Research Article

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

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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^{-1}. 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 $\log_{10}$ of the spores per gram of fruit was achieved by all CFS in grapes inoculated with *Aspergillus ochraceus* and by 0.94 $\log_{10}$ 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.

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.[1] Fungi are the main agent in this deterioration.[2] One of the most affected industries is the grape sector – the most extended horticultural crop in the world.[3] Fungi causes losses to the viticultural industry of millions of euros annually.[4] 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,[5] or even affect the health of consumers by producing secondary toxic metabolites.[6]

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.[7] This leads to the second problem: rising consumer demand to reduce the presence of antifungal compounds in food.[6] Finally, the abuse of these antifungal treatments is associated with rising resistance of mould to these compounds.[8] 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*.[9]

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

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literature that prove their ability to preserve food against fungi.[10-12] 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.[6,8,12-14]

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.[15,16]

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^{-1}) 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 ($MgSO_4$) 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^{-1}–10^{-7}) 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.*[17] The method was performed with MALDI-TOF MS using a Microflex L20 (Bruker Daltonics, Billerica, MA, USA) mass spectrophotometer equipped with an N_2 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.[18] The lyophilized CFS was suspended in PDB to a concentration of 200 g L^{-1}. 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.*[19] 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^{-1} in PDB medium, then 100 μL from a suspension of 5×10^4 spores mL^{-1} 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.

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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^{-1} 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^{-1}. 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 $MgSO_4$, 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 $MgSO_4$, 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^{-1}. 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_2), 8.0 L min^{-1}; 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.[20] 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, Bellafonte, 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^{-1}. 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^{-1} 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-picryl-hydrazil (DPPH) free-radical-scavenging

Table 1. Results of agar diffusion test of the CFS fermented by LAB diluted at 400 g L^{-1} 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

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

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Table 2. Results of MIC-MFC method of CFS fermented by LAB against *Aspergillus* spp. and *Botrytis cinerea*. Concentrations are expressed as g L^{-1}

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

assay according to Khan *et al.*[21] 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^{-1} 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.*,[22] with a few modifications. First, the CFS were diluted in water 1/4 (v/v^{-}). 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 $NaCO_3$ at 20% (w/v^{-}) 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^{-1}. Results were given as milligrams of gallic acid equivalents per milligram (mg GAE L^{-1}).

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.*[19] 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 10^4 spores mL^{-1} 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^{-1} 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^{-1}) and (b) phenolic compounds (mg L^{-1}) produced by LAB in CFS

	CFS			
(a) Organic acids	BN16	BN17	E3	E4
Lactic acid	15.38 ± 1.55a	15.88 ± 1.44a	18.01 ± 3.31b	17.44 ± 3.58ab
Acetic acid	nd	nd	0.94 ± 0.38a	0.50 ± 0.15a
	CFS			
(b) Phenolic compounds	BN16	BN17	E3	E4
Dihydrocaffeic acid	8.87 ± 1.49a	5.09 ± 2.76a	7.31 ± 1.38a	6.08 ± 1.11a
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
p-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.

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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.[23] 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

	CFS				
VOC	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
n-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-*tert*-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.

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BN17 showed the largest inhibition halos. These results are similar to the findings of other papers, such as Ouiddir *et al.*,[24] were an *L. plantarum* strain evidenced inhibitor halos against fungi from *Aspergillus* and *Penicillium* genera; or Zamani-Zadeh *et al.*,[25] 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^{-1} for *Aspergillus* and from 3.1 to 6.3 g L^{-1} for *Botrytis*. Treatments from LAB E3 and E4 evidenced the highest antifungal activities, achieving MFCs from 50 to 100 g L^{-1} 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^{-1}. All preparations proved to have antifungal properties against *B. cinerea* CECT 20973, showing MFC from 6.3 to 25 g L^{-1}, 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[2]; 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.*,[26] who show MFC of 250 g L^{-1}, or de Melo *et al.*,[27] with MFC from 125 to 250 g L^{-1} – 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.*,[28] in which an extract from bryophyte reached an MFC of 7.81 μg L^{-1} against *A. flavus*; or Li *et al.*,[29] who evidenced MFC concentrations of 2 μg L^{-1} 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.*,[30] where extracts from fermented media from *Stereum hirsutum* presented an MFC of 20 μg L^{-1} 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

