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Control of spoilage fungi by lactic acid bacteria

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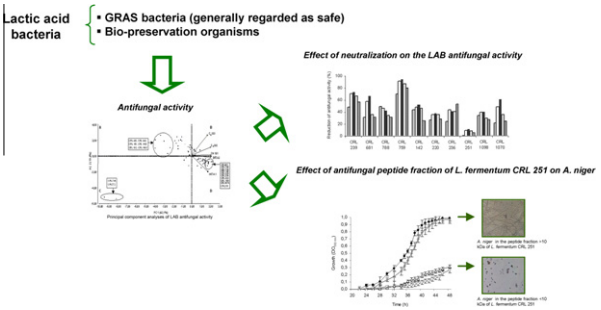
HIGHLIGHTS

- Ten LAB strains were selected from a total of 91 due to their high antifungal effect.
- Lactic, acetic and phenyllactic acids (PLA) were responsible for antifungal activity.
- This is the first report on antifungal peptide/s produced by *Lactobacillus fermentum*.
- This peptide/s was thermostable, <10 kDa, active at pH 4–7 and sensitive to trypsin.

GRAPHICAL ABSTRACT



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ABSTRACT

The evaluation of the potentiality of lactic acid bacteria (LAB) strains isolated from different origins to inhibit mould growth and to identify and characterize the antifungal metabolites were the aims of this study. From a total of ninety-one LAB strains tested, ten were selected due to their high inhibitory effect (>80%). The antifungal activity of the majority of the selected LAB strains was lost after the neutralization treatment determining the acidic nature of the antifungal metabolites. Lactic, acetic and phenyllactic (PLA) acids were identified as being responsible for antifungal effect in the 10 cell-free supernatants (CFS) evaluated. Amongst the strains evaluated, only *Lactobacillus fermentum* CRL 251 produced fungus inhibitory peptide/s, smaller than 10 kDa, thermostable, active in the pH range of 4–7 and sensitive to trypsin. This is the first report on antifungal peptide/s produced by a *L. fermentum* strain.

1. Introduction

Fungi are robust organisms capable of growing on all kinds of foods, including cereals, meats and fruits. They are important spoilage organisms in different foods causing significant economic losses in the industry. Several strategies have been used, to extend the shelf life of vegetables and food such as heat treatments, irradiating the goods with infrared rays or microwaves, using modified atmospheres during packaging, or by adding chemical preservatives (such as sorbic, benzoic, and propionic acids) (Gould, 1996). However, some fungi are able to adapt to the presence of certain preservatives (Brul and Coote, 1999; Hesse et al., 2002; Viljoen, 2001). In addition, consumers are increasingly demanding higher quality vegetable products and foods that are free of chemical pesticides with extended shelf life. This last feature is an important aspect to consider when discussing the need for new preservation methods to inhibit the growth of undesirable contaminating fungi.

The bio-preservation or the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods has gained the interest of producers due to consumers' demands (Stiles, 1996). Lactic acid bacteria (LAB) have been used for centuries as bio-preservation organisms in foods preventing the growth of spoilage microorganisms through the production of lactic acid; moreover, LAB are able to produce different kinds of bioactive molecules, such as organic acids, fatty acids, hydrogen peroxide and bacteriocins. In recent years, considerable effort has been directed

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to harness the antifungal activity of LAB in order to reduce fungal spoilage of foods (Gerez et al., 2010a,b; Mauch et al., 2010; Ryan et al., 2008; Valerio et al., 2009; Voulgari et al., 2010). Various screenings have been undertaken aimed at identifying LAB with antifungal properties isolated from different sources e.g. cereals (Ndagano et al., 2011; Rouse et al., 2008), sourdough (Coda et al., 2011; Corsetti et al., 1998; Gerez et al., 2009; Lavermicocca et al., 2000), vegetables (Gerez et al., 2010b; Magnusson and Schnürer, 2001) and dairy food (Schwenninger et al., 2005; Voulgari et al., 2010). Antifungal compounds produced by LAB that have been characterized include organic acids such as phenyllactic acid (Gerez et al., 2009; Lavermicocca et al., 2000), proteinaceous compounds (Magnusson and Schnürer, 2001; Rouse et al., 2008), reuterin (Chung et al., 1989), cyclic dipeptides (Ström et al., 2002; Yang and Chang, 2010), and fatty acids (Sjogren et al., 2003).

Certain authors have shown that only a limited number of fungal genera and species are able to grow and contaminate a determined type of food (Dijksterhuis and Samson, 2002; Filtenborg et al., 1996), e.g. the most common spoilage fungi of cereal-based products belong to the genera *Penicillium* (*P*), *Aspergillus* (*A.*), *Fusarium* (*F.*) (Keshri et al., 2002); while *Penicillium digitatum* and *Penicillium italicum* are the main postharvest pathogens agents in citrus fruits (Fogliata et al., 2000). In a previous work, we selected antifungal LAB strains able to inhibiting the growth of fungal contaminants of bread or citrus. Four LAB strains isolated from sourdough (*Lactobacillus plantarum* CRL 778, *Lactobacillus reuteri* CRL 1100, *Lactobacillus brevis* CRL 772, and *L. brevis* CRL 796) were able to inhibit the mycelial growth of spoilage molds found in small bakeries (Gerez et al., 2009). More recently, a ready-to-use antifungal starter formulated with *L. plantarum* CRL 778 significantly extended the shelf life of packaged bread (Gerez et al., 2010a). In addition, the effect of LAB on postharvest pathogenic fungi of lemon was determined using the Well Diffusion Agar method and the effectiveness of the metabolites involved was also evaluated (Gerez et al., 2010b).

