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ORIGINAL ARTICLE

Lactobacillus sakei CRL1862 improves safety and protein hydrolysis in meat systems

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Abstract

Aims: The capacity of *Lactobacillus sakei* CRL1862 to prevent the growth of pathogens and its ability to degrade sarcoplasmic and myofibrillar proteins in pork meat systems was evaluated. In addition, basic safety aspects of *Lact. sakei* CRL1862 such as production of biogenic amines and antibiotic susceptibility were addressed.

Methods and Results: The bacteriocin-producing *Lact. sakei* CRL1862 showed respectively bactericide and bacteriostatic effect against *Listeria monocytogenes* and *Staphylococcus aureus* in beaker sausage assay during 9 days of storage at 22°C. The hydrolytic effect of *Lact. sakei* CRL1862 on protein extracts was evaluated by SDS-PAGE and reverse phase HPLC. A more pronounced proteolysis was evidenced in inoculated sarcoplasmic proteins compared with myofibrillar extracts with the generation of predominantly hydrophilic peptides and increase of total free amino acids concentration. *Lactobacillus sakei* CRL1862 produced neither histamine nor tyrosine and exhibited no resistance to the antibiotics assayed.

Conclusions: *Lactobacillus sakei* CRL1862 effectively controlled the growth of *L. monocytogenes* and *Staph. aureus*; moreover, it was able to hydrolyse pork meat extracts generating peptides and amino acids, which may improve hygienic and sensorial attributes of fermented meat products.

Significance and Impact of the Study: The use of an integrated approach to evaluate the major traits of *Lact. sakei* CRL1862 showed it can be applied as an autochthonous functional starter in meat fermentation.

Introduction

Microbial populations that develop in meat and meat ecosystems are the result of the prevailing environmental conditions on the growth of microorganisms initially present in the raw materials and/or introduced during food processing. Meat ecosystems support the growth of highly specific microbial associations, and it has been established that spoilage is caused by a dominating fraction of the initial microbiota in which high species diversity from a few bacterial genera was reported (Nychas *et al.* 2007). Although meat is a selective agent for aerobic bacteria, the competition between facultative anaerobic Gram (+) biota results in lactic acid bacteria (LAB), as a significant portion of the spoilage microbiota (Nychas *et al.* 2008). A few *Lactobacillus* species have been reported as the predominant population in the different meat ecosystems (Vignolo *et al.* 2010). Among them, *Lactobacillus sakei* exhibits a combination of several adaptation strategies to grow and survive on meat and meat products, as was revealed by *Lact. sakei* 23K genome analysis (Chaillou *et al.* 2005). Other species such as *Lactobacillus plantarum* and *Lactobacillus curvatus* also identified as part of fermented sausages microbiota lacks the meat specialization found in *Lact. sakei* (Hebert *et al.* 2012; Siezen *et al.* 2012).

Because fresh meat as well as their fermented and processed products provides an excellent environment for the growth of pathogenic and spoilage organisms, the presence of the emergent food poisoning *Listeria monocytogenes*,

Staphylococcus aureus and *Escherichia coli* O157:H7 during manufacturing has increased the awareness in the general public opinion demanding pathogen-free foods with minimal processing, fewer chemical preservatives, high nutritional value and intact sensory quality. In the last decades, antimicrobial peptides produced by LAB have received great attention as natural food preservatives, leading to the discovery and characterization of a large number of these compounds. LAB currently associated with different meat ecosystems have shown great potential for bacteriocin production (Vignolo *et al.* 2012). The use of bacteriocinogenic LAB to inactivate pathogens such as *L. monocytogenes* and *Staph. aureus* in fermented sausages proved to be highly competitive (Ravyts *et al.* 2008; Liu *et al.* 2010). In addition, lactic acid production by LAB from added sugars exerts a preservative effect against bacteria little resistant to low pH such as *E. coli*, and contributes to the development of the typical organoleptic characteristics of the fermented sausages (Vignolo *et al.* 2010).

On the other hand, the hydrolysis of meat proteins to small peptides and amino acids by LAB during sausage fermentation has become an important issue due to their contribution to their typical taste and aroma. The role of muscle proteinases in the breakdown of sarcoplasmic and myofibrillar proteins as initial main agents of proteolysis is well recognized; nevertheless, LAB are endowed with proteolytic activity mainly intracellular amino, di- and tri-peptidases (Sanz and Toldrá 2002; Fadda *et al.* 2010). The implication of LAB in protein changes is highly dependent on the hydrolytic capacity of the strains used as starter cultures. Several studies have been conducted on the proteolytic activity of the common meat-borne lactobacilli using meat proteins extracts as substrates; indeed, many peptidases have been characterized and purified essentially from *Lact. sakei* (Montel *et al.* 1995; Sanz and Toldrá 2002). Besides the focus on functional characteristics of potential new starter cultures, negative aspects such as antibiotic resistance and undesirable metabolites formation, such as biogenic amines, should not be overlooked. The high load of endogenous bacteria in meat raw material and the inoculation with starters may represent a problem concerning the spreading of antibiotic resistance (Gevers *et al.* 2003; Ammor *et al.* 2007, 2008). In addition, during the ripening of fermented sausages, biogenic amines such as tyramine, histamine, tryptamine, cadaverine, putrescine and spermidine may be formed by microbial decarboxylation of amino acids and thus are usually present in a wide range of foods, fermented sausages being one of the major sources. The use of selected starter cultures is one of the best technological measures to control aminogenesis during meat fermentation (Latorre-Moratalla *et al.* 2012).

