Antibiosis of vineyard ecosystem fungi against food-borne microorganisms

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Abstract

Fermentation extracts from fungi isolated from vineyard ecosystems were tested for antimicrobial activities against a set of test microorganisms, including five food-borne pathogens (*Staphylococcus aureus* EP167, *Acinetobacter baumannii* (clinical isolated), *Pseudomonas aeruginosa* PAO1, *Escherichia coli* O157:H7 (CECT 5947) and *Candida albicans* MY1055) and two probiotic bacteria (*Lactobacillus plantarum* LCH17 and *Lactobacillus brevis* LCH23). A total of 182 fungi were grown in eight different media, and the fermentation extracts were screened for antimicrobial activity. A total of 71 fungi produced extracts active against at least one pathogenic microorganism, but not against any probiotic bacteria. The Gram-positive bacterium, *Staphylococcus aureus* EP167, was more susceptible to antimicrobial fungi broths extracts than Gram-negative bacteria and pathogenic fungi. Identification of active fungi based on internal transcribed spacer rRNA sequence analysis revealed that species in the orders *Pleosporales*, *Hypocreales* and *Xylariales* dominated. Differences in antimicrobial selectivity were observed among isolates from the same species. Some compounds present in the active extracts were tentatively identified by liquid chromatography-mass spectrometry. Antimicrobial metabolites produced by vineyard-ecosystem fungi may potentially limit colonization and spoilage of food products by food-borne pathogens, with minimal effect on probiotic bacteria.

Keywords

Antimicrobial screening; fungi; vineyard ecosystem; food-borne microorganisms; secondary metabolites

1. Introduction

Foods are commonly contaminated by pathogenic bacteria and yeasts that may cause food spoilage and food-borne diseases in humans (Ray, 1996; Vazquez et al., 1993; Velusamy et al., 2010), especially in hospital environments. In contrast, occurrence of probiotic bacteria in food, such as bifidobacteria and lactobacilli, confers health benefits to the host (Lebeer et al., 2008). New trends recommend the reduction of the use of chemically synthesized preservatives in favour of natural alternatives that guarantee sufficiently prolonged shelf-life of foods and ensure food safety with respect to food-borne pathogens. In the search for use molecules, microorganisms have emerged as an effective source of natural substances that could be used as preservatives in order to ensure food preservation and safety (Wiyakrutta et al., 2004).

Fungi are well-known to produce both beneficial and deleterious natural products for human health and nutrition (Demain, 2000) and continue to be investigated as useful sources of natural products -secondary metabolites- (Hoffmeister et al., 2007) for their potential medical, industrial and agricultural use (Bills et al., 1994; Calvo et al., 2002; Li et al., 2005; Liu et al., 2008). Natural products screening programmes have often focused on searching of antibiotics (Basilio et al., 2003; González del Val et al., 2001; Peláez et al., 1998; Suay et al., 2000), although some antibiotically-active molecules could have unexpected alternative applications (Demain, 1998). Antibiotic screening does not only provide candidate compounds useful for a target application, but also antibiotic activity may be indicative of complementary bioactivities and suggests high priority status for broad-based pharmacological, microbiological, molecular biological, and agricultural testing of the fungi-originated compound or mixture compounds (Demain, 1998).

Grapevines (*Vitis vinifera* L.) are one of the most important fruit species worldwide because their fruit is the basis of wine production (Ali et al., 2009). In their natural

environment, grapevine trunks are a host to a number of fungi and yeasts. The fungi most frequently isolated from grapevine ecosystem are *Fusarium* spp, *Cylindrocarpon* spp, *Alternaria* spp, *Penicillium* spp, *Trichoderma* spp, and *Pestalotiopsis* spp (Halleen et al., 2003). Regarding secondary metabolites produced by grapevine fungi; most of the previous studies have focused on pathogenic fungi such as *Botryosphaeria obtusa*, *Botrytis cinerea* and *Eutypa lata* (Djoukeng et al., 2009; González Collado et al., 2007; Jiménez Teja et al., 2006; Molyneux et al., 2002). We have built a collection of fungi associated from these environments to explore for potential applications of their metabolic products, including their enzymes, small molecular weight metabolites, and genomic DNAs.

