

# Potential application of lactic acid bacteria in the biopreservation of red grape from mycotoxigenic fungi

Victor Dopazo,<sup>a†</sup> Carlos Luz,<sup>a†\*</sup>  Juan M Quiles,<sup>a</sup> Jorge Calpe,<sup>a</sup> Raffaele Romano,<sup>b</sup> Jordi Mañes<sup>a</sup> and Giuseppe Meca<sup>a</sup> 

## Abstract

**BACKGROUND:** Filamentous fungi are the main contamination agent in the viticultural sector. Use of synthetic fungicides is the regular answer to these contaminations. Nevertheless, because of several problems associated with the use of synthetic compounds, the industry demands new and safer methods. In the present work, the biopreservation potential of four lactic acid bacteria (LAB) strains was studied against the principal grape contaminant fungi.

**RESULTS:** Agar diffusion test evidenced that all four culture-free supernatant (CFS) had antifungal properties against all tested fungi. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) test values evidenced that media fermented by the *Lactobacillus plantarum* E3 and *Lactobacillus plantarum* E4 strains showed the highest antifungal activity, resulting in an MFC from 6.3 to 100 g L<sup>-1</sup>. Analysis of CFS evidenced the presence of different antifungal compounds, such as lactic acid, phenyllactic acid and pyrazines. In tests on red grapes, an average reduction of 1.32 log<sub>10</sub> of the spores per gram of fruit was achieved by all CFS in grapes inoculated with *Aspergillus ochraceus* and by 0.94 log<sub>10</sub> for *L. plantarum* E3 CFS against *Botrytis cinerea*.

**CONCLUSION:** The antifungal activity of the fermented CFS by *L. plantarum* E3 reduced the growth of *B. cinerea* and *A. ochraceus* in grapes, which are the main contaminant and main producer of ochratoxin A in these crops, respectively. Therefore, based on the results obtained in this work, use of the strain *L. plantarum* E3 could be an interesting option for the biopreservation of grapes.

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**Keywords:** lactic acid bacteria; antifungal activity; grape; *Aspergillus*; *Botrytis*

## INTRODUCTION

Worldwide, around 14% of food production is lost along the supply chain due to different causes, such as inadequate harvesting time, climatic conditions, pre-harvest and post-harvest practices, storage conditions, poor practices during food transportation, shelf life in the shop and poor in-home storing.<sup>1</sup> Fungi are the main agent in this deterioration.<sup>2</sup> One of the most affected industries is the grape sector – the most extended horticultural crop in the world.<sup>3</sup> Fungi causes losses to the viticultural industry of millions of euros annually.<sup>4</sup> Product deterioration can occur by various means, such as alteration of the organoleptic quality of the product (aroma, aspect, taste and texture), 'mould flavours' in wine,<sup>5</sup> or even affect the health of consumers by producing secondary toxic metabolites.<sup>6</sup>

Nowadays, the use of antifungal compounds of synthetic origin is the standard response to fungal contamination of food, but this carries some problems. These are very stable compounds that are difficult to eliminate from food and nature; in addition, some of them are toxic to humans and animals.<sup>7</sup> This leads to the second

problem: rising consumer demand to reduce the presence of antifungal compounds in food.<sup>6</sup> Finally, the abuse of these antifungal treatments is associated with rising resistance of mould to these compounds.<sup>8</sup> Therefore, a study of new methods of preservation against fungi is being undertaken, such as the use of natamycin for the conservation of mandarin oranges against *Botrytis cinerea*.<sup>9</sup>

One of the most promising techniques under study is the use of lactic acid bacteria (LAB). There are many examples in the

\* Correspondence to: CL Mínguez, Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy University of València Av. Vicent Andrés Estellés s/n 46100 Burjassot, Spain. E-mail: carlos.luz@uv.es

† These authors have contributed equally to the study, and thus may be regarded as the first author.

a Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Burjassot, Spain

b Department of Agriculture, University of Napoli Federico II, Portici, Italy

literature that prove their ability to preserve food against fungi.<sup>10–12</sup> It is believed that this biopreservation effect originates from the pool of antifungal compounds produced by LAB (organic acids, volatile organic compounds, phenolic acids and bioactive peptides). Also, different application methods have proven effective against fungal food contamination, such as fermentation of the product, the use of bioprotective cultures, culture-free supernatants (CFS) or purified molecules from their CFS.<sup>6,8,12–14</sup>

Following the trend, the objectives of this study were (i) to evaluate the antifungal activity of the CFS of a few LAB using *in vitro* assays, (ii) to identify and characterize the antifungal compounds present in the CFS and (iii) to find a novel application of CFS produced by LAB for the biopreservation of red grapes for wine production (*Vitis vinifera*) against the genera *Aspergillus* and *Botrytis* – the most typical producers of rottenness in these fruits.<sup>15,16</sup>

## MATERIALS AND METHODS

### Chemicals

The culture media used in this study – potato dextrose agar (PDA), potato dextrose broth (PDB), and Man–Rogosa–Sharpe agar (MRS-A) and broth (MRS-B) – were acquired from Liofilchem (Teramo, Italy). Deionized water (<18 MΩ cm<sup>-1</sup>) was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Acetonitrile, ethyl acetate, formic acid and methanol were from VWR Chemicals (Radnor, PA, USA). Ammonium formate, C18, magnesium sulfate (MgSO<sub>4</sub>) and sodium chloride (NaCl) were acquired from Sigma-Aldrich.

Gallic acid, chlorogenic acid, caffeic acid, syringic acid, vanillic acid, *p*-coumaric acid, hydroxybenzoic acid, vanillin, hydroxycinnamic acid, sinapic acid, benzoic acid, phenyllactic acid, dihydrocaffeic acid, 3,4-dihydroxyhydrocinnamic acid and *DL-p*-hydroxyphenyllactic acid were acquired from Sigma-Aldrich (Dublin, Ireland). Phenyllactic acid was obtained from BaChem (Weil am Rhein, Germany). Ferulic acid was purchased from MP Biomedicals, and protocatechuic acid came from HWI Pharma Services (Ruelzheim, Germany). Lactic acid was from Sigma-Aldrich (St Louis, MO, USA) and acetic acid from Fisher Scientific (Waltham, MS, USA). All analytes had a purity of 95%.