In this study, the capacity of *Lact. sakei* CRL1862 isolated from meat products to prevent the growth of *L. monocytogenes* and *Staph. aureus* and its ability to hydrolyse sarcoplasmic and myofibrillar proteins in meat systems was evaluated. In addition, basic safety aspects such as the production of biogenic amines and antibiotic susceptibility were addressed in view of its application as functional starter culture for sausage fermentation.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus sakei CRL1862 and CRL1424 isolated from artisanal dry sausages (Castellano *et al.* 2004 and Castro *et al.* 2011; respectively) were cultured in Man, Rogosa and Sharp (MRS; Scharlau Chemie, Barcelona, Spain) broth at 30°C for 18 h and then maintained at −70°C in 15% (v/v) glycerol. *Listeria monocytogenes* FBUNT (Facultad de Bioquímica Química y Farmacia, Universidad Nacional de Tucumán) and *Staph. aureus* FBUNT were grown in trypticase soy broth (TSB; BBL, Cockeysville, MD, USA) supplemented with 0·5% (w/v) yeast extract, and brain heart infusion (BHI; Britania, Argentina) at 30 and 37°C, respectively. *Kocuria varians* GV822 was isolated from artisanal fermented sausages produced in Argentina (Vignolo *et al.* 1986), activated in a medium composed (w/v) of 0·3% meat extract and 0·5% peptone, pH 6·6 and incubated 18 h at 30°C. Cell counts were carried out as following: (i) total aerobic bacterial on Plate Count Agar incubated for 48 h at 30°C; (ii) LAB on MRS agar incubated for 48 h at 30°C; (iii) *L. monocytogenes* on PALCAM selective media (Difco Laboratories, Inc., Detroit, MI, USA) incubated for 48 h at 30°C and (iv) *Staph. aureus* on Baird-Parker agar supplemented with Egg Yolk Tellurite Emulsion (Oxoid, Hampshire, UK) incubated for 48 h at 37°C.

Beaker sausage preparation and inoculation with *Lactobacillus sakei* CRL 1862

A sausage mix was prepared using 210 g of pork meat added with 0·01% $NaNO_2$, 3·0% NaCl, 0·75% sucrose and 0·75% glucose. The meat and curing salts were thoroughly mixed, divided into 25-g portions and placed in 50-ml beakers. Beaker sausages were separately (three treatments) inoculated with 250 μl of physiological solution (0·89% w/v NaCl) containing *L. monocytogenes* + *Staph. aureus* (10^4 CFU ml^{-1} each), *L. monocytogenes* + *Staph. aureus* (10^4 CFU ml^{-1} each) + *Lact. sakei* CRL1862 (10^9 CFU ml^{-1}), and *L. monocytogenes* + *Staph. aureus* (10^4 CFU ml^{-1} each) + *Lact. sakei* CRL1424 (10^9 CFU ml^{-1}). A noninoculated control was assayed

simultaneously. The sausage mixtures were incubated for 9 days at 22°C, and two samples from each treatment were collected at 0, 2, 4, 7 and 9 days for further analyses.

Antimicrobial activity and pH determinations

A semiquantitative modified well-diffusion assay (Castellano *et al.* 2010) was used to determine *Lact. sakei* CRL1862 bacteriocin activity in the beaker sausages. Samples were stomached for 1 min (Stomacher Lab-Blender 400; A.J. Seward Laboratory, London, UK) in a stomacher bag added with 225-ml physiological solution. Each homogenate (1 ml) was centrifuged (4000 ***g***, 10 min) to obtain a cell-free supernatant (CFS) followed by a filtration through a 0·22-μm-pore-size cellulose acetate filter (Sartorius, Goettingen, Germany). Samples were adjusted to pH 6·5 with 1 N NaOH (Merck, Argentina) to rule out acid inhibition. Five micro litres of each sample were spot placed in semi-solid TSB plates overlay inoculated with *L. monocytogenes*; positive bacteriocin activity was evidenced as an inhibition zone on the indicator organism lawn. The measurement of pH was performed using a pH-meter (Crison Instrument S.A., Barcelona, Spain) in beaker sausage homogenates.