Using components of this collection, we set out to test the hypothesis that vineyard ecosystem fungi might produce natural products able to selectively inhibit food-borne pathogens without limiting the growth of beneficial probiotic bacteria. The pathogenic microorganisms evaluated were: *E. coli* O157:H7 (CECT 5947), *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* EP167, *Acinetobacter baumannii* (clinical isolated) and *Candida albicans* MY1055, and the probiotic bacteria *Lactobacillus plantarum* LCH17 and *Lactobacillus brevis* LCH23. DNA from the fungi whose extracts showed selective antimicrobial activity against pathogens was purified and their internal transcribed spacer rRNA regions were amplified and sequenced for molecular identification. Additionally, active extracts were also analysed by liquid chromatography-mass spectrometry (LC-MS) for identification of active compounds.

2. Materials and methods

2.1. *Isolation of fungi from grapevine plants and soils*

Vineyard soil and plants were sampled at two locations in the province of Madrid (Villamanrique del Tajo (VT) and Escuela de la Vid (EV)), one location in the province of Guadalajara (Tortuero, (T)) and one location in the province of Ciudad Real (Membrilla, (M)), all in Central Spain. To isolate endophytic fungi, grapevine stems were cut from grapevine plants, place in clean paper envelopes, and transported to the laboratory at ambient temperature in the same day. Samples were stored at 4 °C up to 48 h before processing. Bark and leaf bud surfaces were disinfected by sequential 30 sec washes in 70% ethanol, 5% sodium hypochlorite, 70% ethanol and sterile water (bark samples), and 70% ethanol and sterile H_2O (leaf bud samples). To obtain xylem samples, grapevine stems were split at the distal end to expose the fresh uncontaminated xylem, and small chips were removed aseptically from the centre of the stem's interior with a sterile scalpel and forceps. After surface decontamination, individual bark fragments, xylem chips and leaf buds were aseptically transferred to each well of 48-well tissue culture plates containing YMC medium [malt extract (Becton Dickinson), 10 g; yeast extract (Becton Dickinson), 2 g; agar (Conda), 20 g; cyclosporin A, 4 mg; streptomycin sulfate, 50 mg; terramycin, 50 mg; distilled water 1 L]. Eighteen 48-well microplates were prepared per plant (six for bark fragments, six for xylem chips and six for leaf buds). Isolation plates were dried briefly in a laminar flow hood to remove excess liquid from agar surfaces, and incubated for two weeks at 22 ºC and 70% relative humidity.

Soil samples were sieved before fungi isolation. Soils aliquots were first washed and separated into particles using a particle filtration method in order to reduce the number of colonies of heavily sporulating fungi (Bills et al., 2004). Washed soil particles were plated

using a dilution-to-extinction strategy (Collado et al., 2007; Sánchez Márquez et al., 2011). Approximately 0.5 cm^2 of washed soil particles were resuspended in 30 mL of sterile H_2O. Ten-microliter aliquots of particle suspensions were pipetted per well into 48-well tissue culture plates containing YMC medium. Nine (three per dilution) 48-well microplates were prepared per sample. Isolation plates were dried briefly in a laminar flow hood to remove excess liquid from agar surfaces, and incubated for two weeks at 22 ºC and 70% relative humidity.

From each type of isolation plate, individual colonies were transferred to YM plates [malt extract, 10 g; yeast extract, 2 g; agar, 20 g; 1 L distilled H_2O] and incubated for 3 weeks. Isolates were classified into 'morphospecies' on the basis of colony morphology (Bills et al., 2004). Morphospecies groupings were re-evaluated and consolidated following analyses of internal transcribed spacer (ITS) sequence data, and representative isolates were selected for screening. Representative strains were preserved as frozen agar plugs in vials containing 10% glycerol at -80 °C. Strains are available from Fundación MEDINA Culture Collection, Granada, Spain (www.medinaandalucia.es).