The aim of this present study was to select efficient antifungal LAB strains isolated from different sources, able to inhibit contaminant fungi of bread and citrus using the Microtitre Plate Well Assay and to identify and characterize the antifungal metabolites.

2. Materials and methods

2.1. Microorganisms and culture conditions

LAB strains (91) isolated from different sources and belonging to the Culture Collection of the Centro de Referencia para Lactobacilos (CRL) (CERELA-CONICET), Tucumán, Argentina, were used (Table 1). The mould strains used in this study were *Aspergillus* (*A.*) *niger* CH 101, *Penicillium* (*P.*) sp. CH 102 and *Fusarium* (*F.*) *graminearum* CH 103, previously isolated from contaminated bread; and *P. digitatum* INTA2 and *Geotrichum* (*G.*) *citri-aurantii* INTA1, isolated from a commercial citrus fruit packing industry of National Agricultural Institute (INTA) Famaillá, Tucumán, Argentina. All fungal strains were used as indicators in the bioassay determinations.

LAB cultures were grown in MRS (De Man et al., 1960) broth (pH 6.5) at 37 °C for 24 h without agitation. Cell-free supernatants (CFS) obtained by centrifugation at 9000*g* for 10 min at 4 °C (IEC model B-22 M, International Equipment Company, USA) were filtered (0.2 μm-pore-size, Sartorius AG, Goettingen, Germany) and stored at −20 °C until used for antifungal assays.

The fungi strains were grown on Czapek-Dox medium (0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.001 FeSO4, 3% sacarose, 0.5% yeast extract 1.5% agar) at 25 °C for 7 days. The conidias were collected in sterile Tween 80 at 0.05% (v v^{-1}) and counted at the microscope in a haemacytometer chamber. Dilutions with collection fluid were used to adjust to different concentrations (10^3–10^6 conidia ml^{-1}).

Table 1
LAB strains evaluated for their antifungal activity.

LAB Strains (CRL)	Source
L. acidophilus 1063, 1064, 1065	Dairy products
L. brevis 376	
L. casei 59, 69, 75, 87, 143, 168, 234, 237, 239, 429, 206, 225, 295, 645	
L. paracasei 717	
L. bulgaricus 142, 406	
L. fermentum 220, 236, 251, 345	
L. plantarum 92, 93, 95, 99, 101, 107, 110, 121, 130, 133, 136, 137, 140,	
182, 183, 358	
L. rhamnosus 186, 201	
Ent. faecium 176	
Ent. mundtii 35	
Lactococcus lactis 1109	
St. thermophilus 414	
L. paracasei 1501	Tomato extract
L. plantarum 1073, 1093	Pea
L. plantarum 681	Sausages
L. curvatus 705	
L. paracasei 686	
L. plantarum 759, 768, 769, 775, 778, 783, 785, 788, 794, 795	Sourdough
L. reuteri 1097, 1098, 1099, 1100	
P. acidilactici 770, 767	
P. pentosaceus 761, 791, 792	
L. curvatus 760	
L. brevis 763, 772, 780, 781, 796	
L. plantarum 725	Bagasse
L. plantarum 353	Ensilage
L. acidophilus 1070	
Pediococcus pentosaceus 908	Cabbage
L. collinoides 1013	Apple juice
L. coryniformis 1001	ATCC 25602
L. paracasei 997	ATCC 25598
L. plantarum 948	ATCC 10241
L. mali 1003	ATCC 27053
Weissella ascens 962	ATCC 12706
L. rhamnosus 932	ATCC 7469
L. rhamnosus 981	ATCC 15820
Leuconostoc mesenteroides 742	NCDO 523

2.2. Antifungal assay

The antifungal activity of the CFS from the different LAB strains was determined by Microtitre Plate Well Assay (Lavermicocca et al., 2003) as described below. Conidial suspensions (10 μl) containing 10^4 conidia per ml were added to 190 μl of the CFS of each LAB strain. The assays were performed in sterile multiwell microdilution plates (96 sterile wells) (Corning Incorporated, USA). Fungal growth was determined by measuring the optical density (OD_{580nm}) at 30 °C after 48 h in a spectrophotometer (VERSAmax, Molecular Devices, USA). Conidia of each strain inoculated in MRS broth were used as control. The antifungal activity of the LAB strains was expressed as the fungal growth inhibition (%) measured as described above. In all trials, the percentages of inhibition [Inhib% = $100 - (\Delta OD_{LAB} \times 100/\Delta OD_{Control})$] was determined.

2.3. Characterization of the antifungal compounds

The LAB CFS (pH 3.5) were subjected to different treatments to determine the nature of the antifungal compounds: exposure to high temperature (100 °C for 10 min), neutralization to pH 7.0 (with 0.1 M NaOH), or subjected to the action of the following enzymes: catalase (Sigma Chemical Company, St. Louis, MO, USA, pH