.036	–
2-Nonanol	$C_9H_{20}O$	Fatty acyls/Lipids and lipid-like molecules	0.760 ± 0.407	2.120 ± 0.637
9-Decen-1-ol, methyl ether (10-Methoxy-1-decene)	$C_{11}H_{22}O$	–	–	0.437 ± 0.185
2-Undecanol, acetate (Ethyl undecanoate)	$C_{13}H_{26}O_2$	Fatty esters/Lipids and lipid-like molecules	–	0.149 ± 0.045
2-Tridecanol	$C_{13}H_{28}O$	Fatty acyls/Lipids and lipid-like molecules	0.134 ± 0.009	–
(Z)-Tetradec-6-en-2-one	$C_{14}H_{26}O$	–	–	0.177 ± 0.134
(Z,E)-Farnesal	$C_{15}H_{24}O$	Sesquiterpenoids	0.098 ± 0.011	–

which showed a colony diameter less than 1 cm when treated with 0.0250 μL/mL of this compound (Duan et al., 2023). Both 1-Octen-3-ol and *cis*-2-octen-1-ol are thought to be responsible for the characteristic musty-fungal odor of certain fungi; the latter compound may be a useful chemical index of fungal growth (Kamiński, Libbey, Stawicki, & Wasowicz, 1972). No information was found about antifungal activity of (Z)-2-Octen-1-ol (*cis*-2-octenol).

Regarding the other compounds, propionic acid and 2-Tridecanol were detected as unique compounds in the co-culture of only one strain but were detected in both single culture and co-culture of the other, and for this reason they were not further investigated. 2-Nonanol, 2-Undecanol acetate and 2-Tridecanol seemed to be derived from the relative ketones. As stated before, two ketones were previously reported as antifungal VOCs against *Botrytis cinerea* and *Monilinia* spp. (Calvo et al., 2020). However, when tested in precedent works 2-Tridecanone exerted weak or no effects on mycelial growth of *P. italicum* (Wang et al., 2021) and *F. oxysporum* f. sp. *cubense* (X. Li et al., 2020). Amyl acetate was detected in the volatile profile of *Candida intermedia* and effectively inhibited the mycelial growth of *B. cinerea*, with IC50 value of 46.5 μL/L (Huang et al., 2011). No information was found about the remaining compounds ((Z,E)-Farnesal (Z)-Tetradec-6-en-2-one, 9-Decen-1-ol, methyl ether; 7-Methyl-1-octene).

3.4. Antifungal activity of the selected pure VOCs

Five VOCs identified in fungal-LAB co-cultures (1-Hexanol, *trans*-2-octenal, 1-Octen-3-one, 3-Octanone, and 2-Nonanol) and three VOCs identified in LAB cultures (acetic acid, 2-Nonanone and 2-Undecanone) were individually tested for antifungal activity. 1-Octen-3-ol was also added to the assay due to its reported activity in literature (Chitarra et al., 2004; Xiong et al., 2017) and because it was a co-formulant of the 1-Octen-3-one used. These nine pure compounds were diluted in absolute ethanol and spotted on filter paper discs in order to obtain different concentrations in the airspace of the plates. Fig. 3 shows the inhibition percentage of mycelial growth of *A. niger* after 5 days of exposition at different concentrations of volatile compounds.

The results showed that three compounds, such as acetic acid, 1-Hexanol and 1-Octen-3-one showed a complete inhibition only at the highest concentrations tested, while little or no inhibition was detected at lower concentrations. For this reason, values of IC50 could not be predicted due to lack of linearity. This behavior was already reported for 1-Hexanol against *A. flavus* CICC 2219 in a previous work, in which 1-Hexanol completely inhibit the mycelial growth in red pepper powder at 80 μL/L while lower doses were ineffective (Li et al., 2022).

VOC treatment reduced mycelial diameter in a dose-dependent manner in six out of nine compounds, such as *trans*-2-octenal, 1-Octen-3-ol, 3-Octanone, 2-Nonanol, 2-Nonanone and 2-Undecanone. These compounds effectively inhibited mycelial growth of *A. niger*, with IC50 values from 2.16 to 37.42 μL/L (Table 5). Among these, *trans*-2-Octenal and 2-Nonanol which were among those antifungal