2.2. *Fungal fermentation and metabolite extraction*

Media formulations, tools and protocols for fermenting fungi in nutritional arrays and extracting metabolites from mycelium have been described previously (Bills et al., 2008, 2009; Duetz et al., 2010; Vicente et al, 2009). Briefly, each strain was grown as a liquid hyphal suspension in tubes. Hyphal suspensions from sets of 80 strains were transferred to the centre 80 wells of a master plate. Inoculum in the master plate was replicated with a pin tool across eight new plates each containing different fermentation medium at 1 mL per well to generate an eight-medium by 80 strain nutritional arrays.

To extract each well of the nutritional array, the mycelia adhering to the well walls were gently dislodged by introducing a block of fixed 200 µL pipette tips five times, and 850 µL of acetone was added to each well. Plates were sealed with a silicone mat and were shaken for 30 min in one direction, and 30 min in the opposite direction, at 220 rpm and 22 ºC. To retain metabolites in solution, 170 µL of dimethyl sulfoxide (DMSO) was added to each well, and after 5 min shaking, the acetone was evaporated in a Genevac HT-24 vacuum centrifuge for 75 min. Plates were opened in a chemical fume hood and air dried for about 2 h more. Extracted mycelium was pushed to the bottom of the wells with a metal plunger (Duetz et al., 2010). About 500 µL of the acetone-medium supernatant from each well was transferred to 800 µL-well assay plates (AB-gene AB-0765). After addition of 165 µL of water, each well contained an aqueous 665 µL sample that was 0.75× the concentration of the original fermentation and contained 20% DMSO. Extracts were stored at -4 ºC for 2 days and were briefly shaken on a MicroMix plate mixer prior to assay.

2.3. *Evaluation of the antimicrobial activity*

Test microorganisms and assay plates. In vitro antimicrobial activity susceptibility was determined by using a panel of seven microorganisms (Table 1). The probiotic strains were kindly provided by Dr. J.M. Rodriguez from the Department of Nutrition and Food and Science Technology, Universidad Complutense de Madrid (Madrid, Spain) and included: *L. plantarum* LCH17 and *L. brevis* LCH23, which were isolated from milk of healthy mothers (Jiménez et al., 2008; Martín et al., 2003). The human pathogenic reference strains, *E. coli* O157:H7 (CECT 5947; virulence factor deleted) was obtained from the Spanish Type Culture Collection (CECT), whereas *Acinetobacter baumannii* (clinical isolated), *Candida albicans* MY1055, *Staphylococcus aureus* EP167 (methicillin-

susceptible *S. aureus*) (Novick, 1990), and *Pseudomonas aeruginosa* PAO1 (Holloway et al., 1979) were from the Fundación MEDINA Culture Collection. Culture maintenance, assay growth conditions and antibiotics used as positive and negative control have been detailed previously (Cueva et al., 2010) and are summarized in Table 1.

Antimicrobial activity assay. For screening the antimicrobial activity of fungal fermentation extracts, antimicrobial activity assay were carried out as described previously Cueva et al. (2010). Inhibition percentage values considered active were: ≥30% for *C. albicans* MY1055, ≥50% for *A. baumanii* 5973, *E. coli* CECT 5947 and *P.aeruginosa* PAO1, and ≥60% for *S. aureus* EP167. The minimal values were selected based on effective fungi extracts resistance of the strains, which is strain-dependent. This approach, commonly used to study of natural products (Bills et al., 2008; Vicente et al., 2009), selects cut-off values high enough in order to obtain a repeatability of results.