### Fungal and bacterial isolation

The LAB used in this study were obtained from different food sources. The strains *L. plantarum* BN16 (BN16) and *L. plantarum* BN17 (BN17) were isolated from tomatoes, and strains *L. plantarum* LIE3 (E3) and *L. plantarum* LIE4 (E4) from fish guts using 0.1% peptone water in ratio 1:10 (w/v) and homogenization with a stomacher. Then, 100 μL of dilutions in peptone water (10<sup>-1</sup>–10<sup>-7</sup>) were plated and incubated at 37 °C for 72 h in an anaerobic atmosphere, using an Anaerocult® system. Morphologically different colony-forming units were inoculated in MRS-A to isolate pure cultures. A Gram stain was performed on isolated cultures to select Gram-positive bacteria. Selected cultures were preserved in cryotubes with MRS-B with 25% glycerol at –20 °C. Restoration was performed by adding the frozen bacteria to MRS-B and incubating them for 24 h at 37 °C, after which time 1 mL was aliquoted to MRS-B and incubated under the same conditions.

Fungi used were six *Aspergillus* (*A. carbonarius* ISPA 5010, *A. niger* CECT 2088, *A. ochraceus* CECT 2093, *A. niger* CECT 2915, *A. tubingensis* CECT 20543 and *A. tubingensis* CECT 20545) and one strain of *B. cinerea* (CECT 20973). They were obtained from the Colección Española de Cultivos Tipo (CECT) in the University

of Valencia and the ITEM Collection from Istituto di Scienze delle Produzioni Alimentari (ISPA). The moulds were frozen in cryotubes at –20 °C in PDB with 25% glycerol for preservation. To prepare moulds for the tests, the frozen tubes were suspended in PDB and incubated at 25 °C for 72 h, then 1 mL was inoculated on PDA and incubated under the same conditions.

### Use of a matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) system for bacterial identification

The identification of the LAB strains was performed by direct extraction of isolated cultures, following the steps described by Maier *et al.*<sup>17</sup> The method was performed with MALDI-TOF MS using a Microflex L20 (Bruker Daltonics, Billerica, MA, USA) mass spectrophotometer equipped with an N<sub>2</sub> laser. All spectra were acquired in positive linear ion mode. Voltage acceleration was 20 kV. The mass range for the analysis was delimited from 2000 to 20 000 Da. For each sample, three spectra were obtained following the method MALDI Biotyper Realtime Classification (RTC). The identification corresponded to the largest log score. Results were compared with the database MBT 7854 y MBT 7311\_RUO (Bruker Daltonics).

### Preparation of CFS

To start, 50 mL MRS-B was inoculated with recovered LAB in a 1/100 proportion (v/v) and incubated at 37 °C for 72 h. After fermentation, the medium was centrifuged at 4 °C and 4000 rpm for 15 min, in an Eppendorf 5810 R centrifuge (Hamburg, Germany). The CFS was recovered and frozen at –80 °C, then lyophilized in a FreeZone 2.5 L, Labconco (Kansas City, MO, USA) and preserved at –20 °C for further analysis.

### Qualitative assay in solid medium

The antifungal activity of the CFS from the different LAB was tested using the agar diffusion method described by de Bauer *et al.* with a few modifications.<sup>18</sup> The lyophilized CFS was suspended in PDB to a concentration of 200 g L<sup>-1</sup>. Plates of PDA were inoculated with fungal spores using a sterile swab; wells were then made in the agar using blue micropipette tips, and 100 μL of the different suspended CFS were aliquoted into the wells. Then, the plates were incubated at 25 °C for 72 h. At the end of the incubation period, antifungal activity was expressed as follows: (+) means <8 mm of inhibition zone between the well and fungal growth; (++) means 8–10 mm of inhibition zone between the well and fungal growth; (+++) means >10 mm of inhibition zone between the well and fungal growth.

### Quantitative assay of antifungal activity

The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the CFS were studied using the method described by Luz *et al.*<sup>19</sup> In one line of a 96-well sterile plate, 100 μL of the CFS was resuspended in concentrations from 100 to 0.4 g L<sup>-1</sup> in PDB medium, then 100 μL from a suspension of 5 × 10<sup>4</sup> spores mL<sup>-1</sup> in PDB were added to each well. A negative control was performed by adding 200 μL PDB to a well and a positive control by adding 100 μL PDB and 100 μL of the spore suspension. Plates were incubated at 25 °C for 72 h. The MIC was considered the lowest concentration of CFS at which fungi did not grow. Four replicas of this assay were performed.

The next step was to plate 10 μL on PDA from each well of the 96-well plate that contained the MIC concentration and higher. After incubation at 25 °C for 72 h, the results were observed.

The MFC was considered as the lowest concentration of CFS at which an irreversible inhibition of fungal viability was achieved at the end of the incubation period.

### Identification of organic acids in the CFS

For the study of organic acids, the CFS was diluted 1/20 (v/v) in MilliQ water, then clarified with a 0.22 µm pore filter. Samples were then injected into a JASCO Analytica (Easton, MD, USA) high-performance liquid chromatography (HPLC) system with an MD 4015 PDA diode array detector along a 20 µL sample injection loop and a quaternary pump. Separation of the different phases was performed using an isocratic mobile phase of water and formic acid at 0.1% moving at a flow rate of 0.8 mL min<sup>-1</sup> for 20 min and a Rezex ROA organic acid (150 × 7.8 mm) reverse-phase column (Phenomenex Inc, Torrance, CA, USA). The chromatogram was set at 214 nm. Data were acquired with ChromNAV 2.0 HPLC software from JASCO Analytica. Calibration of the method was performed using lactic acid and acetic acid in MRS broth, diluted 1/20 (v/v), at a final concentration from 0.125–1 g L<sup>-1</sup>. Results were expressed in grams per litre.

### Identification of phenolic compounds in CFS

To study phenolic acids, first a QuEChERS extraction was performed to purify the CFS. A solution of 4 g MgSO<sub>4</sub>, 1 g NaCl, 1% formic acid (v/v) and 10 mL ethyl acetate was prepared in 50 mL tubes, then 10 mL CFS was added. Tubes were vortexed for 1 min. After a 1 min incubation on ice, the solution was centrifuged for 10 min at 4 °C and 3000 rpm. The supernatant was mixed with 150 mg C18 and 900 mg MgSO<sub>4</sub>, vortexed for 1 min and centrifuged under the same conditions. Finally, the supernatant from this step was evaporated under nitrogen flow.

For the analysis, the purified CFS was suspended in 1 mL water with acetonitrile in a proportion of 90/10 (v/v). Chromatographic determination was performed using an Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) with an autosampler, a binary pump and a vacuum degasser. A Gemini C18 (50 mm × 2 mm, 100 Å, 3 µm particle size; Phenomenex) was used as the column. The column was equilibrated prior to analysis. The mobile phase flow rate was 0.3 mL min<sup>-1</sup>. The solvents consisted of water (A) and acetonitrile (B), and the elution gradient was 0 min, 5% B; 30 min, 95% B; and 35 min, 5% B. Both solvents were acidified with formic acid at 0.1%.