Preparation of *Lactobacillus sakei* CRL1862 cell suspensions and meat proteins extracts

Whole-cell (WC) suspensions (150 ml) from *Lact. sakei* CRL1862 in logarithmic growth phase were collected by centrifugation (10 000 ***g*** for 20 min, 4°C), washed twice in 20 mmol l^{-1} phosphate buffer (pH 7·0) and resuspended in the same buffer (20% of initial volume). The cell-free extracts (CFE) were obtained by the procedure described by Sanz *et al.* (1999). Meat protein extracts were obtained from porcine *longissimus dorsi* sarcoplasmic and myofibrillar proteins according to Fadda *et al.* (1999). The protein content of sarcoplasmic and myofibrillar extracts determined by the method of Bradford (1976) using bovine serum albumin as standard were 2·50 and 0·60 mg ml^{-1}, respectively.

Protein hydrolysis, peptides and free amino acids analysis

For each protein extract (sarcoplasmic or myofibrillar), a combination (1 : 1) of *Lact. sakei* CRL1862 WC and CFE suspensions were used as enzymatic source. The reaction mixture consisted of 3 ml of each (WC and CFE) aseptically added to 30 ml of protein extract. The mixtures were incubated at 30°C in a shaken water bath and sampled initially (0 h) and after 96 h for further analyses. In each case, control samples without the addition of any bacterial enzymes were assayed simultaneously.

Proteins were analysed by SDS-PAGE according to Laemmli (1970) using a Mini Protean II (Bio-Rad Laboratories, Hercules, CA, USA) electrophoresis equipment. Gels were run with Precision Plus Protein Standards (Bio-Rad) of known molecular weight (6·5, 14, 20, 24, 29, 36, 45, 55, 66, 97, 116 and 200 kDa) being subsequently stained for 1 h in a 0·1% (w/v) solution of Coomassie Brillant Blue R in methanol : acetic acid : water (40 : 10 : 50) and then destained for 2 h. The analysis of generated peptides was performed by RP-HPLC as follows: 2 ml of each extract were deproteinized with 5 ml of methanol, centrifuged, supernatants concentrated by evaporation to dryness and resuspended in deionized water. Peptides were analysed at day 0 and after 96 h of incubation, using a 1050 high-performance liquid chromatography (Agilent, Palo Alto, CA, USA) equipped with a photodiode array detector and an automatic injector. A Symmetry C18 (4·6 × 250 mm) (Waters, Milford, MA, USA) column was used. The mobile phase consisted of solvent A (0·1% trifluoroacetic acid in water) and solvent B (acetonitrile-water-trifluoroacetic acid [60 : 40 : 0·085, v/v/v]). The elution was performed as follows: an isocratic phase in 1% solvent B for 5 min, followed by a linear gradient from 1 to 100% solvent B for 55 min, at a flow rate of 1·0 ml min^{-1} at 40°C. Peptides were detected at 214 nm.

For amino acids analysis, samples were extracted and deproteinized using the method described by Aristoy and Toldrá (1991). The amino acids were derivatized to their phenylthiocarbamyl derivatives in accordance with the method of Bidlingmeyer *et al.* (1987) and analysed by reverse phase RP-HPLC using a Novapack C18 column (3·9 × 300 mm) (Waters) as described by Flores *et al.* (1997). Norleucine was used as internal standard. The samples were recorded by measuring the absorbance at 254 nm, and amino acids were identified by their retention times in comparison with standards.

Qualitative detection of biogenic amines and antibiotic susceptibility

Lactobacillus sakei CRL1862 was investigated for its ability to produce histamine and tyramine by decarboxylation of the corresponding precursor amino acid (L-histidine and L-tyrosine) using *K. varians* GV822 as amino acid decarboxylase-positive strain as described by Fadda *et al.* (2001). Antibiotic susceptibility test was performed by applying the disk diffusion assay according to CLSI guidelines (CLSI 2006) using Mueller Hinton agar (Becton Dickinson, USA) and test disks for penicillin G

(10 units), ampicillin (10 μg), tetracycline (30 μg), vancomycin (5 μg), clindamycin (2 μg), erythromycin (15 μg), oxacillin (1 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), streptomycin (300 μg), cefoxitin (30 μg) and rifampicin (5 μg).

Statistical analysis

Each experiment was repeated on two independent replications. The microbial counts and pH values were analysed using one-way analysis of variance (MINITAB STATISTIC Program, release 8.21; Minitab Inc., Philadelphia, PA, USA).