2.4. *Liquid chromatography-mass spectrometry (LC-MS) and database matching of known antimicrobial metabolites*

Metabolites in fermentation extracts were matched to a proprietary reference library of fully characterized fungal metabolites and authentic samples using an in-house developed application where the diode array signal, retention time, positive and negative mass spectra of the active samples were compared to those of library (Bills et al., 2009; Vicente et al., 2009). Active extracts (2 μL) were analysed with an Agilent (Santa Clara, CA) 1100 single Quadrupole LC-MS, coupled to a Zorbax SB-C8 column (2.1 x 30 mm), maintained at 40 ºC at a flow rate of 300 μL/min. Solvent A consisted of 10% acetonitrile, 90% H_2O, 1.3 mM trifluoroacetic acid and 1.3 mM ammonium formate, while solvent B was 90% acetonitrile, 10% water, 1.3 mM trifluoroacetic acid and 1.3 mM ammonium formate. The gradient started at 10% B and went to 100% B in 6 minutes, kept at 100% B

for 2 min and returned to 10% for 2 min to initialize the system. Full diode array UV scans from 100 to 900 nm were collected in 4 nm steps at 0.25 sec/scan. The eluting solvent was ionized using the standard Agilent 1100 ESI source adjusted to a drying gas flow of 11 L/min at 325 ºC and a nebulizer pressure of 40 psig. The capillary voltage was set to 3500 V. Mass spectra were collected as full scans from 150 m/z to 1500 m/z, with one scan every 0.77 sec, in positive and negative modes. The possible origin of metabolites from medium components was exclude by analysing negative controls, i.e. extracts of the sterile media treated identically to cultures, in parallel.

2.5. *Molecular identification*

DNA extraction. Approximately 1 mL of fungi inoculum from each tube was transferred into 96-well plates with a Transfer Tube (Spectrum Laboratories, Rancho Dominguez, CA, USA). Total genomic DNA from the different microorganisms was isolated using a Master Pure ™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies) following manufacturer's instruction; slight modifications were made in order to improve fungi DNA extraction. The modifications carried out were as following: a) some centrifugation steps were made twice (the first step of Gram Positive DNA Purification Protocol and the seventh step in the DNA Precipitation), b) the volume of isopropanol added for DNA precipitation was 300 μL, followed of drying step in a Genevac HT-24 vacuum centrifuge at 45ºC for 15 min, and c) DNA extracts were resuspended in 100 μL of Milli-Q water.

PCR amplification. DNA extracted was used for PCR amplification. DNAs were subjected to PCR reactions with primers ITS1 and ITS4 (White et al., 1990). Reactions were performed in a final volume of 50 μL containing 0.2 mM of the four dNTPs (Applied Biosystems), 0.05 μM of each primer, 5 μL of the extracted DNA (about 10 ng/μl) and 0.5

U *Taq* polymerase (Appligene, Illkirch, France) with its appropriate reaction buffer. Controls without fungi DNA were included for each PCR experiment. Amplifications were performed in a Thermocycler PCR PTC-200 (Bio-Rad), according to the following profile: 40 cycles of 1 min at 95ºC, 1 min at 51ºC and 2 min at 72ºC. Amplifications products were visualized by electrophoresis in 1% agarose gels (Invitrogen E-Gel® 48 1 % (GP) G8008-01) using an Invitrogen E-Base. PCR products were purified using Ilustra GFX 96 PCR Purification Kit (Amersham Biosciences).

DNA sequencing and sequence analysis. PCR primers ITS1 and ITS 4 were used for the amplification of the ITS1-5.8S-ITS2 region of the wild-type isolates. The PCR products were purified and used as a template in sequencing reactions with the primers ITS1 and ITS4. Amplified and cloned DNA fragments were sequenced by using an ABI Prism Dye terminator cycle sequencing kit (Amersham Biosciences). Sequences were aligned using CLUSTAL W (Thomson et al., 1994). The analysis was complemented with ITS1-5.8S-ITS2 sequences of fungal species available in GenBank and with similarity searches using BLAST.

3. Results and discussion

3.1. *Isolation of fungi*

Following surface disinfection methods, a total of 290 strains were isolated from samples of vineyard soil and plants from four locations of Spain: 30 from Villamanrique del Tajo (VT), 97 from Escuela de la Vid (EV), 101 from Membrilla (M), and 62 from Tortuero (T). An initial visual screening was carried out to discard identical isolates. As a result, a total of 182 fungal isolates were selected for the antimicrobial activity assays. The large number of fungal species confirmed that vineyard environment was an important source of potentially-active fungi.