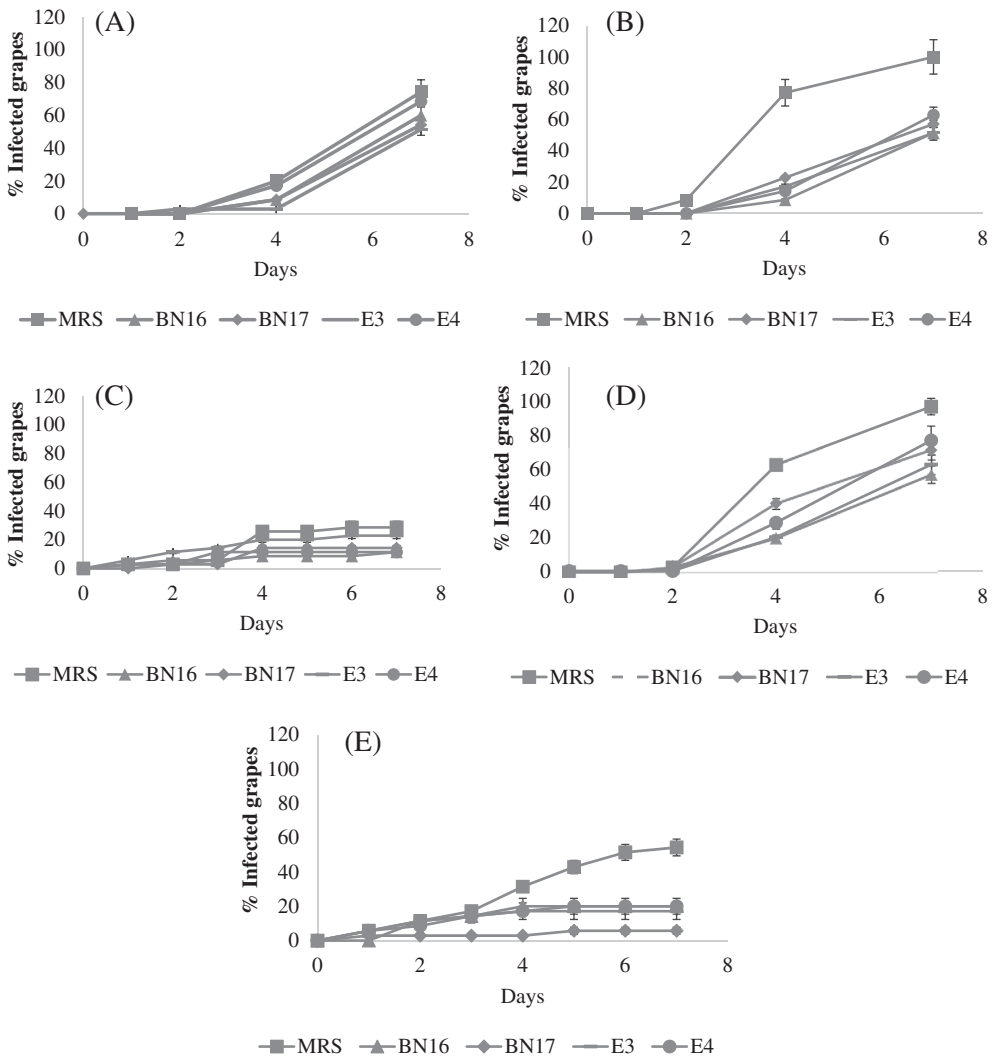

Figure 1. Results of the biopreservation test on red grape contaminated with *A. carbonarius* ISPA 5010 (A), *A. niger* CECT 2088 (B), *A. ochraceus* CECT 2093 (C), *A. tubingensis* CECT 20545 (D) and *B. cinerea* CECT 20973 (E).

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Table 5. Total polyphenol content (mg GAE L^{-1}) 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^{-1})
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.[31]

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^{-1} and 0.50 to 0.94 g L^{-1}, 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^{-1}, respectively. The presence of phenyllactic acid has been linked to some antifungal activities, as reported in Omedi *et al.*[11] or Dagnas *et al.*[32] 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.*[33] report MIC values of lactic acid of 2.5–300.0 mmol L^{-1}, acetic acid 0.3–120.0 mmol L^{-1} and phenyllactic acid 0.02–6.0 mmol L^{-1} against *Fusarium graminearum* and *A. niger*. The average concentration of lactic acid (184 mmol L^{-1}), acetic acid (12 mmol L^{-1}) and phenyllactic acid (0.05 mmol L^{-1}) 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 biopreservative against *B. cinerea*, 1-octen-3-ol and 2,5- dimethyl pyrazine against fungal pathogen *Phaeomoniella chlamydospore* involved in grapevine trunk diseases, and pyrazine derivatives produced by *Paenibacillus* as potential biocontrol agents in agriculture.[34-37]

