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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* (clinically 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 was 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 *S. aureus* EP167 was more susceptible to antimicrobial fungi broth 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.
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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 upon the host (Lebeer et al., 2008). New trends recommend a reduction in the use of chemically synthesized preservatives in favor 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 useful 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 and Fang, 2000) and continue to be investigated as useful sources of natural products – secondary metabolites – (Hoffmeister and Keller, 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 product screening programs have often focused on searching for antibiotics (Basilio et al., 2003; González del Val et al., 2001; Peláez et al., 1998; Suay et al., 2000), although some antibiotically active molecules

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could have unexpected alternative applications (Demain, 1998). Antibiotic screening not only provides candidate compounds useful for target applications. Indeed, antibiotic activity may be indicative of complementary bioactivities, suggesting high priority status for broad-based pharmacological, microbiological, molecular biological, and agricultural testing of fungi-originated compounds and 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 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 search 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: *Escherichia coli* O157:H7 (CECT 5947), *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* EP167, *Acinetobacter baumannii* (clinical isolate) 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 (ITS) rRNA regions were amplified and sequenced for molecular identification. Additionally, active extracts were also analyzed 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, placed in clean paper envelopes and transported to the laboratory at ambient temperature on 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 s 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 center 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 a 48-well tissue culture plate 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 °C and 70% relative humidity.

Soil samples were sieved prior to isolation of fungi. Soil 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 °C 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 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 center 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 media 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

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