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Evaluation of a bacteriocin-producing strain of *Pediococcus acidilactici* as a biopreservative for "Alheira", a fermented meat sausage

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ABSTRACT

This study was conducted to evaluate the ability of *Pediococcus acidilactici* HA-6111-2, a PA-1 bacteriocin-producing lactic acid bacterium (LAB), isolated from "Alheira" to inhibit a cocktail of *Listeria innocua* strains during production and shelf-life of these products. The bacteriocinogenic culture reduced the *Listeria* population below the detection limit (1.5log CFU/g) and had no effect on the growth of the natural LAB flora or on the pH. Pathogenic organisms were not detected in any sample. The presence of some virulence factors and antibiotic resistances of the strain to be used as a bioprotective culture were investigated. *P. acidilactici* HA-6111-2 did not produce any of the biogenic amines tested; no formation of biofilms was observed; more L(+)lactic acid was produced than its isomer D(−); no gelatinase, DNase or lipase activity was recorded; no structural genes for the haemolysin, enterococcal surface protein, hydrolytic compounds, aggregation protein and cell-wall adhesins were detected, no significant antibiotic resistances were found. *P. acidilactici* HA-6111-2 appears to have potential as a bioprotective culture during "Alheira" fermentation. Moreover, a trained panel considered the protected product to be sensorially acceptable.

1. Introduction

"Alheira" is a traditional naturally fermented meat sausage typical of Trás-os-Montes (Portugal). The relevant steps in the production process are the boiling of various meats (pork and/or poultry) in lightly salted and spiced water; soaking thinly sliced bread in some of the broth (bread represents about 25% of the total raw material), formed during the boiling of the meats, until it is soft enough; adding meat in small pieces, spices and olive oil and/or fat dripping to the bread/broth mixture; fermentation without addition of starter cultures; stuffing the paste into cellulose-based casings when everything is completely mixed and the salt and spices adjusted to the desired taste (variable); smoking the formed horseshoe-shaped sausages (ca. 15 cm long; ±60 mm) at low but uncontrolled temperature (below 37 °C) and uncontrolled humidity, for a maximum of 8 days. A wide variety of microorganisms have already been isolated from "Alheiras" by traditional methods. These are mainly LAB and *Micrococcaceae* (Ferreira et al., 2006). Pathogenic organisms such as *Listeria monocytogenes*, *Salmonella* and *Staphylococcus aureus*, have already been isolated from market samples of these products (Ferreira et al., 2006). Ferreira et al. (2006) also characterized "Alheiras" in respect to their chemical status and showed that pH, salt content and moisture *per se*, do not assure the microbiological safety of these products (means of 5.0, 1.3% and 52.3%, respectively).

The shelf life of "Alheiras" is about 1 month if stored at 4 °C in air or longer if the sausages are packed under vacuum or modified atmosphere. It is known that *L. monocytogenes* can survive the commercial dry sausage manufacturing process despite the various hurdles such as low pH, salt and nitrites (Le Marc, Hucket, Bourfeois, Guyonnet, & Mafart, 2002) and proliferate at refrigeration temperatures. In a previous study, more than 60% of the lots of "Alheiras" analysed were contaminated with *L. monocytogenes* in concentrations higher than 100 CFU/g (Ferreira et al., 2007). While "Alheiras" are cooked before consumption either by frying, grilling roasting, boiling or microwaving according to regional traditions or consumer preferences, Felício (2008) demonstrated that cooking methods might not be sufficient to inactivate *L. monocytogenes* in "Alheiras".

Several publications have reported that bacteriocinogenic LAB, especially pediococci strains, could be used as bioprotective cultures for food manufacturing processes in attempts to control *L. monocytogenes* (Dicks, Mellett, & Hoffman, 2004; Nieto-Lozan, Reguera-Useros, Peláez-Martínez, & de la Torre, 2006). PA-1 is a bacteriocin produced by *Pediococcus acidilactici* HA-6111-2 isolated from "Alheira" with antimicrobial activity against *L. monocytogenes* and several strains of *L. inoccua* (Albano et al., 2007b). This bacteriocin is stable over a wide range of temperature and pH

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conditions, and sensitive to a number of digestive proteases (Albano et al., 2007b) suggesting that it might be a promising alternative to chemical preservatives in some applications.