Results

Antibacterial activity of *Lactobacillus sakei* CRL1862 in beaker sausages

The bacteriocinogenic strain *Lact. sakei* CRL1862 was able to grow from 6·95 to 10·00 log CFU g^{-1} after 9 days of incubation at 22°C (Fig. 1a,b). The growth of LAB naturally present in the pork meat (control) and *Lact. sakei* CRL1424 reached a final cell count of 6·56 and 9·20 log CFU g^{-1}, respectively at the end of the incubation period (data not shown). When the beaker sausage was inoculated with *Staph. aureus* FBUNT, its population increased 6·80 log cycles after 9 days of incubation, while in the presence of the protective culture, a bacteriostatic effect was observed (Fig. 1a). On the other hand, cell counts for *L. monocytogenes* after the addition of *Lact. sakei* CRL1862 showed a dramatic decrease from 2·90 log CFU g^{-1} (day 0) to undetectable values from day 4 up to the end of the incubation period; an increase by 3·70 log cycles was determined in untreated samples (Fig. 1b). The beaker sausage inoculated with the nonbacteriocin-producing control strain (*Lact. sakei* CRL1424) showed no significant differences in the cell counts of *Staph. aureus* and *L. monocytogenes* when compared to the growth of both micro-organisms alone (Fig. 1a,b). Changes in the pH values of the beaker sausages due to the growth of *Lact. sakei* CRL1862 were not significantly different from those in samples inoculated with the nonbacteriocin-producing strain (Table 1). The presence of inhibitory activity in the beaker sausage was detected from the second day until the end of incubation period only for the samples containing the bioprotective culture. Homogenates from the control series and that inoculated with *Lact. sakei* CRL1424 did not exhibit any inhibitory activity, indicating that the antimicrobial compound produced by *Lact. sakei* CRL1862 was responsible for pathogen inhibition in treated beaker sausages (Table 1).

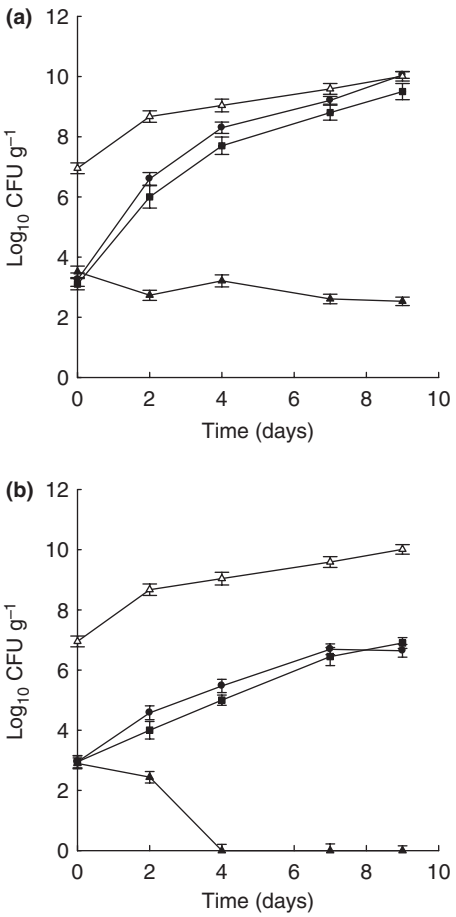

Figure 1 Growth of *Staphylococcus aureus* and *Listeria monocytogenes* FBUNT in mixed culture with *Lactobacillus sakei* CRL1862 in beaker sausages incubated at 22°C during 9 days. (a) Growth of *Lact. sakei* CRL1862 (Δ) and *Staph. aureus* (●) alone, and *Staph. aureus* (■) co-inoculated with *Lact. sakei* CRL1424 and (▲) co-inoculated with *Lact. sakei* CRL1862 (b) Growth of *Lact. sakei* CRL1862 (Δ) and *L. monocytogenes* (●) alone, and *L. monocytogenes* (■) co-inoculated with *Lact. sakei* CRL1424 and (▲) co-inoculated with *Lact. sakei* CRL1862.

Protein degradation, peptide and amino acids formation from pork meat extracts by *Lactobacillus sakei* CRL1862

Cell counts in both sarcoplasmic and myofibrillar protein extracts inoculated with *Lact. sakei* CRL1862 WC and CFE suspensions showed an initial cell number of approximately 10^9 CFU ml^{-1}, a decline to about $1{\cdot}5 \times 10^8$ CFU ml^{-1} and undetectable levels were found in sarcoplasmic and myofibrillar extracts, respectively, after 96 h of incubation. The registered pH values ranged between 6·80–7·00 for myofibrillar protein extract and 5·00–5·50 for sarcoplasmic protein extract after incubation at 30°C during 96 h (data not shown).