MS analysis was performed with a Q-TOF-MS (6540 Agilent ultra-high-definition accurate mass spectrometer), equipped with an Agilent Dual Jet Stream electrospray ionization interface in

negative ionization mode under the following conditions: drying gas flow (N<sub>2</sub>), 8.0 L min<sup>-1</sup>; nebulizer pressure, 30 psig; gas drying temperature, 350 °C; capillary voltage, 3.5 kV; fragmentor voltage, 175 V; and scan range, *m/z* 20–380. The collision energies used to perform the MS/MS experiment were 10, 20 and 40 eV. Integration and data elaboration were managed using Masshunter Qualitative Analysis Software B.08.00.<sup>20</sup> Results were expressed as milligrams per litre.

### Analysis of the main volatile organic compounds (VOCs) from CFS

Analysis of VOCs was performed by gas chromatography with a single-quadrupole mass spectrometer detector (GC/MS). Samples were prepared for analysis by adding 200 g lyophilized CFS to 2 mL water in a vial; they were then transferred to a 55 °C bath for 45 min with constant stirring with a crystal rod throughout the whole incubation period. The extraction of VOCs from the headspace was performed by solid-phase microextraction (SPME). The SPME holder (Supelco, Bellefonte, PA, USA) contained a fused-silica fibre coated with a 50/30 µm layer of divinylbenzene-carboxen-polydimethylsiloxane. The fibre was introduced into the Agilent 6890N GC system, and thermal desorption of the analytes was performed at 250 °C for 5 min. After extraction, the fibre was inserted in splitless mode into an Agilent 6890N GC system, and thermal desorption of the extracted samples was performed at 250 °C for 5 min. The capillary column (J&W Scientific, Folsom, CA, USA), with an HP-5MS (30 m × 0.25 mm, 0.25 µm 5% diphenyl-95% dimethylpolysiloxane) was used for the analysis. The carrier gas was 99.999% helium, flowing at a rate of 1 mL min<sup>-1</sup>. The program started at 40 °C for 2 min, then increased to 160 °C at 6 min; finally, the temperature was raised to 260 °C at 10 °C min<sup>-1</sup> and remained constant for 40 min. Flow in the column was transferred to an Agilent 5973 MS detector. The ion source temperature was set at 230 °C, the ionizing electron energy was 70 eV and the mass range was 40–450 Da in full-scan acquisition mode. Compound identification was performed with an NIST Atomic Spectra Database version 1.6 (Gaithersburg, MD, USA), using 95% spectral similarity. Three replicas of each analysis were carried out. Results were given as a percentage of each VOC in the CFS by dividing each analyte area by the total area.

### In vitro study on antioxidative activities of CFS

For the study of antioxidant activity, the method used was the 1,1-diphenyl-2-picrylhydrazil (DPPH) free-radical-scavenging

**Table 1.** Results of agar diffusion test of the CFS fermented by LAB diluted at 400 g L<sup>-1</sup> against *Aspergillus* spp. and *Botrytis cinerea*. The antifungal activity was expressed as follows: (+) means <8 mm of inhibition zone between the well and fungal growth; (++) means 8–10 mm of inhibition zone between the well and fungal growth; (+++) means >10 mm of inhibition zone between the well and fungal growth

| Fungi                 | Strain     | CFS  |      |      |    |    |
|-----------------------|------------|------|------|------|----|----|
|                       |            | CTRL | BN16 | BN17 | E3 | E4 |
| <i>A. carbonarius</i> | ISPA 5010  | –    | +    | +    | +  | +  |
| <i>A. niger</i>       | CECT 2088  | –    | +    | +    | +  | +  |
| <i>A. ochraceus</i>   | CECT 2093  | –    | +    | +    | +  | +  |
| <i>A. niger</i>       | CECT 2915  | –    | ++   | ++   | ++ | ++ |
| <i>A. tubingensis</i> | CECT 20543 | –    | +    | +    | +  | +  |
| <i>A. tubingensis</i> | CECT 20545 | –    | ++   | ++   | +  | +  |
| <i>B. cinerea</i>     | CECT 20973 | –    | ++   | ++   | ++ | ++ |

**Table 2.** Results of MIC-MFC method of CFS fermented by LAB against *Aspergillus* spp. and *Botrytis cinerea*. Concentrations are expressed as g L<sup>-1</sup>

| Fungi                 | Strain     | CFS    |        |      |        |       |        |       |        |
|-----------------------|------------|--------|--------|------|--------|-------|--------|-------|--------|
|                       |            | BN16   |        | BN17 |        | E3    |        | E4    |        |
|                       |            | MIC    | MFC    | MIC  | MFC    | MIC   | MFC    | MIC   | MFC    |
| <i>A. carbonarius</i> | ISPA 5010  | >100.0 | >100.0 | 25.0 | 50.0   | 25.0  | 50.0   | 25.0  | 50.0   |
| <i>A. niger</i>       | CECT 2088  | 50.0   | 100.0  | 50.0 | >100.0 | 100.0 | >100.0 | 100.0 | >100.0 |
| <i>A. ochraceus</i>   | CECT 2093  | 50.0   | >100.0 | 12.5 | 100.0  | 12.5  | 100.0  | 12.5  | 100.0  |
| <i>A. niger</i>       | CECT 2915  | 25.0   | 100.0  | 25.0 | >100.0 | 25.0  | 100.0  | 25.0  | 100.0  |
| <i>A. tubingensis</i> | CECT 20543 | 50.0   | >100.0 | 12.5 | 100.0  | 25.0  | 50.0   | 25.0  | 50.0   |
| <i>A. tubingensis</i> | CECT 20545 | >100.0 | >100.0 | 50.0 | >100.0 | 25.0  | >100.0 | 25.0  | >100.0 |
| <i>B. cinerea</i>     | CECT 20973 | 3.1    | 25.0   | 3.1  | 6.3    | 6.3   | 6.3    | 6.3   | 12.5   |

assay according to Khan *et al.*<sup>21</sup> with some modifications. First, CFS was diluted in water in a proportion of 1/4 (v/v). Then, 200 µL of the diluted samples were added to 1 mL of a 0.04 mmol L<sup>-1</sup> suspension of DPPH in methanol and then vortexed for 1 min. After a 2 h incubation in the dark, samples were centrifuged at 11 000 rpm for 5 min. Finally, the absorbance of the samples was recorded at 517 nm in a spectrophotometer. Control of the assay was performed with methanol. Triplicates of each sample were performed. Results were given as a percentage of the antioxidant activity *D* obtained using the equation  $D (\%) = [(B - M)/B] \times 100$ , where *B* is the absorbance of the control and *M* is the absorbance of the samples. Results were given as a percentage of inhibition of DPPH.