In a previous study, Albano et al. (2007a) demonstrated, in *in situ* assays, the inhibitory effect of a bacteriocinogenic *Pediococcus* spp. on *L. innocua* in a sterilized paste of "Alheira", but not stuffed or smoked. In the present study the ability of the PA-1 producing strain *P. acidilactici* HA-6111-2, to control a cocktail of *L. inoccua* in "Alheiras" during processing and storage, was investigated. The presence of virulence factors and antibiotic resistance of the putative bioprotective strain were assessed.

2. Materials and methods

2.1. Bacterial strains and media

P. acidilactici HA-6111-2 and *P. acidilactici* HA-2485-3 were previously isolated from "Alheira" (Albano et al., 2007a). *L. innocua* 2030c PHLS (Public Health Laboratory Service, Colindale, London), *L. innocua* NCTC 11280 (National Collection of Type Cultures, Central Public Laboratory Service, London, UK) and *L. innocua* NCTC 10528, were included in the cocktail of *L. innocua* used in the assay.

P. acidilactici was grown in de Man, Rogosa Sharpe (MRS) broth (Lab M, Bury, UK) at 30 °C for 24 h; *Enterococcus* spp., *S. aureus* and *Listeria* spp. were grown in Tryptone Soy Broth (TSB, LabM) at 37 °C for 24 h. All strains were stored at −80 °C in the presence of 15% (v/v) glycerol.

2.2. Characterization of P. acidilactici HA-6111-2 strain

2.2.1. Antibiotic susceptibility testing

The minimal inhibitory concentrations (MICs (μg/ml)) for strain *P. acidilactici* HA-6111-2 were determined by the agar microdilution method, according to National Committee for Clinical Laboratory Standards (NCCLS, 2004). Each test was carried out on Muller-Hinton Agar (MHA) with cation adjusted (BioMérieux, Marcy l'Etoile, France) for penicillin G (Sigma, Steinheim, Germany) and ampicillin (Fluka, Steinheim, Germany), brain heart infusion (BHI) agar (Difco, Heidelberg, Germany) for vancomycin (Fluka) and on MHA medium, for the other antibiotics investigated, gentamicin, kanamycin, streptomycin and oxacilin (all from Sigma), chloramphenicol and nitrofurantoin (both from Fluka), ciprofloxacin, rifampicin and tetracycline (kindly supplied by Labesfal, Tondela, Portugal). *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 25213 were used as quality control strains. The inoculum was prepared from an overnight culture on MRS agar, by suspension in sterile Ringer's solution (LabM) in order to obtain turbidity equivalent to 0.5 McFarland standards. For each antibiotic susceptibility determination, at least duplicate experiments were performed.

2.2.2. Determination of biogenic amine-forming capacity

P. acidilactici HA-6111-2 was screened for the production of histamine, tyramine, putrescine and cadaverine, according to the method described by Bover-Cid and Holzapfel (1999). The LAB strain was sub-cultured seven times in MRS broth with 0.1% of each precursor amino-acid (all from Sigma), in order to promote enzyme induction. Then, all strains were spotted in duplicate on the Bover-Cid medium plates with and without (as control) each amino-acid and incubated at 37 °C for 4 days under aerobic conditions. Positive reaction was confirmed when a purple colour occurred or tyrosin precipitate disappeared around the colonies.

2.2.3. Production of gelatinase, lipase and DNase

The production of extracellular enzymes was assayed in modified Luria–Bertani broth (MLB) agar supplemented with 2.0 g/l of $CaCl_2$ and 10 g/l of Tween-80. A positive reaction was indicated by a clear halo around the colonies.