Electrophoretic analysis of pork sarcoplasmic and myofibrillar proteins hydrolysis by *Lact. sakei* CRL1862 are

Table 1 pH values (±SD) and bacteriocin activity in beaker sausage incubated at 22°C for 9 days

	pH					Bacteriocin activity				
	Days of storage					Days of storage				
	0	2	4	7	9	0	2	4	7	9
L. monocytogenes + *Stap. aureus*	5·53 ± 0·19a	5·60 ± 0·15a	5·37 ± 0·18c	5·28 ± 0·21c	5·24 ± 0·23c	–	–	–	–	–
L. monocytogenes + *Staph. aureus* + *Lactobacillus sakei* CRL1862	5·50 ±0·16a	4·41 ± 0·18b	4·24 ± 0·14b	4·39 ± 0·17b	4·28 ± 0·19b	–	+	+	+	+
L. monocytogenes + *Staph. aureus* + *Lact. sakei* CRL1424	5·49 ±0·16a	4·42 ± 0·18b	4·31 ± 0·14b	4·35 ± 0·17b	4·38 ± 0·22b	–	–	–	–	–
Control	5·53 ± 0·20a	5·72 ± 0·21a	5·65 ± 0·13a	5·88 ± 0·22a	5·84 ± 0·24a	–	–	–	–	–

–, no inhibition; +, inhibition halo <10 mm.
Means with different letters in the same column differ significantly ($P < 0·05$).

shown in Fig. 2. Although no detectable differences in protein band patterns between 0 and 96 h was observed in control samples, the activity of both WC + CFE on sarcoplasmic proteins produced drastic proteolytic changes hydrolysing all bands, which decreased their intensity throughout the incubation period (Fig. 2a). On the other hand, the proteolytic pattern for myofibrillar proteins displayed a decrease of the band intensity corresponding to myosin (200 kDa) at 96 h of incubation when compared with the noninoculated control (Fig. 2b), myosin hydrolysis being followed by the appearance of new faint protein bands in the range of 50–97 kDa. No degradation of the protein band around 42–45 kDa attributed to actin was evidenced neither by *Lact. sakei* CRL1862 WC and CFE action nor in control extracts.

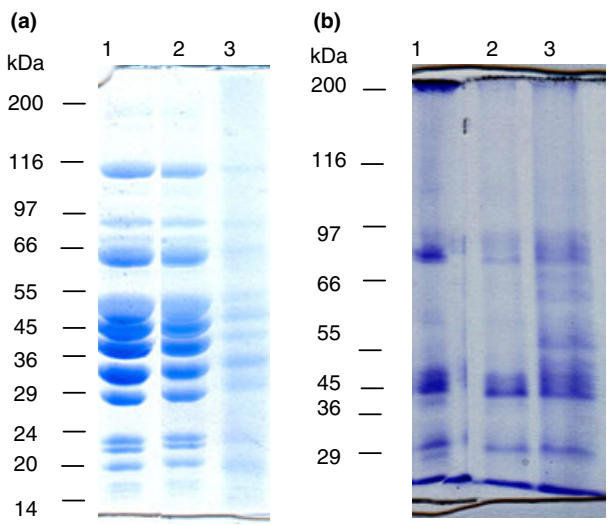

Figure 2 SDS-PAGE of (a) sarcoplasmic and (b) myofibrillar pork proteins hydrolysis by *Lactobacillus sakei* CRL1862 [whole-cells plus cell-free extract (WC + CFE)] during 96 h of incubation at 30°C. (a) lane 1: control at 0 h; lane 2: control at 96 h; lane 3: samples containing WC + CFE at 96 h (b) lane 1: control at 0 h; lane 2: control at 96 h; lane 3: samples containing WC + CFE at 96 h.

The resulting RP-HPLC peptide patterns from the proteolytic activity of *Lact. sakei* CRL1862 on sarcoplasmic and myofibrillar proteins are shown in Figs 3 and 4, respectively. In control samples, peptide profiles from sarcoplasmic extracts showed minor changes between 0 and 96 h (Fig. 3a,b), while that obtained from the combination of WC and CFE from *Lact. sakei* CRL 1862 displayed several new peaks particularly in the range from 20 to 45 min. New peaks eluting at 38, 39, 41 and 42 min of retention time appeared, while others disappeared (47 min) after 96 h of incubation (Fig. 3c,d). On the other hand, when myofibrillar protein extracts were analysed, control samples showed few peaks and minor changes at 96 h compared with day 0 (Fig. 4a,b). The addition of WC and CFE from *Lact. sakei* CRL 1862 resulted in more notable changes after 96 h with the generation of two new peaks eluting at 9 and 12 min (Fig. 4c,d).

Changes in the concentration of free amino acids and natural dipeptides resulting from the activity of *Lact. sakei* CRL1862 WC and CFE on sarcoplasmic proteins are shown in Table 2. In general, the content of almost all analysed amino acids increased with the incubation time both in control and inoculated samples, while a decline in dipeptides concentration was observed in uninoculated extracts. In the presence of *Lact. sakei* CRL1862 WC and CFE, significant increases were observed for leucine, alanine, phenylalanine, β-alanine, histidine and in a lesser extent for isoleucine, tyrosine and arginine when compared with control extracts. As regards to myofibrillar protein extracts, control and inoculated (WC + CFE) samples exhibited a poor amino acid generation, a significant release of alanine and tyrosine being only detected in inoculated samples (data not shown).