3.2. *Antimicrobial activity of fungi extracts*

Each fungal isolate was grown in eight different media to promote the development of each strain's full capacity to produce secondary metabolites. Therefore, a total of 1456 extracts were assayed for antimicrobial activity against five food-borne pathogenic strains (*S. aureus* EP167, *A. baumannii* (clinical isolated), *P. aeruginosa* PAO1, *E. coli* O157:H7 (CECT 5947) and *C. albicans* MY1055) and two probiotic strains (*L. plantarum* LCH17 and *L. brevis* LCH23). A total of 71 fungi isolates met or exceeded the minimal antimicrobial activity in at least one of the eight growth conditions and resulted inactive against the two probiotic bacteria. Among these active fungi, 13 of them showed antimicrobial activity against two pathogenic strains or more (Table 2). However, the majority of them inhibited selectively the growth of only one pathogenic strain (Table 3).

Regarding antibacterial resistance, Gram-positive *S. aureus* EP167 was the most susceptible to fungal extracts, whereas *A. baumannii*, *E. coli* O157:H7 and *P. aeruginosa* PAO1 (Gram negative) were more resistant. These results are consistent with the fact that

Gram negative bacteria are characterised by an outer membrane that provides the cell with a hydrophilic surface that is able to exclude certain hydrophobic molecules, therefore imparting intrinsic resistance of these bacteria to antimicrobial compounds (Perry et al., 2009).

Bacterial and fungal food infections pose a health threat, most notably in immunocompromised subjects. *S. aureus* is an opportunistic human pathogen, causing major problems in the food sector as well as the clinic (Rode et al., 2007). Regarding Gram negative strains, *E. coli* O157:H7 is a pathogen that causes haemorrhagic colitis, haemolytic uremic syndrome and thrombotic thrombocytopaenic purpura (Charimba et al., 2010); *P. aeruginosa* is a nosocomial pathogen that causes urinary tract infections, respiratory system infections, dermatitis, and gastrointestinal infections; *A. baumannii* is implicated in a variety of nosocomial infections, including bacteremia, urinary tract infection and pneumonia (Bergogne-Bérézin et al., 1996). On the other hand, dietary contamination with the yeast *C. albicans* may cause opportunistic mouth infections (Rauha et al., 2000). In contrast, the occurrence of probiotic bacteria in food, such as *Lactobacillus plantarum* LCH17 and *Lactobacillus brevis* LCH23 confer a health benefit to the host. One of the mechanisms exerting health promoting effects in human beings is pathogen inhibition and restoration of microbial homeostasis through microbe-microbe interactions. This capacity has been a major principal for food preservation (Lebeer et al., 2008). Although the techniques to preserve food safety are improving, pathogenic microorganisms such as used in this study may be ingested in the human body through contaminated food, which could provoke serious problems on human health, especially in hospitalized patients whose immune system are debilitated.

3.3. *Molecular identification*

The sequences from each strain were compared with sequences in the GenBank database to approximate fungi identification. Best GenBank Blast match identifications and GenBank accession numbers of fungi causing antimicrobial activity are provided in Tables 2 and 3. These fungi belonged mostly to the *Dothideomycetes* and *Sordariomycetes* classes. The most frequently active fungi were *Alternaria* spp, Coelomycete strains, *Aspergillus* spp, *Fusarium* spp, *Discostroma* spp, *Penicillium* spp, *Leptosphaeria* spp and *Pestalotiopsis* spp. The distribution of fungal tax is consistent with the report of Halleen et al. (2003). These fungi apparently were not restricted to any particular location. The common phytophatogens, *Botryosphaeria* sp and *Phaeoacremonium* sp were also found. These pathogens are associated with black dead arm and esca disease respectively (Djoukeng et al., 2009; Larignon et al., 1997; Sánchez-Torres et al., 2008). Sequences of some fungi could not be identified (unidentified fungus) because of lack of comparative sequences in the Genbank database (results not shown). Antifungal activities were most frequently detected in strains from the order *Pleosporales*, and the most potent antibacterial activity was found in the strains of the order *Hypocreales*. Some of the results reported in this study are consistent with those from earlier studies in other fungi sources. For example, it is known that members of group *Eurotiales*, *Hypocreales* and *Pleosporales* consistently produce antibacterial and antifungal metabolites (Peláez et al., 1998; Suay et al., 2000).

