Available online at www.sciencedirect.com
ScienceDirect
Meat Science 79 (2008) 603–610
MEAT SCIENCE
www.elsevier.com/locate/meatsci

Lactobacillus role during conditioning of refrigerated and vacuum-packaged Argentinean meat

S. Fadda [a,*], C. Chambon [b], M.C. Champomier-Vergès [c], R. Talon [d], G. Vignolo [a]

[a] *Centro de Referencia para Lactobacillos (CERELA), CONICET, Chacabuco 145, T4000 ILC, Tucumán, Argentina*
[b] *INRA Clermont Ferrand-Theix, UR370-Plateforme Proteomique, 63122 Saint-Genès Champanelle, France*
[c] *INRA Jouy en Josas, Unité Flore Lactique et Environnement Carné (FLEC), Domaine de Vilvert, 78350 Jouy en Josas, France*
[d] *INRA Clermont Ferrand-Theix, Unité Microbiologie, 63122 Saint-Genès Champanelle, France*

Received 31 January 2007; received in revised form 27 March 2007; accepted 2 April 2007

Abstract

The role of *Lactobacillus* strains with bioprotective and technological potential on raw beef during 15 days of storage under vacuum at 7 °C was investigated. The assayed strains were able to grow on the meat, *Lactobacillus curvatus* CRL705 and *Lactobacillus sakei* 23K showing the highest competitiveness. A net increase of amino acids was determined in inoculated samples when compared to the control, this being maximal for *Lactobacillus plantarum* CRL681. Although an important endogenous activity of meat sarcoplasmic proteins was observed, the disappearance of protein bands and the generation of a new one were detected as a consequence of *Lactobacillus* growth. A synergistic effect of *Lactobacillus* in combination with the muscle proteolytic enzyme complex can be suggested. From the studied strains, the bacteriocin producer *L. curvatus* CRL705 may be considered as a good candidate to contribute to meat ageing by means of small peptides and free amino acids generation while improving shelf life.

Keywords: LAB; Proteolytic activity; Meat proteins; Meat quality

1. Introduction

Animal muscles turn into meat by a number of chemical and structural processes. By definition, meat conditioning starts at the moment of animal death and ends with the exhaustion of degradable energy-rich compounds such as ATP, creatine and glycogen. Biochemical changes undergoing during refrigerated conditioning cause muscle structural alterations which are of paramount importance for meat quality. Immediately after death, skeletal muscle is soft but it soon becomes very tough and unpalatable from *rigor mortis*. As a result of animal death the supply of energy-rich compounds and oxygen stops. However, these compounds continue being anaerobically degraded in the muscle cell conducting to a pH fall between 5.3 and 5.8 and the onset of *rigor mortis* in the meat. These changes produce a shortening in muscles fibers with an adverse effect on meat tenderness (Honikel, 1992). Muscle toughness gradually decreases due to protein degradation, and meat texture is improved during *postmortem* conditioning. Beef immediately after slaughter has been reported to have sourness and little beefy flavor even if it is cooked. However, *postmortem* storage of beef at low temperature gives it a more beefy and palatable flavor (Nishimura, 1998, 2002).

Postmortem ultra structural modifications in meat cytoskeletal proteins network will result in a weakening of myofibrils. Proteolysis plays an important role on meat tenderization and further flavor development. It has been attributed mainly to endogenous enzymes, particularly calcium dependent enzymes, calpains and acidic cathepsins (Toldrá et al., 1992; Koohmaraie, 1994). The initial degradation of actin and myosin into peptides is due to cathepsin

* Corresponding author. Tel.: +54 381 4310465x116.
E-mail address: sfadda@cerela.org.ar (S. Fadda).