Biogenic amines and antibiotic susceptibility tests

When histidine and tyrosine decarboxylation were assayed, results showed *Lact. sakei* CRL1862 failed to

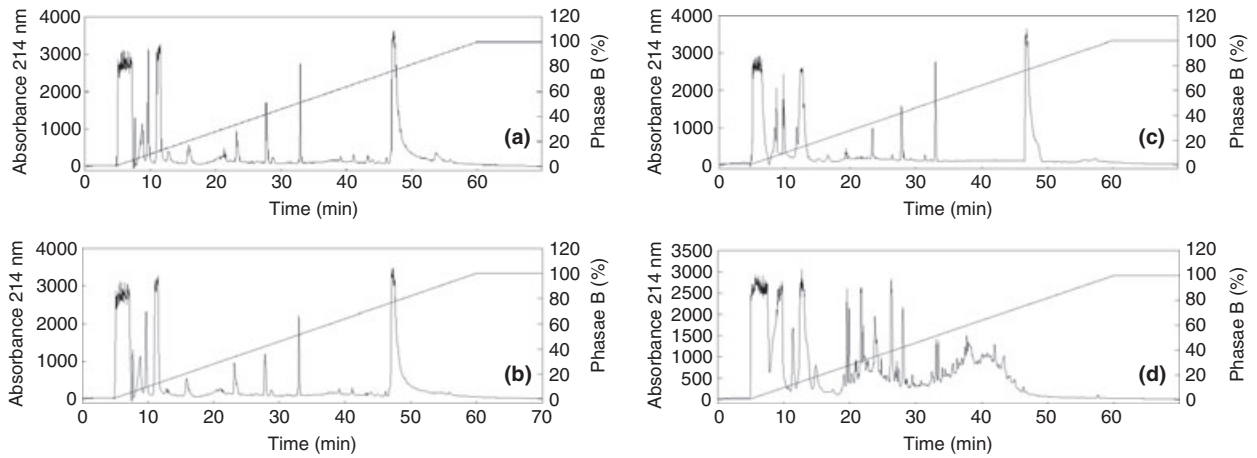

Figure 3 RP-HPLC patterns of soluble peptides contained in sarcoplasmic protein extract control samples at 0 (a) and 96 h (b) of incubation and samples inoculated with *Lactobacillus sakei* CRL1862 (whole-cells plus cell-free extract) at 30°C at 0 (c) and 96 h (d) of incubation.

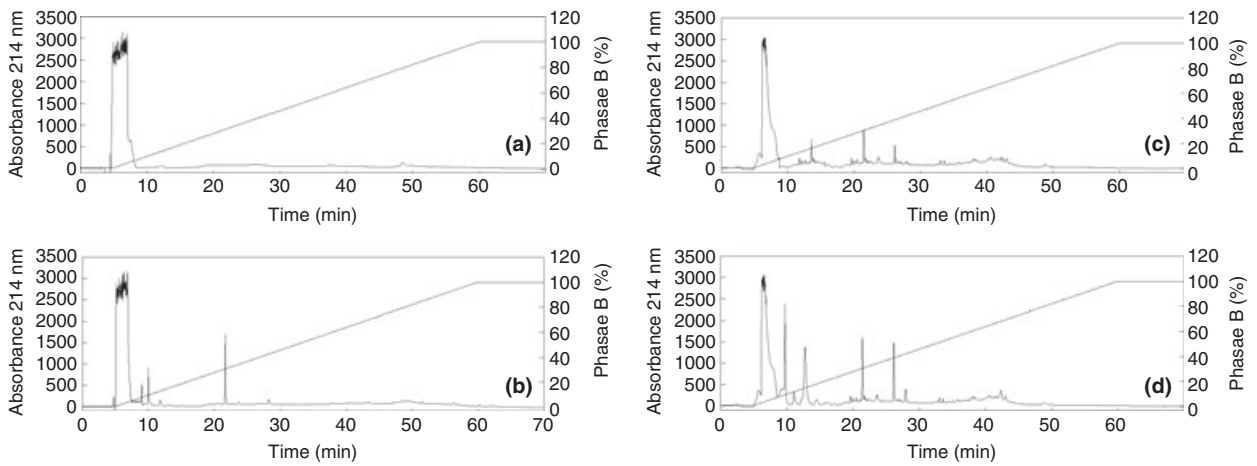

Figure 4 RP-HPLC patterns of soluble peptides contained in myofibrillar protein extract control samples at 0 (a) and 96 h (b) of incubation and samples inoculated with *Lactobacillus sakei* CRL1862 (whole-cells plus cell-free extract) at 30°C at 0 (c) and 96 h (d) of incubation.

produce neither histamine nor tyrosine. As regard as antibiotic susceptibility test, this strain showed to be sensitive to the assayed antibiotics and particularly to those clinically important such as ampicillin, tetracycline and vancomycin (data not shown).