The two fungi whose extracts showed activity against all the pathogens were *Aspergillus niger* (E-000535890) and *Epicoccum nigrum* (E-000535780) (Table 2). Other strains of *E. nigrum* (E-000535735) were active but only against *A. baumannii* (Table 3) As seen for other isolates, antimicrobial selectivity of fungi extracts varied slightly among strains of the same species (Table 2 and 3). Similar intraspecific variability was also

described by Möller et al. (1997) for *Chaunopycnis alba* and by Peláez et al. (1998) for *Pseudodiploidia* sp and *Sporomiella intermedia*.

3.4. *Identification of bioactive metabolites*

An attempt to confirm the antimicrobial potential of extracts from vineyard ecosystem fungi, known antimicrobial metabolites in the active extracts were identified by LC-MS database matching, taking into account that these metabolites might not be the agent responsible for the antimicrobial activity of the extracts. Liquid chromatography-mass spectrometry identified thirteen known metabolites considered of broad antimicrobial spectra (Tables 2 and 3). In addition, other yet uncharacterized metabolites were recognized (results not shown), which are a candidates for purifying of antibacterial and antifungal metabolites.

Most of the extracts containing the compounds identified in Tables 2 and 3 showed antimicrobial activity against *S. aureus* EP167. As described previously Peláez et al. (1998), when the antimicrobial activity exerted by extracts are specific to one pathogen it is more probably that a single compound was responsible for this activity, however, when the inhibition acts across different type of pathogen microorganisms, it is unclear whether the activity detected is caused by a single inhibitor of against both types of microorganisms, or rather a mixture of compounds with different specificities.

Previous studies have been reported that some of the compounds identified in our study have biological activities. Thus, asterric acid have reported that performed such as endothelin binding inhibitor (Ohashi et al., 1992) and inhibitor of vascular endothelial growth factor (VEGF) (Lee et al., 2002); enniantins, roridins and ergosterol have showed anticarcinogenic properties (Amagata et al., 2003; Dornetshuber et al., 2007; Wätjen et al., 2009; Yazawa et al., 2000) and equisetin have showed inhibition of recombinant integrase

enzyme (Singh et al., 1998) and inhibition of the substrate anion carriers of the mitochondrial inner membrane in rats (Konig et al., 1993). However, most of these compounds are likely to be toxic and their use to control food pathogens would be inappropriate. Recently Strobel (2003) and Liu et al. (2008) described the ability of *Xylaria* sp YX-28 fungi to produce an antimicrobial compound (7-amino-4-methlcoumarin) with the potential to be used as a food additive. Therefore, further investigation should focus on the causes of antibiosis from extracts from unknown fungal strains.

In summary, our results confirm that grapevine environments are abundantly populated with fungi producing bioactive secondary metabolites that could have an interesting application in the food industry. A significant number of fungi strains whose extracts have proven antimicrobial effects against food-borne pathogens but not against any probiotic bacteria, were isolated and identified by molecular approaches. This opens the possibility of fungi metabolites to be used as antimicrobials preventing food deterioration and infections in human beings. However, further investigations are needed in order to identify the active compounds produced by the grapevine environment fungi.

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Table 1. Strains and growth conditions of the microorganisms.

