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Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production

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ABSTRACT

In this study, 13 lactic acid bacteria (LAB) strains (including 5 *Lactobacillus casei*, 2 *Lactobacillus rhamnosus*, 2 *Lactobacillus fermentum*, 1 *Lactobacillus acidophilus*, 1 *Lactobacillus plantarum*, 1 *Lactobacillus sakei*, and 1 *Lactobacillus reuteri* species) were assessed for both their antifungal activity against four food spoilage molds (*Colletotrichum gloeosporioides*, *Botrytis cinerea*, *Penicillium expansum*, and *Aspergillus flavus*) and their capability to produce the novel antimicrobial compound 3-phenyllactic acid (PLA). Results demonstrated that all molds were sensitive to varying degrees to the cell-free supernatants (CFS) from LAB fermentations ($p < 0.05$), with growth inhibitions ranging from 2.65% to 66.82%. The inhibition ability of CFS was not affected by a heating treatment (121 °C, 20 min); however, it declined markedly when the pH of CFS was adjusted to 6.5. With the exception of *L. plantarum* NRRL B-4496 and *L. acidophilus* ATCC-4495, all other LAB strains produced PLA ranging from 0.021 to 0.275 mM. The high minimum inhibitory concentration for commercial PLA (3.01–36.10 mM) suggests that it cannot be considered the only compound related with the antifungal potential of studied LAB and that synergistic effects may exist among other metabolism products.

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1. Introduction

Molds are capable of growth on all kinds of foods, including cereals, meats, fruits, and vegetables (Gerez et al., 2013). Food spoilage by molds causes extensive economic losses to the industry and may involve health risks to consumers due to both the toxicity and pathogenicity of some species, causing infections or allergies in susceptible individuals (Gerez et al., 2010). Currently, the use of food-grade chemical antifungal agents has become increasingly unpopular with consumers, who look for foods that do not contain them. Also, some authors reported high mutation frequencies of target microorganisms when using common antifungal agents, resulting in increased resistance (Gerez et al., 2010; Wang et al., 2012). In recent years, biopreservation (the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods) has attracted interest due to consumer demand for reducing potential negative effects, including direct or indirect impact of chemically-synthesized products on the environment (Ström et al., 2002; Schnürer and Magnusson, 2005; Mu et al., 2009; Prema et al., 2010; Wang et al., 2012).

Lactic acid bacteria (LAB) represent a large group of microorganisms that have long been used as natural or selected starter cultures for food fermentations, not only because they significantly contribute to the acidification and flavor production but also because they have antagonistic properties which offer protection against food spoilage molds and bacteria (Messens and De Vuyst, 2002; Kim et al., 2009). Such antagonistic properties have been associated with a wide variety of active antimicrobial compounds produced during bacterial fermentation—for instance, lactic, acetic and benzoic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, hydroxyl fatty acids, reuterine, and bacteriocins (Lavermicocca et al., 2000; Messens and De Vuyst, 2002; Ström et al., 2002; Lind et al., 2007; Hassan and Bullerman, 2008; Dalié et al., 2009; Kim et al., 2009). All these antimicrobial compounds might be used as an integral part of hurdle technology (Ananou et al., 2007). In this regard, 3-phenyllactic acid (PLA) has gained interest in recent years due to its effective antimicrobial activity. PLA was first described as a LAB metabolite in the study by Lavermicocca et al. (2000), where it was related with the inhibition of the conidial germination of *Penicillium expansum*, *Penicillium roqueforti*, *Aspergillus flavus*, *Aspergillus niger*, *Monilia sitophila*, and *Fusarium graminearum*, among others. PLA is a by-product of phenylalanine metabolism in LAB, where the first step involves its transamination by a non-specific aminotransferase. The α-amino group is then transferred to a suitable acceptor such as α-ketoglutarate, yielding phenyl pyruvic acid (PPA) and the corresponding amino acid. Finally, PPA can then be reduced by hydroxyl acid dehydrogenases to PLA (Vermeulen et al., 2006; Mu et al., 2012b; Rodríguez et al., 2012).