Discussion

The bacteriocin-producing *Lact. sakei* CRL1862 grew rapidly in pork meat beaker sausages and produced sufficient amount of antimicrobial compounds to completely inactivate *L. monocytogenes* and *Staph. aureus* FBUNT when incubated during 9 days at 22°C. In previous work, Castro *et al.* (2011) reported that the inhibitory compound produced by *Lact. sakei* CRL1862 was heat stable and sensitive to proteolytic enzymes. Although bacteriocin production under the stringent sausage fermentation conditions has been previously reported (Hughes *et al.* 2002; Kouakou *et al.* 2009), in this study, the presence of meat-curing additives ($NaNO_2$ and NaCl) seemed not to have affected neither the bacteriocinogenic strain growth nor the antimicrobial action of the produced bacteriocin. The complete inhibition of *L. monocytogenes* by the bacteriocinogenic *Lact. sakei* CRL1862 at day 4 after inoculation of the beaker sausages is in agreement with those reported using different bacteriocinogenic *Lactobacillus* species during fermented sausage production (Ravyts *et al.* 2008; Liu *et al.* 2010). Although a bacteriostatic effect of the bacteriocin-producing *Lact. sakei* CRL1862 on *Staph. aureus* was found in this study, a more

Table 2 Net increments (expressed as mg g^{-1} of meat ± SD) in free amino acids and natural dipeptides of porcine sarcoplasmic extract after 96 h of incubation at 30°C without (control) and with *Lactobacillus sakei* CRL1862 (WC + CFE)

	Sarcoplasmic protein extract	
Amino acid or dipeptide	Control	WC + CFE
LEU	78·0 ± 13·6	132·5 ± 5·6
ALA	125·0 ± 2·4	157·4 ± 4·8
PHE	43·6 ± 7·5	79·3 ± 2·8
HIS	45·8 ± 0·8	66·7 ± 1·3
ILE	46·3 ± 9·6	53·7 ± 0·1
TYR	28·3 ± 0·5	38·4 ± 3·8
ARG	11·2 ± 0·5	24·1 ± 0·6
ASP	4·9 ± 5·6	−12·4 ± 4·2
LYS	24·7 ± 1·2	−5·0 ± 4·8
MET	19·7 ± 0·6	−28·6 ± 13·9
GLY	42·3 ± 10·9	7·4 ± 3·3
SER	36·0 ± 12	4·0 ± 10
PRO	74·4 ± 11·1	14·2 ± 18·9
GLN	−19·5 ± 13	5·4 ± 12·3
GLU	57·5 ± 12·9	56·2 ± 10·5
ASN	9·6 ± 4·4	0·3 ± 5·0
THR	34·0 ± 1·4	34·3 ± 1·0
TAU	33·1 ± 3·1	28·4 ± 2·4
VAL	55·6 ± 6·4	59·5 ± 7·7
TRP	11·5 ± 8·2	0·3 ± 6·4
ORN	6·3 ± 6·3	−3·0 ± 5·1
BALA*	31·6 ± 1·7	68·8 ± 6·3
Carnosine	−353·5 ± 66	218·9 ± 71
Anserine	−14·8 ± 3·0	6·1 ± 4·8

WC + CFE, whole-cells plus cell-free extract.
*Nonstandard amino acids.

significant reduction of this pathogen was reported by the bacteriocinogenic *Lact. pentosus* 31-1 during ripening of fermented sausages (Liu *et al.* 2010). The suppression of *L. monocytogenes* and *Staph. aureus* growth in this study was due to the bacteriocin produced by *Lact. sakei* CRL1862 because the beaker sausage inoculated with a nonbacteriocinogenic Bac^- strain of *Lact. sakei* CRL1424 was unable to reduce the viable counts to the same extent as did the bioprotective culture. Moreover, the similar pH values observed for the beaker sausages containing the bacteriocin-producing and the Bac^- strain indicated that acid production was not responsible for pathogens inhibition.

Among the phenomena leading to flavour formation, it is well known that small peptides and free amino acids released by starter or endogenous proteases during ripening, directly participate in the basic taste of dry fermented sausages, and indirectly contribute to the development of their typical flavour because they are precursors of many volatile and nonvolatile compounds. In view to apply *Lact. sakei* CRL1862 as a functional culture, the evaluation of its protein hydrolytic ability and amino acid generation from sarcoplasmic and myofibrillar proteins was carried out by a biochemical approach using proteolytic enzymes available in both WC and intracellular CFE. Similarly, CFE from different microorganisms as an additional enzyme source were also applied to achieve high availability of small peptides and amino acids in dry fermented sausages (Flores and Toldrá 2011). Several studies were reported on the proteolytic activity of meat LAB, particularly *Lactobacillus* and *Pediococcus* on protein extracts, showing *Lact. sakei* as having the highest degradation ability (Candogan and Acton 2004; Toledano *et al.* 2011). In this study, proteolytic activity of *Lact. sakei* CRL1862 WC and CFE showed a more pronounced hydrolysis of sarcoplasmic proteins compared with myofibrillar extracts. Similarly, studies performed using a sausage-like system reported a higher activity towards sarcoplasmic fraction when strains of *Lact. sakei, Lact. plantarum* and *Lact. curvatus* from meat origin were used as starter cultures (Fadda *et al.* 2002; Villani *et al.* 2007). Notably, noninoculated sarcoplasmic proteins failed to undergo proteolytic degradation highlighting a weak activity of endogenous proteases, in disagreement with those previously reported (Fadda *et al.* 2002; Hughes *et al.* 2002). Nevertheless, proteolytic changes on myofibrillar proteins denoted the action of both, *Lact. sakei* CRL1862 and muscle enzymes. Also, Hughes *et al.* (2002) and Martin *et al.* (2001) reported degradation of myofibrillar proteins by indigenous and bacterial enzymes in meat products inoculated with different starter cultures. Unlike the findings in this study, a lack of variation in myofibrillar patterns from uninoculated control of fermented sausages was reported by Casaburi *et al.* (2007).