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.*[38] and Sirin *et al.*,[39] reported similar results for DPPH radical-scavenging activity, around 30% higher

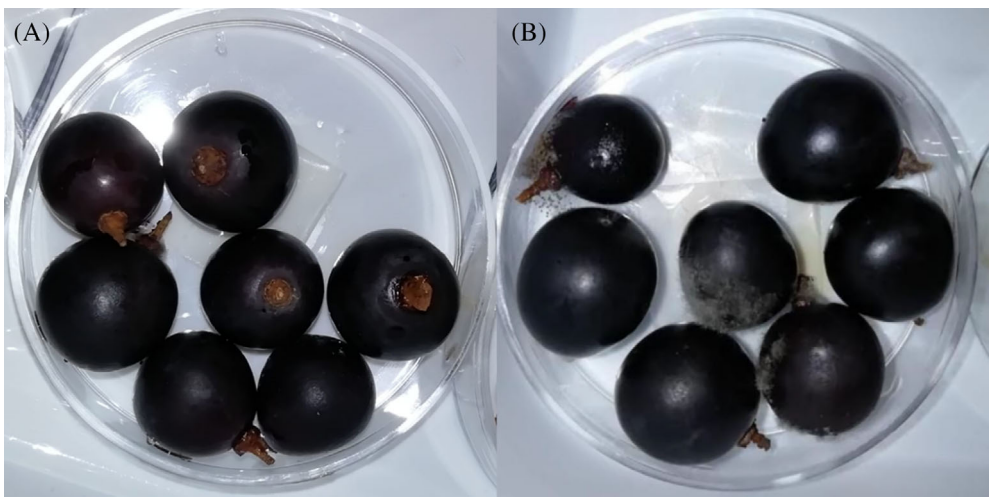

Figure 2. Fungal growth of *B. cinerea* CECT 20973 on grapes treated with *L. plantarum* BN17 CFS (A) and unfermented MRS-B (B) after 3 days of incubation.

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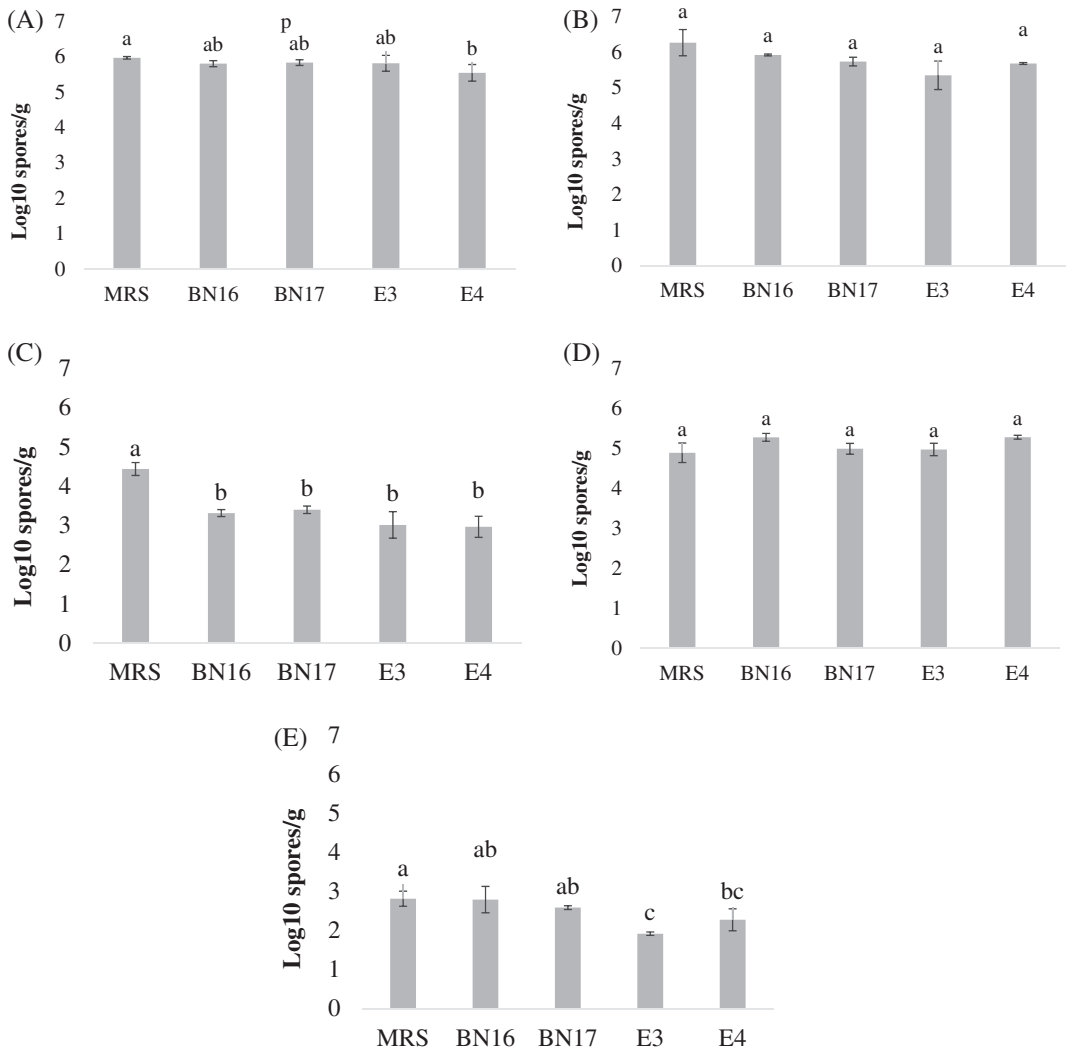