PLA is an antimicrobial compound with a wide activity spectrum against some yeast such as *Candida pulcherrima* and *Rhodotorula mucilaginosa* (Schwenninger et al., 2008) and molds including some mycotoxigenic species such as *Aspergillus ochraceus*, *Penicillium*

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verrucosum, and *Penicillium citrinum* (Valerio et al., 2004). In addition, PLA has been found to inhibit a range of Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Providencia stuartii*, and *Klebsiella oxycota* (Dieuleveux et al., 1998; Lavermicocca et al., 2000; Valerio et al., 2004). Several studies have reported the ability of many LAB to produce PLA, including species such as *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus sanfranciscensis*, *Lactobacillus acidophilus*, *Lactobacillus alimentarius*, *Lactobacillus amylovorus*, *Lactobacillus casei*, *Lactobacillus lactis*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, and *Pediococcus acidilactici*, although its production has been found to be species- and strain- dependent (Lavermicocca et al., 2000; Valerio et al., 2004; Li et al., 2007; Gerez et al., 2009, 2010, 2013; Valerio et al., 2009; Prema et al., 2010; Ryan et al., 2011; Mu et al., 2012a). Additionally, PLA production has been reported for *Geotrichum candidum* (Dieuleveux et al., 1998) and some propionibacteria (Lind et al., 2007).

Recently, several LAB have been screened for their antifungal potential and their ability to produce PLA (Gerez et al., 2010; Prema et al., 2010; Ryan et al., 2011; Gerez et al., 2013), but additional studies are required in this field due to the wide diversity of both LAB and food spoilage molds. Therefore, the aim of this work was both to assess the antifungal activity of thirteen LAB strains against four food spoilage molds (*Colletotrichum gloeosporioides*, *Botrytis cinerea*, *P. expansum*, and *A. flavus*) and to study their capability to produce the antimicrobial compound 3-phenyllactic acid and its relationship with the antifungal activity of studied LAB.

2. Materials and Methods

2.1. Lactic acid bacteria and fungal strains

Lactobacillus cultures used in this work were *L. plantarum* NRRL B-4496; *L. casei* NRRL B-1922, 21/1, ATCC334, DPC3968 and 12A; *Lactobacillus sakei* NRRL B-1917; *L. rhamnosus* NRRL B-442 and 13075; *Lactobacillus fermentum* NRRL B-1932 and ATCC 11976; *L. reuteri* NRRL 14171; and *L. acidophilus* ATCC 4495. Strains were obtained from collections at the Instituto Tecnológico de Veracruz (Veracruz, México) and Universidad de las Américas Puebla (Puebla, México). Stock cultures were maintained at −70 °C in 15% (v/v) glycerol. Working cultures were prepared from frozen stocks by subculture in MRS broth (de Man-Rogosa-Sharpe, Difco™) at 37 °C for 24 h.

Strains of *A. flavus*, *P. expansum*, *B. cinerea*, and *C. gloeosporioides* were obtained from the microbial collection at the Universidad de las Americas Puebla (Puebla, México). Molds were individually grown on PDA (Potato Dextrose Agar, Bioxon) slants at 25 °C for 7–15 days until they sporulated. The spores were then collected from slants using sterile peptone water (0.1% w/v). The spore count was determined using a haemocytometer and suspensions were adjusted to 10^4 spores/mL.

2.2. Preparation of cell-free supernatants (CFS)

In order to obtain CFS, LAB cultures were propagated twice for 18 h at 37 °C in 10 mL of MRS broth. An aliquot (200 μL) of activated culture was then inoculated into fresh sterile MRS broth (20 mL) and allowed to grow at 37 °C without shaking for 72 h. Finally, cells were removed by centrifugation (7200×g, 10 min), and the CFS obtained were filter-sterilized (0.45-μm pore-size Millipore filter) and immediately screened for their antifungal activity.

