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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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A B S T R A C T

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.

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.

Materials and methods

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.

Characterization of P. acidilactici HA-6111-2 strain

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.

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.

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.

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).

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).

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, *efa*A_{fm} and *efa*A_{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,

Ann Arbor, USA), *E. faecalis* F2 (*agg*), P1 (*efa*A_{fs}), P36 (*gel*E, *esp*) and *E. faecium* P11 (*efa*A_{fm}) (Culture collection of Tracy Eaton, Division of Food Safety Sciences, Institute of Food Research, Norwich, UK).

Experimental procedures

Anti-listerial activity of bac+ P. acidilactici HA-6111-2 putatively-protective culture in "Alheira"

The paste, which is normally stuffed into sausage skins in the production of "Alheira", was the starting point in these experiments. This paste was produced by an industrial meat company and, on the day of its production, transferred to the laboratory at 4 °C. The antagonistic effect of the bacteriocinogenic *P. acidilactici* HA-6111-2 strain on a cocktail of *L. innocua* was studied at a pilot plant, the non-bacteriocinogenic *P. acidilactici* strain HA-2485-3 was used as control. The organisms were sub-cultured twice (24 h at 30 °C) in 10 ml MRS (HA-6111-2 and HA-2485-3) or TSB broth (*L. innocua*), using a 1% v/v inoculum. An aliquot (25 ml) of each bacterial suspension (10^9 CFU/ml for LAB strains and 10^6 CFU/ml for each *L. innocua*) was added to 2.5 Kg of paste of "Alheira" contained in sterile bags. After assuring good mixing of the inoculum with the paste (manually massaging of the exterior of the bags) the pastes were stuffed into casings. Fermentation occurred during smoking for 3 days (see below) and then "Alheiras" were vacuum packed in a Multivac-Gastrovac (Multivac Sepp Haggenmüller KG, A300/41/42, Germany). Packs were stored for 54 days at 5 °C and analysed immediately after smoking (day 0) and during storage at days 11, 34 and 54. The experimental conditions were: (C) uninoculated "Alheira" as control, (L) "Alheira" inoculated with a cocktail of *L. innocua*, (P) "Alheira" inoculated with *P. acidilactici* HA-6111-2, (P + L) "Alheira" inoculated with *P. acidilactici* HA-6111-2 and cocktail of *L. innocua*, (CP) "Alheira" inoculated with *P. acidilactici* HA-2485-3 and cocktail of *L. innocua*. Two batches were produced.

Smoking equipment and operational conditions

The smoking process requires temperatures between 15 and 37 °C. For this reason a smoking chamber (AGK, Type 135/12, Wallersdorf, Germany) was coupled to a thermostat (lae® electronic, MTR12, Oderzo, Italy) that was set up for 30 °C. During the drying-smoking process an induced draught was necessary to decrease the humidity in the smoker chamber and moisture in the product. Additionally, an electrical heater, situated at the bottom of the smoking chamber, was switched on (when necessary) to raise the temperature inside the smoker chamber (but to no higher than 37 °C).

During smoke generation, no heat production inside the smoke chamber was made. The heat produced by the electrical heater for smouldering the wood chips of oak and olive (smoke generator) was high enough; therefore there was no need for an extra source of heat. Moreover, the induced draught was changed for natural draught and the smoke generator outlet opened to one quarter, so the smoke did not reach the smoker chamber as rapidly and the temperatures were lower. The inlet damper of the smoker chamber was kept opened both in the drying and during smoke generation, until the smoke process was concluded.

Microbiological analyses

Twenty-five grams samples were added to 225 ml of sterile buffered peptone water (Merck, Darmstadt, Germany), and homogenized in a stomacher for 2 min. Appropriate decimal dilutions were prepared in Ringer's solution for microbial enumeration: lactic acid bacteria on MRS Agar and incubated at 30 °C for 72 h; total counts at 30 °C according to ISO 4833 (ISO, 2003); coagulase-positive staphylococci on Baird Parker RPF Agar (bioMérieux), incubated at 37 °C for 48 h according to NF V08 057-2 (AFNOR, 2004); *Enterobacteriaceae* according to Portuguese Standard NP 4137 (IPQ, 1991). Enumeration of *Listeria* spp. was performed on PALCAM Agar (Merck) and incubated at 30 °C for 72 h. Detection of *Escherichia. coli* O157 was performed by Immuno-Magnetic Separation using Dynabeads (Dynal A.S., Oslo, Norway) coated with absorbed and affinity-purified anti-*E. coli* O157 antibodies according to the International Standard, ISO 16654 (ISO, 2001). *Salmonella* spp. were detected by the VIDAS method (AFNOR, 1994).