Analysis of the total polyphenolic compounds in the CFS was performed by the Folin–Ciocalteu method, as described in Kschonsek *et al.*,<sup>22</sup> with a few modifications. First, the CFS were diluted in water 1/4 (v/v<sup>-</sup>). Then, 780 µL water was mixed with 130 µL of the diluted CFS and 130 µL Folin–Ciocalteu reagent and vortexed for 1 min. Then, 130 µL of a solution of NaCO<sub>3</sub> at 20% (w/v<sup>-</sup>) was added and the mix vortexed for 1 min. After an incubation period of 2 h in the dark at 30 °C, the absorbance of the samples was read at 750 nm. The method was calibrated with gallic acid at concentrations from 8.5 to 140 mg L<sup>-1</sup>. Results were

given as milligrams of gallic acid equivalents per milligram (mg GAE L<sup>-1</sup>).

### Biopreservation of red grapes

To study their antifungal properties, the CFS were tested on grapes contaminated with *A. carbonarius* ISPA 5010, *A. niger* CECT 2088, *A. ochraceus* CECT 2093, *A. tubingensis* CECT 20545 and *B. cinerea* CECT 20973. Grapes were obtained from the Cooperativa Agrícola de Villar del Arzobispo (Valencia, Spain).

Each assay used a total of 35 grapes divided into groups of five in different Petri plates as replicas. Steps followed were adapted from Luz *et al.*<sup>19</sup> To start, grapes were sanitized by submersion in a 1% hypochlorite solution (w/v), then in a 70% ethanol solution (v/v) and finally cleaned with sterilized water. Afterwards, grapes were wounded with a needle, and 1 mL of a solution of 10<sup>4</sup> spores mL<sup>-1</sup> was sprayed on each replica. In the last step, a solution of sterilized water with CFS was sprayed at a final concentration of 33.3 g kg<sup>-1</sup> grapes. Between each step, grapes were left in a laminar flow hood for 1 h to dry. Control was performed using unfermented MRS-B at the same concentration. Finally, Petri plates with the grapes were locked in sterilized boxes and incubated at room temperature for 7 days. The shelf life of the product was studied by visual observation of fungal growth in each grape.

**Table 3.** Identification and quantification of (a) organic acids (g L<sup>-1</sup>) and (b) phenolic compounds (mg L<sup>-1</sup>) produced by LAB in CFS

| (a) Organic acids | CFS         |               |               |               |
|-------------------|-------------|---------------|---------------|---------------|
|                   | BN16        | BN17          | E3            | E4            |
|                   | Lactic acid | 15.38 ± 1.55a | 15.88 ± 1.44a | 18.01 ± 3.31b |
| Acetic acid       | nd          | nd            | 0.94 ± 0.38a  | 0.50 ± 0.15a  |

| (b) Phenolic compounds  | CFS                 |              |              |              |
|-------------------------|---------------------|--------------|--------------|--------------|
|                         | BN16                | BN17         | E3           | E4           |
|                         | Dihydrocaffeic acid | 8.87 ± 1.49a | 5.09 ± 2.76a | 7.31 ± 1.38a |
| Benzoic acid            | 3.96 ± 5.60a        | 4.76 ± 3.23a | 9.01 ± 3.84a | 5.19 ± 0.10a |
| Caffeic acid            | 0.16 ± 0.05a        | 0.31 ± 0.11a | 0.34 ± 0.09a | 0.36 ± 0.02a |
| Phenyllactic acid       | 9.55 ± 0.56a        | 8.15 ± 0.44a | 8.87 ± 0.09a | 8.67 ± 0.68a |
| <i>p</i> -Coumaric acid | 7.50 ± 2.06a        | 4.98 ± 3.83a | 4.7 ± 0.38a  | 4.62 ± 0.56a |
| Syringic acid           | 0.21 ± 0.06a        | 0.08 ± 0.06a | 0.2 ± 0.15a  | 0.12 ± 0.17a |

Different letters represent a significant difference among treatments (*P* < 0.05). The experiment was carried out in triplicate (*n* = 3). Results are expressed as mean ± standard deviation; nd, not detected.

### Determination of the fungal population on grapes

Five replicas were homogenized at random, in a ratio of 1/10 (w/v), with buffered peptone water Tween 80 at a concentration of 0.1% using a Stomacher (IUL, Barcelona, Spain) for 30 s. From the homogenate, two replicas of a serial dilution were performed and plated on PDA.<sup>23</sup> After an incubation period at 25 °C for 48 h, the colonies were counted. Results were reported as spores per gram of fruit.

### Statistical analysis

Results were analysed using InfoStat software version 2008. Tukey's HSD post hoc test for multiple comparisons was employed, with a significance level of  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Identification of LAB strains

Four different bacterial strains were isolated and identified by the MALDI-TOF MS method and classified by the MBT 7854 y MBT 7311\_RUO database. The method confirmed the identity of the different LAB to species level: *L. plantarum* E3, *L. plantarum* E4, *L. plantarum* BN16 and *L. plantarum* BN17.

### Antifungal activity *in vitro*

As shown in Table 1, all CFS showed antifungal activity in the agar diffusion method against the fungi studied. *Aspergillus niger* CECT 2915 and *B. cinerea* CECT 20973 were less resistant to the treatments. Overall, CFS from *L. plantarum* BN16 and *L. plantarum*