2.3. Determination of antifungal activity

Cell-free supernatants of LAB obtained as previously described were tested for their antifungal activity as reported Wang et al. (2012) with some modifications. Briefly, CFS was mixed with PDA (pH 4) to achieve a final concentration of 10% (for *P. expansum*, *A. flavus*, and *B. cinerea*) and 5% (for *C. gloeosporioides*) (v/v) and poured into Petri dishes (20 mL per plate). Resulting media was centrally inoculated with 5 μL of the previously prepared spore suspensions and incubated at 25 °C. Control plates containing PDA media mixed with sterile MRS broth in the same proportions as above were also prepared and inoculated. After a 4- to 8-day incubation period (depending on the required time by each mold in the control media to grow and fill the plate), the area of mycelial growth in both treated (A_T) and control (A_C) plates was determined from the mean perpendicular diameter measurements assuming a circular growth. The percentage of growth inhibition (I) was calculated as

$$I = 100 \times \frac{A_C - A_T}{A_C}$$

In order to qualitatively evaluate the chemical nature of the possible antifungal compounds present in each CFS, CFS were also subjected to heating (121 °C, 20 min) or pH adjustment (pH 6.5 with 46% NaOH aqueous solution, w/v) treatments. The remaining antifungal activity of treated CFS was further assessed as described above.

2.4. Identification of PLA in CFS

PLA was identified in CFS as described by Valerio et al. (2004) and Gerez et al. (2010) with minor modifications. CFS obtained as described in Section 2.2 were adjusted to pH 2.0 with formic acid (10 M) and extracted three times with 20 mL of ethyl acetate. The extracts were dried using Na_2SO_4 and concentrated in a rotary evaporator (Büchi model R210/215, Flawil, Switzerland). The dried residues were reconstituted with 5 mL of 2.5 mM H_2SO_4 and analyzed in an HPLC system (Waters, Milford, MA, USA) fitted with an Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm, Bio Rad) at 50 °C, using 2.5 mM H_2SO_4 as the mobile phase at 0.7 mL/min and a UV-visible detector set at 210 nm (Empower version 2.0). Commercial PLA (Sigma Chemical Co., St Louis, MO, USA) was used as reference (retention time = 32.16 min). Since PLA determination in CFS involves a partial purification step, its recovery yield was assessed by mixing pure PLA with sterile MRS broth until a final concentration of 2.0 mM was achieved. The resulting mixture was incubated at 37 °C for 72 h and PLA was further extracted and quantified as previously described.

2.5. Determination of the minimum inhibitory concentration (MIC) of PLA

To determine the MIC for PLA, melted PDA agar (pH 4.0) was mixed with PLA at different concentrations (0.6–7.0 mg/mL) and poured into Petri dishes (20 mL per plate) (Wang et al., 2012). Resulting media were centrally inoculated with 5 μL of the fungal spore suspensions (10^4 spores/mL) and incubated at 25 °C. By the end of incubation period (4–9 days), the mycelial growth for each mold was recorded. MIC was defined as the lowest PLA concentration that inhibited visible growth of tested molds. MIC values of two reference antifungal agents (sodium benzoate and sodium propionate) were used as positive controls.

2.6. Growth and PLA production curves

Relationship between cell growth and appearance of PLA was further assessed only with the highest PLA producer (*L. casei* 21/1). LAB culture was propagated twice for 18 h at 37 °C in 10 mL of MRS broth before use. Activated culture (1000 μL) was inoculated into fresh sterile MRS broth (100 mL) and allowed to grow at 37 °C without shaking for 72 h. Aliquots (1000 μL) of this culture were taken during fermentation and viable cell counts were made in MRS agar by the pour plate technique, serial 10-fold dilutions were plated in MRS agar and incubated at 37 °C for 48 h. PLA production curve was determined in a separate experiment where several culture tubes were prepared with fresh sterile MRS broth (20 mL) inoculated with activated culture (200 μL) and incubated with the same aforementioned growth conditions. Cultures

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