0309-1740/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.meatsci.2007.04.003

D, while the later decomposition of peptides into free amino acids is done by muscle and microbial enzymes (Verplaetse, 1994). Recent investigations demonstrated that lactic acid bacteria (LAB), particularly *Lactobacillus sakei* and *Lactobacillus curvatus* currently isolated from meat, have the ability to hydrolyze muscle sarcoplasmic proteins and, in a lesser extent, myofibrillar proteins (Díaz, Fernández, García de Fernando, de la Hoz, & Ordoñez, 1997; Rodríguez, Nuñez, Córdoba, Bermúdez, & Asensio, 1998; Fadda, Vignolo, Ruiz Holgado, & Oliver, 1998; Sanz et al., 1999). The intracellular enzymes of *L. curvatus* and *L. sakei* were reported to be responsible for the generation of small peptides and amino acids which contribute to the process either as direct flavor enhancers or as precursors of other flavor compounds during the ripening of dry-fermented sausage (Fadda et al., 1999a, 1999b; Sanz et al., 1999). However, these studies were assessed *in vitro* using experimental meat systems and the hydrolytic action of *Lactobacillus* on raw meat has not been assayed so far.

On the other hand, meat is an excellent substrate for bacterial growth and if some methods to restrict their presence are not applied, meat becomes contaminated. A widely used practice in meat marketing is the vacuum packaging of primal cuts for distribution and extension of storage life followed by removal of the meat from the package for preparation of retail cuts. As a result of these practices meat can become contaminated with pathogens of public health concern such as *Listeria monocytogenes*. Gas-impermeable packaging constitutes an additional hurdle to limit meat spoilage because it restricts the growth of *Pseudomonas* spp., so LAB, *Brochothrix thermosphacta* and *Enterobacteriaceae* become the major components of the spoilage microbiota (Hansen & Bautista, 2000; Sakala et al., 2002). Biopreservation systems such as bacteriocinogenic LAB cultures and/or their bacteriocins have received increasing attention and new approaches to control pathogenic and spoilage microorganisms have been developed. Many studies have demonstrated bacteriocin antagonism against spoilage LAB, *B. thermosphacta* and *L. monocytogenes* in cooked and raw meat products (Bredholt, Nesbakken, & Holck, 2001; Vermeiren, Devlieghere, & Debevere, 2004; Castellano, Holzapfel, & Vignolo, 2004; Castellano & Vignolo, 2006). To be successful in biopreservation, LAB culture must survive during storage at refrigeration temperatures, compete with the relatively high indigenous microbial load of raw meat, actively inhibit pathogenic and spoilage bacteria and do not alter the sensory properties of meat.

The new trends of meat industry involve the use of a *new generation* starter cultures with industrial or nutritional important functionalities (Leroy, Verluyten, & De Vuyst, 2006). The so-called "functional starter cultures" contribute to food safety by producing antimicrobial compounds such as bacteriocins and also provide sensorial, technological, nutritional and/or health advantages.

The objective of the present work was to investigate the proteolytic events and the role of *Lactobacillus* strains, with proved bioprotective and technological potential (Vignolo, Ruiz Holgado, & Oliver, 1988; Fadda et al., 1998; Castellano et al., 2004; Castellano & Vignolo, 2006) in meat conditioning during refrigerated vacuum-packaged storage. Identification of susceptible proteins and hydrolytic products was achieved by matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS).

2. Materials and methods

2.1. Strains and growth conditions

Lactobacillus curvatus CRL705 and *Lactobacillus plantarum* CRL681 were isolated from Argentinean dry fermented sausages and belong to the CERELA-CONICET collection. *Lactobacillus sakei* 23K was isolated from meat in France. Strains were at −70 °C in MRS agar for lactic acid bacteria and activated before use in the same medium. A single colony was grown for 24 h in MRS broth at 30 °C, transferred to a chemical defined medium (MCD) (Lauret et al., 1996) and grown for 16 h at 30 °C. This subculture was then used to inoculate 60 ml of MCD medium at $DO_{600\,nm}$: 0.1. The cells were collected by centrifugation after 6 h of growth at 30 °C (logarithmic growth phase). The pellet was washed twice in sodium phosphate buffer, 20 mM pH 7.0, resuspended in 500 μl of the same buffer and used for the inoculation of meat discs surfaces.