Chemical analysis

Chloride and moisture contents were determined following the Portuguese Standard NP 1845 (IPQ, 1982) and NP 1614 (IPQ, 2002), respectively. pH was determined directly with a Crison MicropH 2002 pH-meter (Crison, Barcelona, Spain). The water activity was measured with a calibrated electric hygrometer, Rotronic DT (Rotronic AG, Bassersdorf, Switzerland), according to the manufacturer's instructions.

Sensorial analysis

"Alheiras" were produced at industrial level (by the same company that supplied the paste) using *P. acidilactici* HA-6111-2 as bio-protective culture. Strains were grown and inoculated as previously described for experiments performed at the pilot plant and inoculated just before stuffing. After smoking "Alheiras" were stored at 5 °C for one month. Immediately after fermentation/smoking process (day 0) and after one-month storage, inoculated and control (uninoculated) "Alheiras" were cooked in an oven, and subjected to sensorial analysis. A 13 member trained sensory panel was selected for their aptitude in the evaluation of sensorial differences between samples using a scoring method. The classification scale varied from 0 to 6, with 0 showing no difference and 6 a large difference. The test used was *Difference from the control*. Sensory analyses were performed for three independent batches.

Statistical analysis

An analysis of variance (two-way ANOVA) was carried out to assess the effects of batch and time of storage on *Listeria* and LAB counts (see Section 2.3.1.). A similar procedure was also used for testing significant batch and time of storage effects on pH values (see Section 2.3.4.). These analyses were performed using statistical tools of Microsoft® Office Excel 2003 (Copyright© 1985–2003 Microsoft Corporation).

Results and discussion

Strain characterization

Although intrinsically non-pathogenic, several studies have reported antibiotic resistance in LAB strains involved in sausage fermentation such as *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* (Gevers, Danielsen, Huys, & Swings, 2003). LAB do have the potential to transfer antibiotic resistances they may possess to pathogenic bacteria. The probability of this occurring is greater in scenarios of close proximity of high concentrations of resistant and non-resistant bacteria such as might be encountered in fermenting meat products.

Table 1 shows the MICs determined for several antibiotics for *P. acidilactici* HA-6111-2. According to microbiological breakpoints established by NCCLS (2004) and the Scientific Committee for Animal Nutrition (SCAN, 2005), *P. acidilactici* HA-6111-2 was considered sensitive to ampicillin, penicillin, gentamicin, rifampicin, erythromycin, kanamycin and chloramphenicol. These antibiotics are usually active against *Pediococcus* species (SCAN, 2005). *P. acidilactici* HA-6111-2 was considered resistant to streptomycin, nitrofurantoin, vancomycin and tetracycline (SCAN, 2005). Pediococci

Table 1
Minimum inhibitory concentration (MIC; µg/ml) of several antibiotics for *Ped. acidilactici* HA-6111-2.

Antibiotic	Penicillin	Ampicillin	Vancomycin	Erythromycin	Kanamycin	Gentamicin	Tetracycline	Oxacilin	Chloramphenicol	Ciprofloxacin	Rifampicin	Streptomycin	Nitrofurantoin
(µg/ml)	1.0	1.0	512.0	4.0	4.0	0.25	64.0	0.25	4.0	2.0	0.25	8.0	16.0

have been described as intrinsically resistant to streptomycin, kanamycin, tetracycline (especially *P. acidilactici*) vancomycin and ciprofloxacin, (SCAN, 2005). There is no information on the break-points for ciprofloxacin and oxacillin. Since resistance to some antibiotics was observed in *P. acidilactici* HA-6111-2 in the present study, it would be necessary to determine the presence of potentially transferable antibiotic resistances.