Figure 3. Results of determination of the fungal population in red grape contaminated with *A. carbonarius* ISPA 5010 (A), *A. niger* CECT 2088 (B), *A. ochraceus* CECT 2093 (C), *A. tubingensis* CECT 20545 (D) and *B. cinerea* CECT 20973 (E).

than the control. Nevertheless, total polyphenol concentrations seemed to be lower than the average compared to results reached by other LAB.[40]

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 $\log_{10}$ spores g^{-1} 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 $\log_{10}$ spores g^{-1}. 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.[41] Other LAB-based treatments exhibited comparable results in grapes inoculated with this fungi, such as Lappa *et al.*,[16] 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.*,[42] 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

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main fungal grape pathogen.[43] Some studies also presented a similar reduction of fungal growth by using a coating of LAB as a biopreservative on lightly damaged grapes;[44] or, like Zhou *et al*.,[45] where the direct application of a strain of *Bacillus amyloliquefaciens* at concentrations of 10^7 CFU mL^{-1} 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.

REFERENCES

1 FAO, *The State of Food and Agriculture 2019: Moving Forward on Food Loss and Waste Reduction*. FAO, Rome (2019).
2 Pitt JI and Hocking AD, *Fungi and Food Spoilage*, 3rd edn. Springer, Berlin (2009).
3 This P, Lacombe T and Thomas MR, Historical origins and genetic diversity of wine grapes. *Trends Genet* **22**:511–519 (2006).
4 Barata A, Malfeito-Ferreira M and Loureiro V, The microbial ecology of wine grape berries. *Int J Food Microbiol* **153**:243–259 (2012).
5 La Guerche S, Dauphin B, Pons M, Blancard D and Darriet P, Characterization of some mushroom and earthy off-odors microbially induced by the development of rot on grapes. *J Agric Food Chem* **54**:9193–9200 (2006).
6 Leyva Salas M, Mounier J, Valence F, Coton M, Thierry A and Coton E, Antifungal microbial agents for food biopreservation: a review. *Microorganisms* **5**:37 (2017).
7 Jabłońska-Trypuć A, Wołejko E, Wydro U and Butarewicz A, The impact of pesticides on oxidative stress level in human organism and their activity as an endocrine disruptor. *J Environ Sci Health B* **52**:483–494 (2017).
8 Gupta R and Srivastava S, Antifungal effect of antimicrobial peptides (AMPs LR14) derived from *lactobacillus plantarum* strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiol* **42**:1–7 (2014).
9 Saito S, Wang F and Xiao CL, Efficacy of natamycin against gray mold of stored mandarin fruit caused by isolates of *Botrytis cinerea* with multiple fungicide resistance. *Plant Dis* **104**:787–792 (2020).
10 Valerio F, Favilla M, De Bellis P, Sisto A, de Candia S and Lavermicocca P, Antifungal activity of strains of lactic acid bacteria isolated from a semolina ecosystem against *Penicillium roqueforti*, *Aspergillus niger* and *Endomyces fibuliger* contaminating bakery products. *Syst Appl Microbiol* **32**:438–448 (2009).
11 Omedi JO, Huang W and Zheng J, Effect of sourdough lactic acid bacteria fermentation on phenolic acid release and antifungal activity in pitaya fruit substrate. *Food Sci Technol* **111**:309–317 (2019).
12 Gajbhiye M and Kapadnis B, Bio-efficiency of antifungal lactic acid bacterial isolates for pomegranate fruit rot management. *Proc Natl Acad Sci India Sect B Biol Sci* **88**:1477–1488 (2018).
13 Varsha KK, Devendra L, Shilpa G, Priya S, Pandey A and Nampoothiri KM, 2,4-Di-*tert*-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp. *Int J Food Microbiol* **211**:44–50 (2015).