**Table 4.** Identification and quantification of the main VOCs from the CFS

| VOC                                      | CFS           |                |               |               |               |
|--|---------------|----------------|---------------|---------------|---------------|
|  | MRS           | BN16           | BN17          | E3            | E4            |
| Alcohols                                 | 38.75 ± 3.36b | 1.94 ± 0.25a   | 1.22 ± 0.11a  | 2.13 ± 0.23a  | 1.23 ± 0.41a  |
| 2-Ethyl-1-hexanol                        | 38.75 ± 3.36  | 1.94 ± 0.25    | 1.22 ± 0.11   | 2.13 ± 0.23   | 1.23 ± 0.41   |
| Aldehydes                                | 19.94 ± 1.1d  | 6.07 ± 0.82abc | 8.74 ± 0.67c  | 4.97 ± 0.87ab | 3.83 ± 0.39a  |
| 3-Methylbutanal                          | nd            | 0.89 ± 0.26    | 1.53 ± 0.37   | 0.8 ± 0.1     | 0 ± 0         |
| Benzaldehyde                             | 10.91 ± 0.82  | 2.17 ± 0.26    | 2.41 ± 0.12   | 1.63 ± 0.24   | 1.55 ± 0.1    |
| Benzene acetaldehyde                     | 6.83 ± 0.05   | 3.02 ± 0.31    | 3.54 ± 0.13   | 2.53 ± 0.53   | 2.27 ± 0.29   |
| Nonanal                                  | 2.19 ± 0.22   | nd             | nd            | nd            | nd            |
| 5-Methyl-2-phenyl-2-hexenal              | nd            | nd             | 1.26 ± 0.06   | nd            | nd            |
| Acids                                    | 6.71 ± 0.29a  | 21.26 ± 0.88c  | 12.09 ± 0.36b | 18.32 ± 0.95c | 20.11 ± 2.16c |
| Acetic acid                              | 6.71 ± 0.29   | 21.26 ± 0.88   | 12.09 ± 0.36  | 18.32 ± 0.95  | 19.66 ± 2.08  |
| <i>n</i> -Decanoic acid                  | nd            | nd             | nd            | nd            | 0.45 ± 0.08   |
| Ketones                                  | nd            | 0.65 ± 0.07b   | 0.49 ± 0.02a  | 0.47 ± 0.05a  | 0.54 ± 0.02a  |
| 2-Nonanone                               | nd            | 0.65 ± 0.07    | 0.49 ± 0.02   | 0.47 ± 0.05   | 0.54 ± 0.02   |
| 2-udecanone                              | nd            | nd             | nd            | nd            | nd            |
| Pyrazines                                | nd            | 65.31 ± 5.53a  | 72.25 ± 2.77a | 69.49 ± 4.86a | 65.21 ± 4.39a |
| Pyrazine, methyl-                        | nd            | 0.85 ± 0.04    | 0.53 ± 0.01   | 1.11 ± 0      | 0.87 ± 0.02   |
| 2,5-Dimethylpyrazine                     | nd            | 5.66 ± 0.85    | 6.08 ± 0.04   | 6.19 ± 0.37   | 4 ± 0.18      |
| 2-Ethyl-6-methylpyrazine                 | nd            | 3.5 ± 0.36     | 3.83 ± 0.21   | 4.34 ± 0.11   | 2.75 ± 0.55   |
| Trimethyl pyrazine                       | nd            | 4.48 ± 0.23    | 1.27 ± 0.01   | 2.59 ± 0.86   | 3 ± 0.2       |
| 3-Ethyl-2,5-dimethylpyrazine             | nd            | 7.94 ± 1.23    | 12.93 ± 0.26  | 10.77 ± 0.51  | 6.11 ± 0.49   |
| 2,3-Dimethyl-5-ethylpyrazine             | nd            | 4.74 ± 0.58    | 2.19 ± 0      | 3.12 ± 0.03   | 4.37 ± 0.4    |
| 2,3-Diethyl-5-methylpyrazine             | nd            | 0.79 ± 0.06    | 1.57 ± 0.21   | 0.97 ± 0.02   | 1.02 ± 0.07   |
| 3,5-Diethyl-2-methylpyrazine             | nd            | 1.55 ± 0.12    | 3.15 ± 0.4    | 2.28 ± 0.03   | 1.61 ± 0.07   |
| 2,3,5-Trimethyl-6-ethylpyrazine          | nd            | 22.17 ± 0.46   | 11.74 ± 0.63  | 15.52 ± 0.56  | 15.85 ± 1.15  |
| 2-Acetyl-3-ethylpyrazine                 | nd            | nd             | 0.48 ± 0.04   | nd            | nd            |
| 3,5-dimethyl-2-isobutylpyrazine          | nd            | 0.44 ± 0       | 0.86 ± 0.01   | 0.41 ± 0.03   | 0.66 ± 0.03   |
| 2-acetyl-3,5-dimethylpyrazine            | nd            | 0.41 ± 0.06    | 0.38 ± 0.01   | 0.27 ± 0.06   | 0.6 ± 0.11    |
| 2-Isoamyl-6-methylpyrazine               | nd            | 0.62 ± 0.02    | 2.47 ± 0.01   | 0.75 ± 0.01   | 1.18 ± 0.12   |
| Trimethylisobutyl pyrazine               | nd            | 1.04 ± 0.32    | 0.84 ± 0.21   | 0.47 ± 0      | 1.26 ± 0.1    |
| 2,6-Dimethyl-3(2-methyl-1-butyl)pyrazine | nd            | 0.29 ± 0.04    | 1.26 ± 0.04   | 0.36 ± 0.02   | 0.7 ± 0.05    |
| 2,5-Dimethyl-3-isopentylpyrazine         | nd            | 2.74 ± 0.34    | 11.49 ± 0.08  | 14.58 ± 1.87  | 5.8 ± 0.12    |
| 2,3-Dimethyl-5-isopentylpyrazine         | nd            | 0.71 ± 0.03    | 1.66 ± 0.18   | 0.6 ± 0.03    | 1.8 ± 0.13    |
| 2,3,5-Trimethyl-6-isopentylpyrazine      | nd            | 7.39 ± 0.78    | 9.53 ± 0.41   | 5.16 ± 0.36   | 13.63 ± 0.62  |
| Others                                   | 34.6 ± 2.55c  | 4.77 ± 0.32a   | 5.2 ± 0.25a   | 4.63 ± 0.37a  | 9.08 ± 0.94b  |
| Toluene                                  | 9.97 ± 2.22   | nd             | nd            | nd            | nd            |
| 7-Methyl-1-naphthol                      | nd            | 0.7 ± 0.03     | 0.55 ± 0.04   | 0.55 ± 0.1    | 0.71 ± 0.09   |
| 9-Methylpoxanthine                       | nd            | 0.58 ± 0.08    | 0.92 ± 0.04   | 0.46 ± 0.04   | 1.44 ± 0.15   |
| 2,4-Di- <i>tert</i> -butylphenol         | 24.63 ± 0.33  | 1.68 ± 0.2     | 2.47 ± 0.13   | 1.88 ± 0.15   | 1.89 ± 0.11   |
| Diethyl phthalate                        | nd            | nd             | nd            | 0.31 ± 0      | 3.09 ± 0.58   |