P. acidilactici HA-6111-2 did not produce any of the BA tested. Addition of "amine-negative" starter cultures has been suggested to prevent amine formation in dry sausages (Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Caro, 2000).

Given their long history of consumption in fermented foods, LAB have been considered to have the status of "generally regarded as safe" (GRAS) by the American Food and Drug Administration. However, LAB strains, including *Pediococcus* sp. have been associated with cases of sepsis, endocarditis, or bacteremia. Therefore, the safety of microorganisms to be deliberately added to should be assessed individually. Gelatinase, DNAse, lipase, enterococcal surface protein (*esp*), haemolysin/bacteriocin (*cyl*), gelatinase (*gel*E), aggregation substance (*agg*) and cell-wall adhesins (*efa*A) genes, virulence factors commonly presented by 'true' pathogens and that have been identified in enterococci (Archimbaud et al., 2002; Creti et al., 2004) were not shown by *P. acidilacticci* HA-6111-2. No biofilm formation was observed. *P. acidilactici* HA-6111-2 produces in more quantity the L(+) lactic acid isomer being in the ratio of production of L(+)/ D(−) lactic acid of 3:1 (9.01 g/l for L-isomer and 3.07 g/l for D-isomer). L(+) lactic acid is more inhibitory than its D(−) counterpart (Benthin & Villadsen, 1995). Since the D(−) isomer is not hydrolyzed by human lactate dehydrogenase and may cause health problems, only strains producing mainly L(+) lactic acid should be selected for addition to foods (Buckenhüskes, 1993).

Insitu behaviour of putatively-protective strain

This study was partially performed in the pilot plant of Escola Superior de Biotecnologia where it is not possible to work with pathogenic organisms. Therefore *L. innocua,* considered a valuable surrogate for *L. monocytogenes*, was used (Friedly et al., 2008; NACMCF, 2004). Indeed some researchers report a greater sensitivity of *L. monocytogenes* towards certain antibacterial compounds than *L. innocua* (Mataragas, Drosinos, & Metaxopoulos, 2003; Çon, Gökalp, & Kaya, 2001). Fig. 1 shows that *P. acidilactici* HA-6111-2 had an antagonistic effect on the growth of the cocktail of *L. innocua* in "Alheira" under vacuum-packed conditions. At the beginning of storage (0 day) the *L. innocua* population was ca. 4.5log CFU/g both in "Alheira" inoculated only with the *L. innocua* cocktail (L) and in those inoculated with the bac– strain of *P. acidilactici* HA-2485-3 plus *Listeria* sp. (CP). In both cases the listerial population decreased until the end of storage, however, after 54 days of storage, the pathogen was still present a lower rate at levels of ca. 2.0log CFU/g.

In the presence of the bac+ strain *Ped. acidilacticii* HA-6111-2 (P + L), the *L. innocua* population was ca. 2.0log CFU/g already at the beginning of storage. Subsequently, a reduction of the listerial population to below the enumeration limit was observed after 11 days of storage. This was maintained until the end of the storage period. In "Alheira" inoculated only with *P. acidilactici* HA-6111-2 (P) and in the control (C, no culture added), *L. innocua* was below the enumeration limit until the end of storage.

In samples inoculated with the *L. innocua* cocktail (CP, L and P + L), a significant reduction ($P < 0.05$) of the *Listeria* population was observed during the time of storage. The LAB population remained constant (ca. 9.5log CFU/g) until the end of storage both in the presence or absence of *L. innocua*, showing no difference ($P > 0.05$) during the time of storage. The pH before smoking