14 Li X, Wang X, Shi X, Wang B, Li M, Wang Q *et al*., Antifungal effect of volatile organic compounds from *Bacillus velezensis* CT32 against *Verticillium dahliae* and *Fusarium oxysporum*. *Processes* **8**:1–14 (2020).
15 Aqueveque P, Céspedes CL, Becerra J, Aranda M and Sterner O, Antifungal activities of secondary metabolites isolated from liquid fermentations of *Stereum hirsutum* (Sh134-11) against *Botrytis cinerea* (grey mould agent). *Food Chem Toxicol* **109**:1048–1054 (2017).
16 Lappa IK, Mparampouti S, Lanza B and Panagou EZ, Control of *Aspergillus carbonarius* in grape berries by *Lactobacillus plantarum*: a phenotypic and gene transcription study. *Int J Food Microbiol* **275**:56–65 (2018).
17 Maier T, Klepel S, Renner U and Kostrzewa M, Fast and reliable MALDI-TOF MS-based microorganism identification. *Nat Methods* **3**:i–ii (2006).
18 Bauer AW, Kirby WM, Sherris JC and Turck M, Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**:493–496 (1966).
19 Luz C, Dopazo V, Quiles JM, Romano R, Mañes J and Meca G, Biopreservation of tomatoes using fermented media by lactic acid bacteria. *LWT – Food Sci Technol* **130**:109618 (2020).
20 Denardi-Souza T, Luz C, Mañes J, Badiale-Furlong E and Meca G, Antifungal effect of phenolic extract of fermented rice bran with *Rhizopus oryzae* and its potential use in loaf bread shelf life extension. *J Sci Food Agric* **98**:5011–5018 (2018).
21 Khan SA, Noreen F, Kanwal S, Iqbal A and Hussain G, Green synthesis of ZnO and Cu-doped ZnO nanoparticles from leaf extracts of *Abutilon indicum*, *Clerodendrum infortunatum*, *Clerodendrum inerme* and investigation of their biological and photocatalytic activities. *Mater Sci Eng C* **82**:46–59 (2018).
22 Kschonsek J, Wolfram T, Stöckl A and Böhm V, Polyphenolic compounds analysis of old and new apple cultivars and contribution of polyphenolic profile to the *in vitro* antioxidant capacity. *Antioxidants* **7**:20 (2018).
23 International Organization for Standardization, Part 1: Colony count technique in products with water activity greater than 0.95, in *Microbiology of Food and Animal Feeding Stuffs: Horizontal Method for the Enumeration of Yeasts and Moulds*. International Organization for Standardization, Geneva, p. 8 (2008).
24 Ouiddir M, Bettache G, Leyva Salas M, Pawtowski A, Donot C, Brahimi S *et al*., Selection of Algerian lactic acid bacteria for use as antifungal bioprotective cultures and application in dairy and bakery products. *Food Microbiol* **82**:160–170 (2019).
25 Zamani-Zadeh M, Soleimanian-Zad S and Sheikh-Zeinoddin M, Biocontrol of gray mold disease on strawberry fruit by integration of *Lactobacillus plantarum* A7 with ajwain and cinnamon essential oils. *J Food Sci* **78**:1–7 (2013).
26 Luz C, Rodriguez L, Romano R, Mañes J and Meca G, A natural strategy to improve the shelf life of the loaf bread against toxigenic fungi: the employment of fermented whey powder. *Int J Dairy Technol* **73**:88–97 (2020).
27 de Melo NT, Luz C, Torrijos R, Quiles JM, Luciano FB, Mañes J *et al*., Potential application of lactic acid bacteria to reduce aflatoxin B1 and fumonisin B1 occurrence on corn kernels and corn ears. *Toxins* **12**:1–16 (2019).
28 Negi K, Asthana AK and Chaturvedi P, GC–MS analysis and antifungal activity of acetone extract of *Conocephalum conicum* (L) Underw (liverwort) against aflatoxins producing fungi. *S Afr J Bot* **131**:384–390 (2020).
29 Li Y, Wang Y, Kong W, Yang S, Luo J and Yang M, *Illicium verum* essential oil, a potential natural fumigant in preservation of lotus seeds from fungal contamination. *Food Chem Toxicol* **141**:111347 (2020).