The combination of WC and CFE from *Lact. sakei* CRL1862 showed to generate predominantly hydrophilic peptides (20–30 min of retention time), while other relatively hydrophobic in nature (30–45 min of retention time) were also observed. Considering that hydrophilic peptides are those correlated with desirable cured-meat flavour, whereas those constituted by hydrophobic residues are associated with bitterness (Sanz *et al.* 1999), the released peptides by *Lact. sakei* CRL1862 will positively contribute to the final organoleptic characteristics of fermented sausages. In addition, inoculated sarcoplasmic and myofibrillar protein extracts were involved in the generation of amino acids, because a clear increase in their total content was produced in the presence of WC + CFE system. In inoculated sarcoplasmic extracts, the amino acids primarily responsible for this increase were leucine, alanine, phenylalanine, β-alanine and histidine, while in a lesser extent, isoleucine, tyrosine and arginine were also released. It has been demonstrated that the proteolytic activity on sarcoplasmic and myofibrillar

proteins of meat-borne *Lactobacillus*, particularly *Lact. sakei*, played an important role in amino acid generation (Sanz *et al.* 1999). In fact, the major release of leucine and alanine in this study agrees with the previously reported aminopeptidase and tripeptidase specificities of *Lact. sakei* CECT4808 (Sanz and Toldrá 2002). Although *Lact. sakei* is able to degrade arginine with ammonia and ATP production by the arginine deiminase pathway (Rimaux *et al.* 2011), the presence of an intracellular arginine aminopeptidase as described by Sanz and Toldrá (2002) may account for the presence of arginine in the sarcoplasmic extract after 4 days of incubation with *Lact. sakei* CRL1862 WC and CFE.

The high amounts of proteins present in fermented sausages and the proteolytic activity during ripening that result in an increase in small peptides and free amino acids provide the precursors for decarboxylase activity. However, under the assayed conditions in this study, *Lact. sakei* CRL1862 did not produce histamine or tyramine in agreement with other LAB strains such as *Lact. sakei* and *Lact. plantarum* frequently described as nonaminogenic (Ansorena *et al.* 2002). However, amino acid decarboxylase activity among LAB from meat products was reported (Curiel *et al.* 2011; Latorre-Moratalla *et al.* 2012). Because it is known that some genera or species are more frequently reported than others to be able to produce specific biogenic amines, the ability to decarboxylate amino acids is a strain-dependent property (Suzzi and Gardini 2003; Latorre-Moratalla *et al.* 2012). Therefore, a case-by-case evaluation of the aminogenic activity of the strains to be selected as amine negative starter culture is essential. On the other hand, concerning antibiotic resistance evaluation of *Lact. sakei* CRL1862, sensitivity towards the assayed clinically relevant antibiotics (ampicillin, tetracycline and vancomycin) was exhibited. In contrast, other meat-borne *Lactobacillus* species such as *Lact. curvatus* and *Lact. plantarum* were reported to be a reservoir for acquired resistance genes (*tet*M) that can be spread to other Gram positive bacteria (Gevers *et al.* 2003; Ammor and Mayo 2007). Although nonenterococcal LAB (with the exception of pathogenic streptococci) have not yet been implicated as a source for isolates causing human disease, recently the European Food Safety Authority (EFSA) has launched a 'qualified presumption of safety' (QPS) concept demanding the investigation of antimicrobial resistance genes among starter or probiotic strains used on the food supply (Franz *et al.* 2010).

Conclusions

The bacteriocin-producing *Lact. sakei* CRL1862 was able to effectively control the growth of *L. monocytogenes* and *Staph. aureus* in the assayed meat systems. In addition, it was able to hydrolyse pork meat proteins generating small peptides and amino acids, which are known to improve sensorial attributes of fermented meat products. On the basis of competitiveness and hygienic aspects such as antimicrobial activity and the lack of biogenic amine production and transferable antibiotic resistance, *Lact. sakei* CRL1862 as functional starter culture could help to increase the quality and safety of fermented sausages.

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