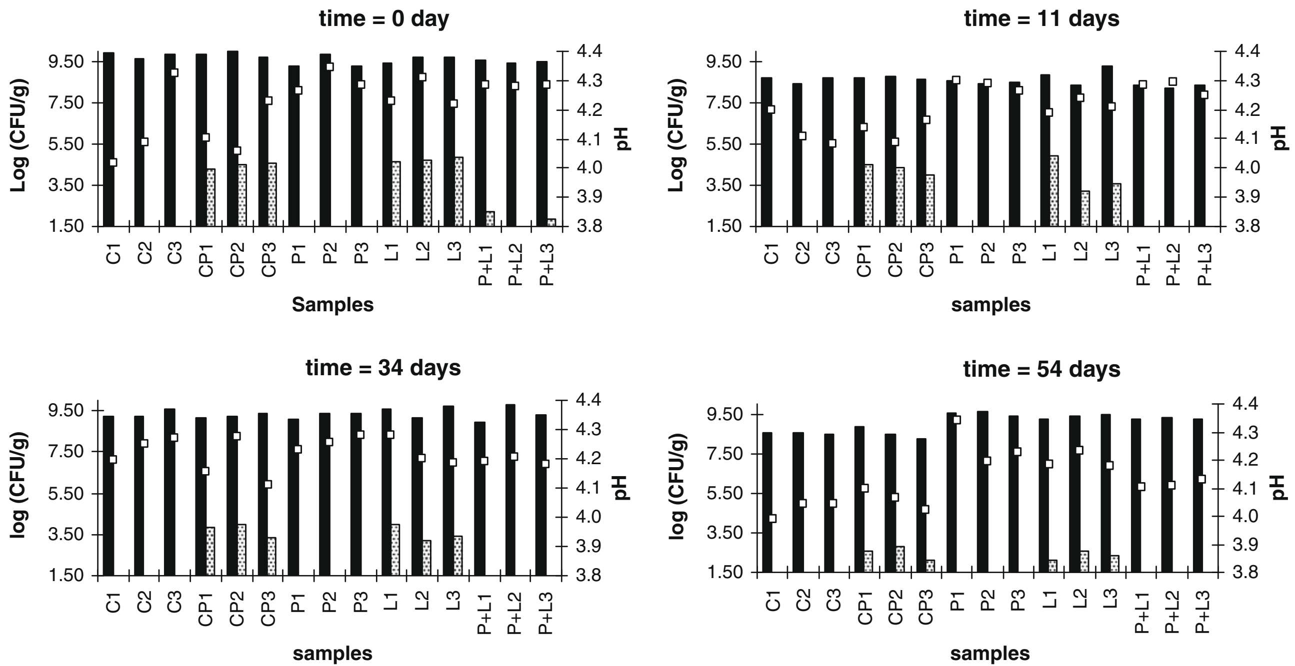

Fig. 1. Growth of lactic acid bacteria on MRS agar (■) and the cocktail of *L. innocua* on PALCAM agar (▒) in "Alheira", after smoking and during storage at 4 °C; (□) pH. Samples: C – "Alheira" non-inoculated; CP – "Alheira" inoculated with a non-bacteriocinogenic *P. acidilactici* HA-2485-3 + cocktail of *L. innocua*; P – "Alheira" inoculated with a bacteriocinogenic *P. acidilactici* HA-6111-2; L – "Alheira" inoculated with cocktails of *L. innocua*; P + L – "Alheira" inoculated with a bactericinogenic *P. acidilactici* HA-6111-2 + cocktail of *L. innocua*. 1, 2, 3 refers to three replicates of analysis of sausages from one batch. Values of 1.5log (CFU/g) means that the population is under the enumeration limit.

process was 4.5 and the average pH throughout storage time was 4.0 (Fig. 1). It is important to note that inhibition was not due to a decrease in pH because *L. innocua* is able to survive in acid conditions with pH values between 4.0 and 4.4 (Char, Guerrero, & Alzamora, 2009; Le Marc et al., 2002; Phan-Thanh, Mahouin, & Alige, 2000; Shabala, McMeekin, Budde, & Siegumfeldt, 2006). Moreover, the pH values were almost identical for both control and inoculated "Alheiras" not being affected by inoculation of any strain ($P > 0.05$). Other factors such as temperature, a_w, nutrients, salt content also constitute hurdles which play a critical role in suppressing the growth of microorganisms. In the present study, the results showed that these factors were not critical in controlling the growth of *L. innocua*, since all the samples had similar values and similar storage conditions (Table 2).

The bacteriocinogenic strain *P. acidilactici* HA-6111-2 was able to inhibit strains of different *L. innocua* in "Alheiras" during storage. A similar antibacterial spectrum for other bacteriocins produced by LAB has been reported in broth or meat (Nieto-Lozan et al., 2006; Teixeira de Carvalho, Aparecida de Paula, Mantovania, & Alencar de Moraes, 2006; Vermeiren, Devlieghere, & Debevere, 2004). The population of LAB was not affected in any of the assays. Previous studies also demonstrated that no influence on LAB populations was observed when inoculated with or without *L. innocua* (Alves, Martinez, Lavrador, & De Martinis, 2006).