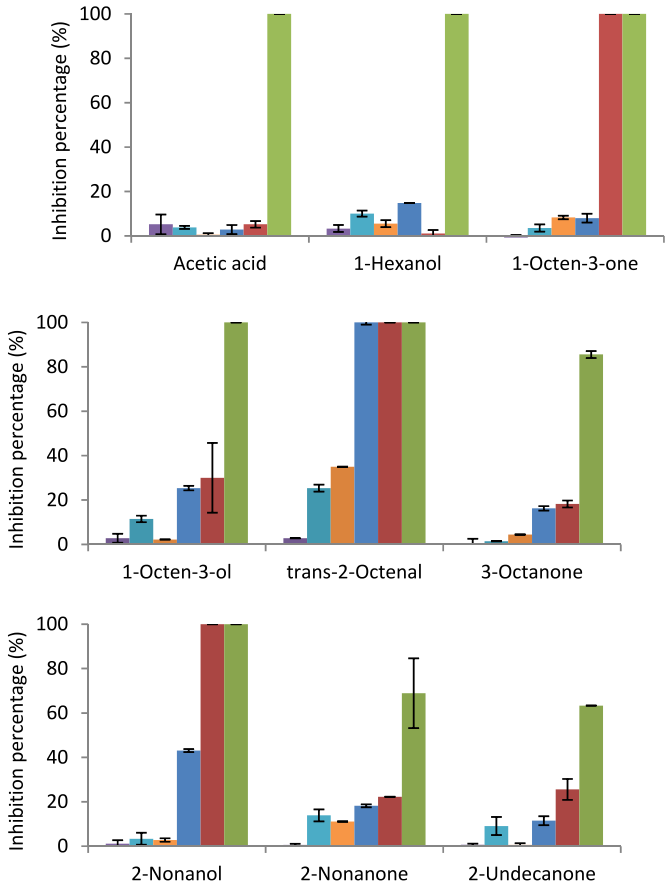

Fig. 3. Synthetic VOCs inhibition of mycelial growth in airtight petri dishes assay. Values are expressed as percentage of radial growth inhibition against *A. niger* CECT 2805 after 5 days of growth at 25 °C. Concentration in airspace (μL/L): 1 (purple), 5 (light blue), 10 (orange), 50 (blue), 100 (red), 500 (green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Values of the 50% inhibition concentration (IC50) of the synthetic volatile organic compounds for mycelial growth inhibition of *A. niger* CECT 2805.

VOC(s)	Detection	IC50 (μL/L)	R2
Acetic acid	LAB cultures	–	–
1-Hexanol	Co-culture UFG 121	–	–
trans-2-Octenal	Co-culture 10A	2.16	0.9682
1-Octen-3-ol	Volatile Self-Inhibitor	22.53	0.9747
1-Octen-3-one	Co-culture 10A	–	–
3-Octanone	Co-cultures both LAB	28.47	0.9927
2-Nonanol	Co-cultures both LAB	4.58	0.9929
2-Nonanone	LAB cultures	33.56	0.9969
2-Undecanone	LAB cultures	37.42	0.9544

compounds detected only in co-cultures had the highest activity against *A. niger*, with IC50 values of 2.16 and 4.58 μL/L, respectively. These IC50 values are roughly ten times lower than those of 2-Nonanone and 2-Undecanone which were detected in LAB single cultures. In previous works, these compounds inhibited the growth of *B. cinerea*, *Monilinia* spp. (Calvo et al., 2020), and *Colletotrichum gloeosporioides* (Zheng, Shi, Shi, Wang, & Li, 2013), but not three *Penicillium* strains tested (Calvo et al., 2020). The antifungal activity of 3-Octanone, detected in co-cultures only, was confirmed, with IC50 value of 28.47 μL/L against *A. niger* CECT 2805. It is worth noticing that 3-Octanone exerted antimicrobial activity on a wide range of bacterial and fungal plant pathogens, and in particular it was able to inhibit germ tube elongation of *A. niger* NBRC105649 but not spore germination (Muto et al., 2023).

4. Conclusions

Both volatile organic compounds (VOCs) and organic acids produced by *L. plantarum* strains have been correlated with their antifungal activity against *A. niger*, while the antifungal 3-phenyllactic acid (PLA) appears to play a minor role. Remarkably, unique VOCs with high antifungal activity were detected only when the fungal target and the tested bacterial strains were co-cultivated, suggesting that metabolic modulation is likely behind the strain-dependent antifungal activity of *L. plantarum* isolates. This co-culture strategy enabled the detection of novel antifungal VOCs candidates, i.e. *trans*-2-Octenal and 2-Nonanol, which are worth testing in future biopreservation approaches to reduce fungal contamination and the associated food waste, health risks and economic losses in food production.

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CRediT authorship contribution statement

Nicola De Simone: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Lucía López:** Methodology, Investigation, Data curation. **Carmen S. Ciudad:** Investigation, Data curation. **Angela Scauro:** Visualization, Investigation, Data curation. **Pasquale Russo:** Writing – review & editing, Supervision, Conceptualization. **Jorge Rodríguez:** Methodology, Investigation, Data curation. **Giuseppe Spano:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Beatriz Martínez:** Writing – review & editing, Supervision, Resources, Conceptualization.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.103824.

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