Strain	Growth medium	Incubation conditions	Positive control	Negative control
A. baumannii (clinical isolated)	MH	20h, 37ºC	Ciprofloxacin	Amphotericin B
C. albicans MY1055	RPMI modified	20h, 30ºC	Amphotericin B	Penicillin G
E. coli CECT 5947	LB + chloramphenicol (25μg/mL)	18h,37 ºC	Ciprofloxacin	Novobiocin
L. brevis LCH23	MRS	30h, 37ºC	Penicillin G	Amphotericin B
L. plantarum LCH17	MRS	24h,37 ºC	Penicillin G	Amphotericin B
P. aeruginosa PAO1	LB	20h, 37ºC	Ciprofloxacin	Amphotericin B
S. aureus EP167	LB + chloramphenicol (34 μg/mL)	20h, 37ºC	Penicillin G	Amphotericin B

Table 2. Fungal strains whose extracts showed antimicrobial activity against two or more of the pathogens tested but were inactive against the probiotic bacteria. The taxa are cited in alphabetic order.

Class	Order	Identified species	GenBank accession no.	Strain codes	Identified metabolites	Substrate type	Origin	Sta	Aci	Pse	Eco	Can
Dothideomycetes	*Pleosporales*	*Camarosporium* spp	JN545797	E-000535734	Penicillic Acid	Xylem	EV		+			+
		Epicoccum nigrum	JN545803	E-000535780		Leaf bud	M	+	+	+	+	+
Eurotiomycetes	*Chaetothyriales*	*Exophiala* spp	n.s.	E-000535881	Ergosterol D	Soil	M	+	+			
	Eurotiales	*Aspergillus niger*	JN545800	E-000535890		Soil	T	+	+	+	+	+
		Aspergillus ustus	JN545824	E-000535884		Soil	T	+				+
		Aspergillus versicolor	JN545821	E-000535879		Soil	M	+				+
		Penicillium expasum	JN545825	E-000535885	Helvolic acid	Soil	T	+	+		+	
Sordariomycetes	*Hypocreales*	*Fusarium* spp	JN545775	E-000535632	Enniatin (A,A1,B1,D), Fusarielin	Bark	VT	+			+	+
		Fusarium spp	JN545779	E-000535644	Beauvericin	Bark	VT	+			+	
		Myrothecium spp	JN545776	E-000535633	Roridin (A, H), Verrucarin	Bark	VT	+				+
		Paecilomyces marquandii	JN545822	E-000535880		Soil	M	+	+			
	Unidentified Hypocreales		JN545830	E-000535914	Ergosterol D	Soil	T		+	+		
Unidentified fungus			JN545794	E-000535724		Bark	EV	+	+			

n.s.: not sequence
VT: Villamanrique del Tajo (Madrid, Spain), EV: Escuela de la Vid (Madrid, Spain), M: Membrilla (Ciudad Real, Spain), Tortuero (Guadalajara, Spain).
Sta: *Staphylococcus aureus* EP167, Aci: *Acinetobacter baumannii* (clinical isolated), Pse: *Pseudomonas aeruginosa* PAO1, Eco: *Escherichia coli* O157:H7 (CECT 5947), Can: *Candida albicans* MY1055.

Table 3. Isolated fungi strains whose extracts showed antimicrobial activity against one of the pathogens tested but not against probiotic bacteria. The taxa are cited in alphabetic order.

Class	Order	Identified species	GenBank accession no.	Strain codes	Identified metabolites	Substrate type	Origin	Sta	Aci	Pse	Eco	Can
Coelomycete			n.s.	E-000535672		Bark	EV	+				
			n.s.	E-000535692	Alternariol analog	Xylem	EV	+				
			n.s.	E-000535745		Xylem	EV					+
			n.s.	E-000535799		Bark	M	+				
			JN545813	E-000535844		Xylem	T	+				
			n.s.	E-000535903		Soil	T	+				
Dothideomycetes	*Botryosphaeriales*	*Botryosphaeria* spp	JN545831	E-000535801		Bark	M		+			
	Dothideales	*Aureobasidium* spp	JN545783	E-000535659		Xylem	EV	+				
	Pleosporales	*Alternaria* spp	JN545790	E-000535675		Bark	EV	+				
		Alternaria spp	JN545791	E-000535676		Bark	EV	+				
		Alternaria spp	JN545793	E-000535706		Bark	EV	+				
		Alternaria spp	JN545801	E-000535766	Alternariol analog	Leaf bud	M	+				
		Alternaria spp	JN545804	E-000535781		Leaf bud	M		+			
		Alternaria spp	JN545805	E-000535794		Bark	M	+				
		Alternaria spp	JN545807	E-000535813		Bark	M	+				
		Alternaria spp	JN545812	E-000535840		Bark	T	+				
		Epicoccum nigrum	JN545798	E-000535735		Xylem	EV		+			
		Leptosphaeria spp	JN545781	E-000535652		Xylem	VT	+				
		Leptosphaeria spp	JN545784	E-000535660		Xylem	EV	+				