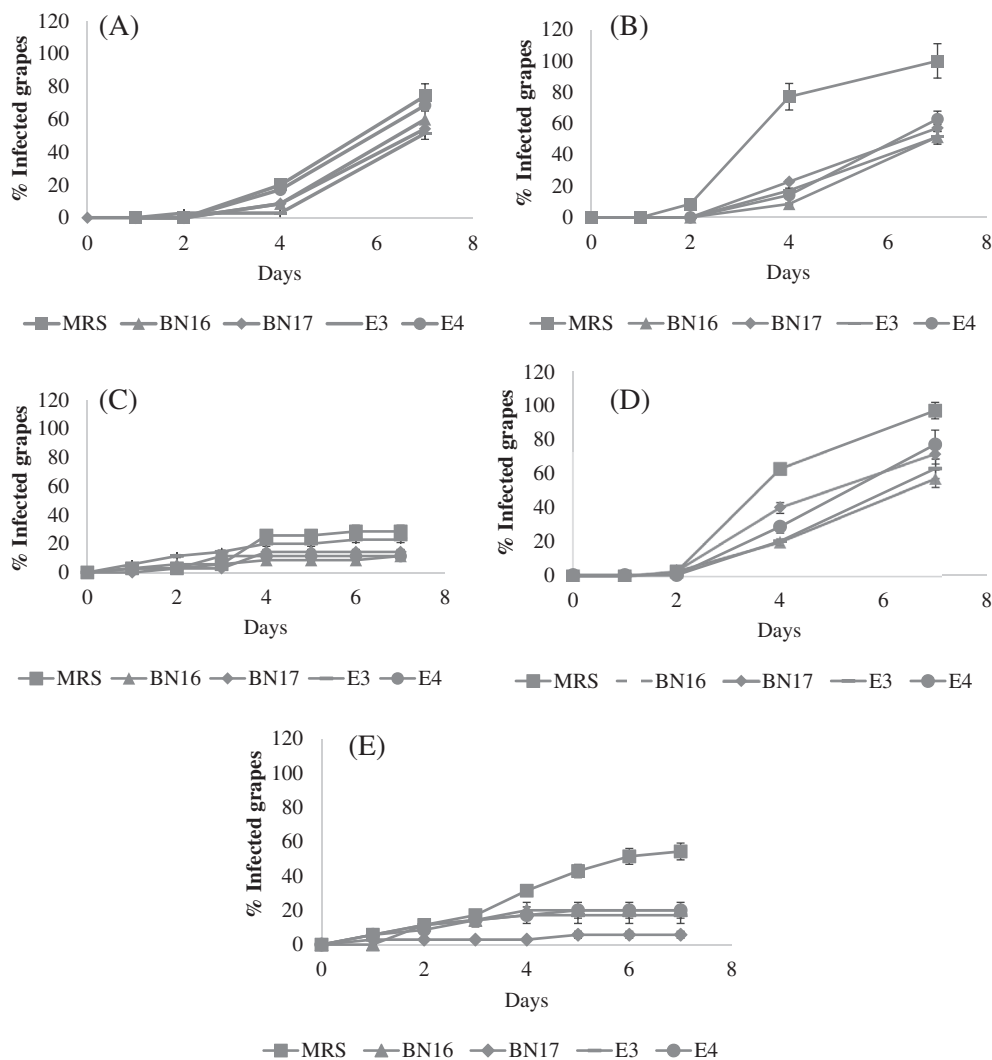
Results are given as a percentage of the total VOCs. Different letters represent a significant difference among treatments ( $P < 0.05$ ). The experiment was carried out in triplicate ( $n = 3$ ). Results are expressed as mean ± standard deviation; nd, not detected.

BN17 showed the largest inhibition halos. These results are similar to the findings of other papers, such as Ouiddir *et al.*,<sup>24</sup> were an *L. plantarum* strain evidenced inhibitor halos against fungi from *Aspergillus* and *Penicillium* genera; or Zamani-Zadeh *et al.*,<sup>25</sup> in which the combined use of essential oils with fermented MRS by a *L. plantarum* strain evidenced a synergic antifungal effect against *B. cinerea*.

The results of MIC-MFC tests are described in Table 2. The CFS from *L. plantarum* BN17, *L. plantarum* BN16, *L. plantarum* E3 and *L. plantarum* E4 showed inhibitory activity against all the fungi tested, reaching MIC from 12.5 to 100 g L<sup>-1</sup> for *Aspergillus* and from 3.1 to 6.3 g L<sup>-1</sup> for *Botrytis*. Treatments from LAB E3 and E4 evidenced the highest antifungal activities, achieving MFCs from 50 to 100 g L<sup>-1</sup> against *A. carbonarius* ISPA 5010, *A. niger* CECT 2088, *A. ochraceus* CECT 2093, *A. niger* CECT 2915 and *A. tubingensis* CECT 20543. In contrast, *A. niger* CECT 2088 and *A. tubingensis* CECT 20545 were the most resistant strains to CFS, showing values of MFC > 100 g L<sup>-1</sup>. All preparations proved to have antifungal properties against *B. cinerea* CECT 20973, showing MFC from 6.3 to 25 g L<sup>-1</sup>, the CFS from *L. plantarum* BN17 and *L. plantarum* E3 being those with the highest activities.

As reported in the literature, most species of *Aspergillus* are highly resistant to antifungal treatments<sup>2</sup>; therefore, the fact that the CFS of these LAB presents antifungal properties against this genus is a promising result. Other articles also evidence activities from LAB against *Aspergillus*; for example, Luz *et al.*,<sup>26</sup> who show MFC of 250 g L<sup>-1</sup>, or de Melo *et al.*,<sup>27</sup> with MFC from 125 to 250 g L<sup>-1</sup> – both higher than the values obtained for *L. plantarum* E3 and *L. plantarum* E4 in this article. Other studies with different biopreservation agents show lower MFCs, such as Negi *et al.*,<sup>28</sup> in which an extract from bryophyte reached an MFC of 7.81 µg L<sup>-1</sup> against *A. flavus*; or Li *et al.*,<sup>29</sup> who evidenced MFC concentrations of 2 µg L<sup>-1</sup> against *A. flavus* using an essential oil extracted from *Illicium verum*.

There are no similar assays to compare the results for *B. cinerea*; this article is probably the first one reporting this activity. Different biopreservation agents found in the literature seem also to have lower MFCs – for example, Aqueveque *et al.*,<sup>30</sup> where extracts from fermented media from *Stereum hirsutum* presented an MFC of 20 µg L<sup>-1</sup> against *B. cinerea* species. In general, other treatments seem to be more effective; nevertheless, extract preparation differs considerably from the one used in this study and, in



**Figure 1.** Results of the biopreservation test on red grape contaminated with *A. carbonarius* ISPA 5010 (A), *A. niger* CECT 2088 (B), *A. ochraceus* CECT 2093 (C), *A. tubingensis* CECT 20545 (D) and *B. cinerea* CECT 20973 (E).

**Table 5.** Total polyphenol content (mg GAE L<sup>-1</sup>) and percent inhibitory activity DPPH of unfermented MRS and CFS fermented by *L. plantarum* strains

| CFS  | % Inhibitory activity DPPH | Total polyphenol content (mg GAE L <sup>-1</sup> ) |
|------|----------------------------|--|
| MRS  | 55.7 ± 4.1a                | 583.6 ± 20.9a                                      |
| BN16 | 88.3 ± 0.5b                | 602.5 ± 30.9a                                      |
| BN17 | 89.0 ± 0.3b                | 599.2 ± 45.1a                                      |
| E3   | 89.0 ± 0.7b                | 539.7 ± 52.6a                                      |
| E4   | 90.1 ± 0.8b                | 610.2 ± 60.3a                                      |

Different letters represent a significant difference among treatments ( $P < 0.05$ ). The experiment was carried out in triplicate ( $n = 3$ ). Results are expressed as mean ± standard deviation.

addition, those extracts cannot be added to food due to the organic solvents used during the purification.