The production of proteases was assayed in MLB broth supplemented with 50.0 g/l of gelatin. Tubes were incubated for 24 h to 72 h and then placed into the refrigerator for approximately 30 min. If the bacteria did not produce gelatinase the medium remained solid. The presence of sufficient gelatinase, turned the medium liquid even when placed in the refrigerator.

The detection of deoxyribonuclease activity was performed on Methyl Green DNAse agar (Difco) (ben-Omar, Castro, Lucas, Abriouel, & Yousif, 2004). A clear halo around colonies after incubation of plates at 37 °C for 48 h was considered a positive result.

S. aureus ATCC 25213 was used as positive control in all tests.

2.2.4. Biofilm plate assay

P. acidilactici HA-6111-2 was tested for production of biofilm using the protocol based on that described by Stepanović, Ćirković, Ranin, and Švabić-Viahović (2004). Bacteria were grown overnight at 37 °C in MRS broth. Polystyrene tissue culture plates (Brand, Wertheim, Germany) were filled with 180 μl of MRS and 20 μl of overnight culture, and the plates were then incubated at 30 °C for 72 h, for the batch and fed-batch assay. For fed-batch assay, 100 μl of medium were discarded every 24 h and filled with 100 μl of fresh culture media. After 72 h, the culture medium was then discarded, and the wells were gently washed three times with 200 μl of sterile deionised water without disturbing the biofilm at the bottom of the wells. Then the attached cells were fixed with 250 μl methanol and the plates were dried at room temperature for 15 min and stained with 2% Hucker's crystal violet for 5 min. Excess stain was removed by rinsing the plates under tap water. Adherent cells were suspended with 300 μl of acetic acid (30%) and quantified by measuring the optical density at 630 nm using a microplate reader (Model 680, Bio-Rad, Richmond, CA).

2.2.5. D-Lactic acid/ L-Lactic acid determination

An overnight culture was centrifuged and placed at 70 °C in a water-bath for 15 min to stop the enzymatic reactions. The supernatant, after centrifugation, was used for the test. The configuration and amount of lactic acid were determined enzymatically by using D-lactate and L-lactate dehydrogenase kit (Boehringer Mannheim GmbH, Mannheim, Germany).

2.2.6. PCR amplification of virulence genes

PCR procedures were performed on total-cell DNA extracted according to the method of Dellaglio, Bottazzi, and Troatelli (1973). The primers used for the amplification of genes *esp*, *agg*, *gel*E, *efaA*$_{fm}$ and *efaA*$_{fs}$ were described by Eaton and Gasson (2001) and primers for the cytolysin genes were developed by Semedo et al. (2003). All the primers were purchased from MWG Biotech AG (Ebersberg, Germany). PCR amplifications were performed in a DNA thermal cycler (My Cycler™ Thermal Cycler Firmware, Bio-Rad) in 0.2 ml reaction tubes with mixtures (25 μl each) using 1× PCR buffer (MBI Fermentas, Mundolsheim, France), 2.5 mM $MgCl_2$ (MBI Fermentas), 0.1 mM deoxynucleoside triphosphates (dNTPs) (Abgene, Surrey, UK), 0.5 μM of each primer, 2 U of Taq DNA polymerase (MBI Fermentas) and 100 ng of DNA. Amplification reactions were as follows: initial cycle of 94 °C for 3 min, 35 cycles of 94 °C for 1 min, the "adequate annealing temperature" for 1 min (55 °C for all genes), 72 °C for 2 min, a final extension step of 72 °C for 7 min and thereafter cooled to 4 °C. A 5 μl aliquot of the amplification mixture was combined with 2 μl of loading buffer and the preparation was electrophoresed on 0.8% (w/v) agarose gel at 90 V for 2 h. A 100-bp PCR DNA ladder (Bio-Rad) was used as a molecular weight marker. The positive controls used were: *E. faecalis* DS 16 (*cyl*) (Culture collection of C.B. Clewell, Department of Oral Biology, School of Dentistry, University of Michigan,