		Leptosphaeria spp	JN545785	E-000535665		Xylem	EV	+		
		Phoma spp	JN545806	E-000535797		Bark	M			+
		Phoma spp	JN545808	E-000535818		Xylem	M			+
		Ulocladium spp	n.s.	E-000535747		Xylem	EV	+		
		Ulocladium chartarum	JN545819	E-000535868		Soil	M	+		
	Unidentified Pleosporales		JN545810	E-000535827		Bark	T			+
	Unidentified Pleosporales		JN545811	E-000535829		Bark	T	+		
	Unidentified Pleosporales		JN545826	E-000535891		Soil	T		+	
Eurotiomycetes	*Eurotiales*	*Aspergillus versicolor*	JN545818	E-000535867		Soil	M	+		
		Penicillium spp	n.s.	E-000535804		Xylem	M	+		
		Penicillium spp	JN545832	E-000535911		Soil	T			+
Sordariomycetes	*Calosphaeriales*	*Phaeoacremonium* spp	JN545782	E-000535655	Equisetin	Xylem	VT	+		
	Hypocreales	*Acremonium* spp	JN545780	E-000535647	Cephalochromin	Xylem	VT	+		
		Acrostalagmus luteoalbus	JN545827	E-000535895		Soil	T	+		
		Fusarium spp	JN545777	E-000535636		Bark	VT	+		
		Fusarium spp	JN545796	E-000535731	Enniatin (A,A1,B1,D)	Bark	EV	+		
		Metarrhizium anisopliae	JN545817	E-000535866	Helvolic acid	Soil	M	+		
		Stachybotrys spp	JN545814	E-000535852		Soil	M	+		
		Stachybotrys spp	JN545829	E-000535904		Soil	T	+		
		Trichoderma spp	JN545774	E-000535629		Bark	VT	+		
	Sordariales	*Chaetomium* spp	JN545828	E-000535897		Soil	T		+	
	Xylariales	*Discostroma* spp	JN545788	E-000535670		Bark	EV	+		
		Discostroma spp	JN545795	E-000535728		Bark	EV	+		

	Discostroma spp	JN545809	E-000535824		Xylem	M		+		
	Pestalotiopsis spp	JN545786	E-000535667		Bark	EV		+		
	Pestalotiopsis spp	JN545816	E-000535861		Soil	M		+		
	Pestalotiopsis spp	JN545820	E-000535876		Soil	M		+		
Unidentified Amphisphaeriaceae		JN545789	E-000535673		Bark	EV				+
		JN545792	E-000535696		Bark	EV				+
Unidentified Ascomycete		JN545778	E-000535639	Dihydrobisdechlorogeodin, asterric acid	Xylem	VT	+			
Unidentified fungus		JN545787	E-000535668		Bark	EV	+			
		n.s.	E-000535721		Bark	EV		+		
		JN545799	E-000535746	Asterric acid	Xylem	EV	+			
		JN545802	E-000535771		Bark	M	+			
		n.s.	E-000535825		Bark	T		+		
		n.s.	E-000535855		Soil	M				+
		JN545815	E-000535860		Soil	M		+		
		JN545823	E-000535882		Soil	M	+			
		n.s.	E-000535892		Soil	T				+

n.s.: not sequence
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