### Identification of antifungal compounds

According to the literature, the antifungal activity of LAB is not due to a single compound but depends on the biocomplex of compounds present in the fermentation medium.<sup>31</sup>

Two organic acids were detected in the CFS, and the results are presented in Table 3(a). Lactic acid and acetic acid were found in concentrations from 15.38 to 18.01 g L<sup>-1</sup> and 0.50 to 0.94 g L<sup>-1</sup>, respectively. Lactic acid was present in all four CFS; nevertheless, acetic acid was only found in CFS fermented by strains E3 and E4. The CFS fermented by strain E4 presented a significant increase in the production of both acids ( $P < 0.05$ ).

The phenolic compounds found in the CFS are shown in Table 3 (b). A total of six different compounds were identified (dihydrocaffeic acid, benzoic acid, caffeic acid, phenyllactic acid, *p*-coumaric acid and syringic acid). No significant differences were found between CFS ( $P < 0.05$ ). Phenyllactic acid, dihydrocaffeic acid and benzoic acid were the most abundant compounds in the CFS, reaching concentrations of 8.15–9.55, 8.87–5.09 and 9.01–3.96 mg L<sup>-1</sup>, respectively. The presence of phenyllactic acid has been linked to some antifungal activities, as reported in Omedi *et al.*<sup>11</sup> or Dagnas *et al.*<sup>32</sup> In both articles, the presence and synergy

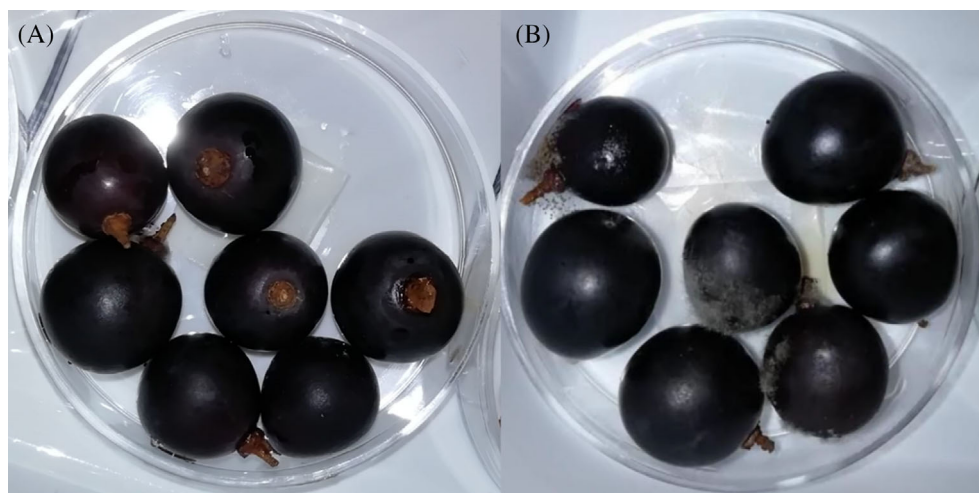
of organic acids and phenolic compounds produced by LAB was linked with antimicrobial activities. There is not unanimous opinion in which the concentrations of these compounds are required to inhibit fungal growth. Gerez *et al.*<sup>33</sup> report MIC values of lactic acid of 2.5–300.0 mmol L<sup>-1</sup>, acetic acid 0.3–120.0 mmol L<sup>-1</sup> and phenyllactic acid 0.02–6.0 mmol L<sup>-1</sup> against *Fusarium graminearum* and *A. niger*. The average concentration of lactic acid (184 mmol L<sup>-1</sup>), acetic acid (12 mmol L<sup>-1</sup>) and phenyllactic acid (0.05 mmol L<sup>-1</sup>) quantified in the CFS are in the order of the MIC reported.

Seventy-eight different VOCs were studied in the samples; 29 of them were present in the CFS fermented by the bacteria – a large number compared with the seven compounds present in the control medium MRS (Table 4). Moreover, the nature of the compounds significantly differed between the control and the CFS. While alcohols, aldehydes and other compounds were more abundant in the MRS, acids, ketones and pyrazines appeared in higher quantities in the media fermented by the LAB. Pyrazines and acids were the most abundant components in the CFS, reaching concentrations of 72–65% and 21–12%, respectively. The inhibition of fungal growth by VOCs produced by bacteria is already well described (Morita *et al.*, 2019). Some articles report activities of pyrazines and volatile acids against fungi, such as the antifungal potential of pulcherriminic acid as a bio-preservative against *B. cinerea*, 1-octen-3-ol and 2,5-dimethyl pyrazine against fungal pathogen *Phaeoemoniella chlamydospore* involved in grapevine trunk diseases, and pyrazine derivatives produced by *Paenibacillus* as potential biocontrol agents in agriculture.<sup>34–37</sup>