2.2. Meat inoculation and sampling

Bovine *semimembranous* muscle was obtained from a commercial beef processor. After removing fat and connective tissue in aseptic conditions, 1 cm thickness steaks were obtained and frozen at −20 °C until utilization. After thawing, meat discs were aseptically obtained (3-cm diameter × 1-cm thick), and inoculated with 100 μl of cell suspensions (50 μl on each side, approximately 10^8 CFU/cm^2). Samples were vacuum-packaged using a film (Cryovac; Argentine) with a diffusion coefficient to oxygen of 6/14 $cm^3\,m^{-2}\,atm^{-1}\,24\,h^{-1}$ at 25 °C and 75% relative humidity. The packages were sealed at a final vacuum of 99% using a Turbovac 320 ST vacuum packaging machine (Howden Food Equipment, Holland). All samples were stored at 7 °C for 15 days. Four meat discs constituted each lot: lot 1: control without microbial inoculation; lots 2–4 inoculated with *L. curvatus* CRL705, *L. plantarum* CRL681 and *L. sakei* 23K, respectively. Samples were taken at 0 and 15 days and were analyzed for microbial viability, pH and protein changes. For each sampling time, two discs were homogenized 1:10 (w/vol) with sterile phosphate buffer 20 mM pH 6.0 using a laboratory blender (Stomacher 400 London, UK) for 8 min. This slurry was then used for bacterial enumeration and for extraction of sarcoplasmic and myofibrillar proteins. The supernatant obtained after centrifugation (10,000 rpm for 20 min at 4 °C) containing among others soluble proteins, consti-

tuted the sarcoplasmic extract. Myofibrillar proteins were extracted from the pellet according to Sanz et al. (1999) using a high-ion strength phosphate buffer (Na_2HPO_4–NaH_2PO_4 0.1 N KI 0.7 M, pH 6.0 containing 0.02% sodium azide). Two independent experiments were carried out.

2.3. Bacterial counts and pH measurement

For bacterial enumeration, decimal dilutions were prepared and plating was carried out on MRS agar (Merck) while for control samples, an additional plating was done on plate count agar, PCA (Merck) and incubated for 48 h at 30 °C and 37 °C, respectively. The pH values of the sarcoplasmic extracts were determined by using a Metrohn 692 pH/Ion Meter.

2.4. Protein changes

2.4.1. Free amino acid (aa) analysis

Free amino acids were measured according to the OPA spectrophotometric assay (Church, Swaisgood, Porter, & Catignani, 1983). One milliliter of 12% trichloroacetic acid (TCA) was added to 0.5 ml of sarcoplasmic or myofibrillar extracts. After protein precipitation the extract was centrifuged (10,000 rpm for 10 min at 4 °C) and 50 µl supernatant aliquot was treated with *o*-phtaldialdehyde reagent. Results were expressed as absorbance at 340 nm. Amino acid concentration (mM) can be calculate from the following relationship: aa (mM) $= \Delta A_{340} F/\varepsilon$, where ΔA_{340} is the experimentally observed change of absorbance at 340 nm using 1 cm light path; F, dilution factor corresponding to the assay procedure; ε, molar absorption coefficient (6000 M^{-1} cm^{-1}). All results are the mean of three replicate assays.

2.4.2. Determination of protein concentration

The protein contents of the sarcoplasmic and myofibrillar extracts were determined by the Bradford method (Bradford, 1976).