The quantity of endogenous microflora (total counts at 30 °C) developed in the same way in all samples (>7log CFU/g) (data not shown). Concerning the pathogenic organisms investigated, (*L. monocytogenes*, *Salmonella*, *S. aureus* and *E. coli* O157-H7 were

Table 2
Mean and standard deviation of physicochemical parameters of "Alheira".

	Sample	Mean ± SD				
		Before fermentation/smoking process	After fermentation/smoking process (days)			
			0	11	34	54
a_w	C	0.991 ± 0.004	0.987 ± 0.004	0.982 ± 0.005	0.985 ± 0.002	0.984 ± 0.001
	CP		0.981 ± 0.003	0.986 ± 0.003	0.983 ± 0.002	0.981 ± 0.002
	P		0.976 ± 0.019	0.985 ± 0.006	0.983 ± 0.002	0.981 ± 0.003
	L		0.974 ± 0.007	0.983 ± 0.003	0.983 ± 0.003	0.983 ± 0.002
	P + L		0.975 ± 0.014	0.984 ± 0.003	0.982 ± 0.004	0.979 ± 0.006
Moisture	C	54.3 ± 0.63	52.4 ± 0.92	52.0 ± 2.03	52.3 ± 1.85	52.5 ± 3.09
	CP		52.2 ± 1.27	53.3 ± 2.76	52.6 ± 1.97	52.4 ± 1.37
	P		53.9 ± 0.75	53.0 ± 1.31	53.5 ± 0.87	51.4 ± 3.56
	L		51.7 ± 0.80	53.2 ± 1.29	53.6 ± 1.91	52.5 ± 1.21
	P + L		52.1 ± 1.66	52.3 ± 1.89	53.6 ± 1.44	51.5 ± 0.73
NaCl	C	1.36 ± 0.66	1.35 ± 0.12	1.28 ± 0.13	1.27 ± 0.13	1.44 ± 0.40
	CP		1.26 ± 0.20	1.23 ± 0.07	1.17 ± 0.13	1.26 ± 0.18
	P		1.46 ± 0.66	1.30 ± 0.16	1.27 ± 0.14	1.26 ± 0.22
	L		1.33 ± 0.22	1.36 ± 0.16	1.24 ± 0.16	1.23 ± 0.16
	P+L		1.29 ± 0.24	1.33 ± 0.14	1.18 ± 0.11	1.26 ± 0.14

not detected in any sample), it can be inferred that "Alheiras" were produced under good hygienic conditions. *Enterobacteriaceae* counts were higher than 3log CFU/g until 11 days of storage; however, it should be pointed out that according to the guidelines for the microbiological quality of fermented meats published by Gilbert et al. (2000), this is considered satisfactory. After this time, the count decreased below the detection limit. There were no significant differences ($P > 0.05$) between the two batches.

In respect to sensorial analysis, data were statistically analysed by *t*-tests. Results showed a small difference ($P < 0.05$) between control and the sample with the bac+ strain in batch 1. For batch 2 there were no significant differences ($P > 0.05$) between samples. For batch 3, a small difference (scores lower than 1) was detected only at the end of storage time ($P < 0.05$). The small differences that were detected by the trained sensory panel were that the samples with the bac+ strain were more tasty and a little bit more acid.

This study demonstrated that *P. acidilactici* HA-6111-2 has several attributes as a potential bioprotective organism against listeriae. Since the listeriae can grow or survive under vacuum, at low temperatures, and this bac+ LAB strain grows rapidly because it is well adapted to growth in these fermented meat products and they can reduce the listeriae numbers, it can be either added as bioprotective with anti-listerial properties in fermented meat products. Moreover, it lacks intrinsic virulence factors, has a good capacity for colonisation of meat and produces sufficient amounts of bacteriocin in these meat mixtures, and no significant antibiotic resistance was detected phenotypically. Moreover, a trained panel considered the product sensorially acceptable.

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