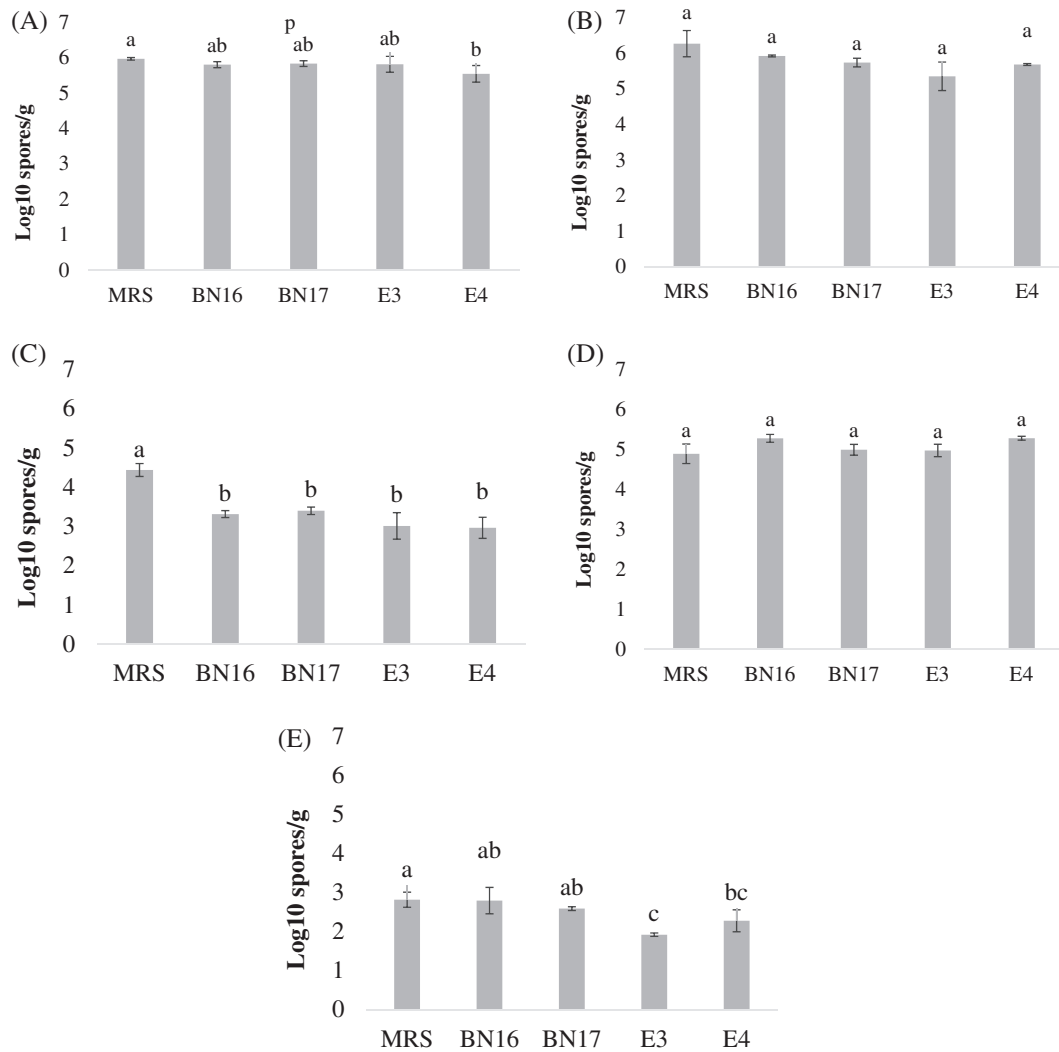
### Antioxidant activity and total polyphenols

The results of the DPPH assay can be seen in Table 5. All CFS showed significantly higher antioxidant activity than the control MRS, with differences ranging from 32% to 34%. Nevertheless, there were no differences between the activity of fermented CFS ( $P < 0.05$ ).

Data acquired from the Folin–Ciocalteu assay are shown in Table 5. All CFS and the control MRS showed similar relative concentrations of polyphenolic compounds ( $P < 0.05$ ). Other articles, such as those by Zhang *et al.*<sup>38</sup> and Sirin *et al.*,<sup>39</sup> reported similar results for DPPH radical-scavenging activity, around 30% higher



**Figure 2.** Fungal growth of *B. cinerea* CECT 20973 on grapes treated with *L. plantarum* BN17 CFS (A) and unfermented MRS-B (B) after 3 days of incubation.



**Figure 3.** Results of determination of the fungal population in red grape contaminated with *A. carbonarius* ISPA 5010 (A), *A. niger* CECT 2088 (B), *A. ochraceus* CECT 2093 (C), *A. tubingensis* CECT 20545 (D) and *B. cinerea* CECT 20973 (E).

than the control. Nevertheless, total polyphenol concentrations seemed to be lower than the average compared to results reached by other LAB.<sup>40</sup>

### LAB CFS in the biopreservation of red grapes

Figure 1 shows the results for the percentage of fruits contaminated per day. Figure 2 shows the antifungal effect of CFS fermented by *L. plantarum* BN17 against *B. cinerea* CECT 20973 in comparison with the treatment with unfermented MRS after 3 days of incubation. After 7 days, a decrease in the contaminated grapes was achieved in the fruits inoculated with *A. niger* CECT 2088, from 37% to 49% less contaminated grapes compared to the MRS control. In grapes inoculated with *B. cinerea* CECT 20973 the fungal growth reduction reached values from 34% to 48% compared to control. No significant reduction was achieved in the number of grapes contaminated with *A. carbonarius* ISPA 5010, *A. ochraceus* CECT 2093 or *A. tubingensis* CECT 20545.

The results of the number of viable spores per gram of grapes are shown in Fig. 3. All four CFS managed to reduce the number of spores per gram of grapes compared with the control in the

fruits inoculated with *A. ochraceus* CECT 2093 and *A. carbonarius* ISPA 5010, from 1.32 and 0.48 log<sub>10</sub> spores g<sup>-1</sup> grapes compared with the MRS control, respectively. In grapes inoculated with *B. cinerea* CECT 20973, the CFS from *L. plantarum* E3 evidenced a decrease of 0.94 log<sub>10</sub> spores g<sup>-1</sup>. Treatments against the other two fungi did not show any significant count spore reduction.

The data obtained regarding *A. carbonarius* is interesting because this species is the principal ochratoxin A producer in grapes. This fungal metabolite is the only mycotoxin with legislated concentration limits in grapes.<sup>41</sup> Other LAB-based treatments exhibited comparable results in grapes inoculated with this fungi, such as Lappa et al.,<sup>16</sup> where two strains of *L. plantarum* reduced fungal growth on damaged grapes by 30–36%. Different biopreservation techniques also seem to have positive results against fungal development, such as Tryfinopoulou et al.,<sup>42</sup> where direct inoculation from a strain of *Saccharomyces cerevisiae* reduced the population of *A. carbonarius* to below detectable levels on non-damaged grapes. In the case of *B. cinerea*, the significant reduction in fungal growth achieved in this study is really positive due to the fact that this genus is the



main fungal grape pathogen.<sup>43</sup> Some studies also presented a similar reduction of fungal growth by using a coating of LAB as a biopreservative on lightly damaged grapes;<sup>44</sup> or, like Zhou *et al.*,<sup>45</sup> where the direct application of a strain of *Bacillus amyloliquefaciens* at concentrations of  $10^7$  CFU mL<sup>-1</sup> per gram of fruit was able to reduce the growth of this fungus. In general, due to differences in the application of the treatments, no true comparisons can be performed between the methods found in the literature and this assay. Nevertheless, the data show that LAB-based treatments seem to be effective against both grape pathogens, so further investigations should be performed to improve and optimize this method of grape biopreservation.

## CONCLUSION

*In vitro* antifungal assays showed promising results for the CFS made by *L. plantarum* E3 and *L. plantarum* E4, which evidenced an inhibition of fungal growth against all *Aspergillus* and *Botrytis* samples tested. A wide pool of different antifungal compounds were detected, including acetic acid, phenyllactic acid and pyrazines. Those compounds were present in higher amounts in the CFS produced by strains *L. plantarum* E3 and *L. plantarum* E4. There was an average 33% increase in antioxidant activity in all CFS compared to the control. Finally, results of *in vivo* studies on red grapes show a possible novel biopreservation application for the reduction of fungal growth from all CFS against *A. ochraceus* and for *L. plantarum* E3 CFS against *B. cinerea*.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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