2.4.3. Gel electrophoresis (SDS-PAGE)

The hydrolysis of muscle proteins was monitored by SDS-PAGE analysis (Fritz, Swartz, & Greaser, 1989) using a Mini Protean 3 gel Unit (BIORAD) at 12% and 10% polyacrylamide gels for sarcoplasmic and myofibrillar proteins, respectively. Wide range protein markers (from 212 to 6.5 kDa) were used as standards (Biolabs Inc., UK). Proteins were visualized by Bio-Safe™ Coomassie staining (BIORAD).

2.4.4. Protein and peptides identification by MALDI-TOF MS

Gels bands of interest were excised and were washed with 100 µl of 25 mM NH_4HCO_3 for 30 min, destained with 100 µl of 25 mM NH_4HCO_3/acetonitrile (v/v) twice 30 min and dehydrated in acetonitrile. Gels bands were completely dried using a speed vac before trypsin digestion. The dried gel volume was evaluated and three volumes of trypsin (V5111; Promega, Madison, WI, USA), 10 ng/µl in 25 mM NH_4HCO_3 were added. Digestion was performed at 37 °C during 5 h. The gels pieces were centrifuged and 12–20 µl of acetonitrile (depending of gel volume) were added to extract peptides. The mixture was sonicated for 5 min and centrifuged. For MALDI-TOF MS analysis, 1 µl of supernatant was loaded directly onto the MALDI target. The matrix solution (5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) was added immediately and allowed to dry at room temperature (Hamelin et al., 2006). A Voyager DE-Pro model of MALDI-TOF mass spectrometer (Perseptive BioSystems, Farmingham, MA, USA) was used in positive-ion reflector mode for peptide mass fingerprinting. Internal calibration was performed using peptides resulting from auto-digestion of porcine trypsin. Monoisotopic peptide masses were assigned and used from NCBI or Swissprot databases searches with the "Mascot" software (http://www.matrixscience.com).

3. Results

3.1. Bacterial counts and pH evolution in vacuum-packaged meat discs

LAB cells were spread onto meat discs surfaces at an initial value ranging from 4 to 7×10^8 CFU/cm^2 (Table 1) and increased between 1 and 2 log after 15 days of storage at 7 °C under vacuum-packaged conditions. Although *L. curvatus* CRL705 cell counts were 1-log higher than *L. sakei* 23K (3.0×10^{10} vs. 3.5×10^9 CFU/cm^2), the ultimate pHs on meat discs were similar, this indicating a higher acidifying potential of the last strain. The pH values

Table 1
Bacterial counts and pH changes of vacuum-packaged beef discs during 15 days of storage at 7 °C

Day	Control		*L. curvatus* CRL705		*L. plantarum* CRL681		*L. sakei* 23K	
	0	15	0	15	0	15	0	15
PCA	$<10^2$[a]	1.3×10^5						
MRS	$<10^2$	8.0×10^4	4.0×10^8	3.0×10^{10}	7.0×10^8	3.8×10^9	4.0×10^8	3.5×10^9
pH	5.70	5.64	5.85	4.90	5.82	5.12	5.78	4.88

[a] CFU/cm^2.

Table 2
Amino acids, resulting from the sarcoplasmic extracts after incubation of *Lactobacillus* strains on vacuum-packaged beef discs during 15 days of storage at 7 °C

Day	Amino acid ($DO_{340\,nm}$)			
	Control	*L. curvatus* CRL705	*L. plantarum* CRL681	*L. sakei* 23K
0	0.280 ± 0.008[a]	0.279 ± 0.008	0.262 ± 0.007	0.293 ± 0.008
15	0.366 ± 0.007	0.448 ± 0.009	0.451 ± 0.009	0.414 ± 0.009
Net increase	0.086	0.169	0.189	0.122

[a] Results are expressed as mean ± SD.

dropped between 0.70 and 0.95 units on the inoculated discs. Indigenous bacterial development was also observed on meat discs with no lactobacilli inoculation (control), although pH values remained almost constant during the 15 days (Table 1).