30 Aqueveque P, Céspedes CL, Alarcón J, Schmeda-Hirschmann G, Cañumir JA, Becerra J *et al*., Antifungal activities of extracts produced by liquid fermentations of Chilean *Stereum* species against *Botrytis cinerea* (grey mould agent). *Crop Prot* **89**:95–100 (2016).
31 Sadiq FA, Yan B, Tian F, Zhao J, Zhang H and Chen W, Lactic acid bacteria as antifungal and anti-mycotoxigenic agents: a comprehensive review. *Compr Rev Food Sci Food Saf* **18**:1403–1436 (2019).
32 Dagnas S, Gauvry E, Onno B and Membré JM, Quantifying effect of lactic, acetic, and propionic acids on growth of molds isolated from spoiled bakery products. *J Food Prot* **78**:1689–1698 (2015).
33 Gerez CL, Torino MI, Rollán G and Font de Valdez G, Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control* **20**:144–148 (2009).
34 Morita T, Tanaka I, Ryuda N, Ikari M, Ueno D and Someya T, Antifungal spectrum characterization and identification of strong volatile organic compounds produced by *Bacillus pumilus* TM-R. *Heliyon* **5**: e01817 (2019).
35 Gore-lloyd D, Sumann I, Brachmann AO, Schneeberger K, Ortiz-Merino RA, Moreno-Beltrán M *et al*., Snf2 controls pulcherriminic acid biosynthesis and antifungal activity of the biocontrol yeast *Metschnikowia pulcherrima*. *Mol Microbiol* **112**:317–332 (2019).
36 Haidar R, Roudet J, Bonnard O, Dufour MC, Corio-Costet MF and Fert M, Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grape vine trunk diseases. *Microbiol Res* **192**:172–184 (2016).
37 Rybakova D, Cernava T, Köberl M, Liebminger S, Etemadi M and Berg G, Endophytes-assisted biocontrol: novel insights in ecology and the mode of action of *Paenibacillus*. *Plant Soil* **405**:125–140 (2016).
38 Zhang DI, Li C, Shi R, Zhao F and Yang Z, *Lactobacillus fermentum* JX306 restrain D-galactose-induced oxidative stress of mice through its antioxidant activity. *Pol J Microbiol* **69**:205–215 (2020).
39 Sirin S and Belma A, Characterization of lactic acid bacteria derived exopolysaccharides for use as a defined neuroprotective agent against amyloid beta1–42-induced apoptosis in SH-SY5Y cells. *Sci Rep* **10**:8124 (2020).
40 Sirilun S, Sivamaruthi BS, Kesika P, Peerajan S and Chaiyasut C, *Lactobacillus paracasei* hii01 mediated fermentation of *Syzygium cumini* l. fruits: assessment of changes in phenolic content and antioxidant capacity. *Asian J Pharm Clin Res* **11**:304–308 (2018).
41 European Commission. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Vol. 2006 (2006).
42 Tryfinopoulou P, Chourdaki A, Nychas GJE and Panagou EZ, Competitive yeast action against *Aspergillus carbonarius* growth and ochratoxin a production. *Int J Food Microbiol* **317**:108460 (2020).
43 Masmoudi F, Ben Khedher S, Kamoun A, Zouari N, Tounsi S and Trigui M, Combinatorial effect of mutagenesis and medium component optimization on *Bacillus amyloliquefaciens* antifungal activity and efficacy in eradicating *Botrytis cinerea*. *Microbiol Res* **197**:29–38 (2017).
44 Marín A, Plotto A, Atarés L and Chiralt A, Lactic acid bacteria incorporated into edible coatings to control fungal growth and maintain postharvest quality of grapes. *HortScience* **54**:337–343 (2019).
45 Zhou Q, Fu M, Xu M, Chen X, Qiu J, Wang F *et al*., Application of antagonist *Bacillus amyloliquefaciens* NCPSJ7 against *Botrytis cinerea* in postharvest red globe grapes. *Food Sci Nutr* **8**:1499–1508 (2020).