3.2. Amino acid contents

Total TCA-soluble amino acids were analyzed at 0 and 15 days of incubation of meat discs at 7 °C. The variation in the concentration of free amino acids resulting from the activity of the assayed *Lactobacillus* strains on sarcoplasmic extracts is shown in Table 2. When compared to the control, increase of amino acids was significantly higher in inoculated samples after 15 days, this being maximal for *L. plantarum* CRL681 ($OD_{340\,nm}$: 0.189). Free amino acids were recovered in the water soluble sarcoplasmic extract, their concentration being negligible in myofibrillar extract (data not shown).

3.3. Sarcoplasmic and myofibrillar protein contents

Fig. 1 shows the variations of sarcoplasmic protein content in the inoculated meat discs during incubation. Proteins decreased between 26% and 49% after 15 days of storage at 7 °C. Control samples presented 26% less proteins at the end of storage while lot 4 inoculated with

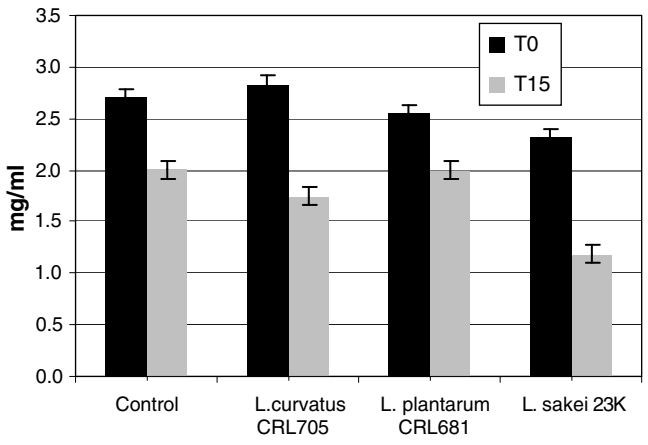

Fig. 1. Effect of *Lactobacillus* strains on sarcoplasmic proteins from vacuum-packaged beef discs after 15 days of incubation at 7 °C.

L. sakei 23K recorded the more pronounced effect (49% of degradation). When the myofibrillar extracts were analyzed, protein content remained almost constant during storage time (data not shown).

3.4. SDS-PAGE analyses

The protein profiles of sarcoplasmic proteins of vacuum-packaged beef discs are shown in Fig. 2. No proteolytic changes were observed for the 119 kDa band during the storage period, this corresponding to an amylo-1,6-glucosidase, 4-α-glucanotransferase isoform according to MALDI-TOF MS analyses. Proteins with molecular weights of 97 and 36 kDa corresponding to myophosphorylase and glyceraldehyde-3-phosphate dehydrogenase respectively, decreased their intensity or disappeared at 15 days in all lots, denoting an important endogenous activity in sarcoplasmic extract since these protein bands were already severely degraded in the control (Fig. 2, lanes 2, 4, 6 and 8). On the other hand, the presence of LAB strains was responsible for the complete (*L. curvatus* CRL705 and *L. sakei* 23K) and partial (*L. plantarum* CRL681) degradation of the band identified by homology as being B-enolase (47 kDa), and the decrease in intensity of the 17 kDa band corresponding to myoglobin (Fig. 2, lanes 4, 6, 8). A slight degradation was also observed in the glucose-phosphate isomerase band (62.8 kDa) in presence of LAB strains (Fig. 2, lanes 4, 6, 8). On the other hand, new bands could be observed after 15 days as result of storage of meat discs. A new band of approximately 30 kDa appeared in all meat samples including the control, while the band corresponding to troponin I (21 kDa) was only generated in inoculated meat discs indicating a major bacterial hydrolysis. This bacterial proteolytic action was also evident by the intensity increase of a single band (approximately 14 kDa) at the bottom of the gel at 15 days corresponding to lighter proteins and peptides, probably degradation products of larger proteins. The sarcoplasmic protein patterns resulting from the hydrolytic action reflected a higher activity of *L. curvatus* CRL705 and *L. sakei* 23K strains. In myofibrillar protein profiles only the intensification of two fragments of ~149–154 kDa after 15 days was recorded. In general, the protein pattern has not been affected by the presence of the lactobacilli strains (data not shown).

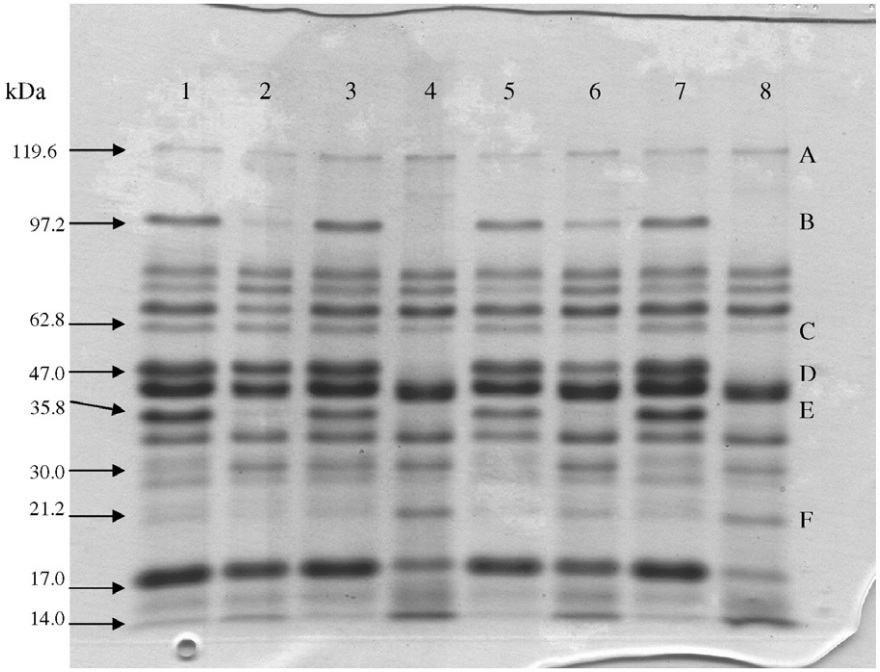

Fig. 2. SDS-PAGE of sarcoplasmic proteins from vacuum-packaged beef discs after 15 days at 7 °C. Lane 1: control, T0; lane 2: control, T15; lane 3: *L. curvatus* CRL705, T0; lane 4: *L. curvatus* CRL705, T15; lane 5: *L. plantarum* CRL681, T0; lane 6: *L. plantarum* CRL681, T15; lane 7: *L. sakei* 23K, T0; lane 8: *L. sakei* 23K, T15. MALDI-TOF MS identified proteins: A: amylo-1,6-glucosidase (119.6 kDa); B: alpha-1,4-glucan orthophosphate glycosyl transferase (97.2 kDa); C: glucose-phosphate isomerase (62.8 kDa); D: B-enolase (46.9 kDa); E: glyceraldehyde-3-phosphate dehydrogenase (35.8 kDa); F: troponin I (21.2 kDa).

Table 3
MALDI-TOF MS identification of the affected vacuum-packaged beef proteins after incubation at 7 °C for 15 days with *Lactobacillus* strains

Protein name		Swiss-Prot or NCBInr accession number/source	M_w (kDa)	PI	Sequence coverage (%)	Recorded effect during storage
Metabolism						
A[a]	Similar to amylo-1,6-glucosidase, 4-α-glucanotransferase isoform 1	gi|76613342_*Bos taurus*	119.571	6.61	28	Unaffected
B	Alpha-1,4-glucan orthophosphate glycosyl transferase; myophosphorylase; glycogen phosphorylase isozyme	gi|1836054_*Bos taurus*	97.232	6.65	40	Affected in all samples (++++)
C	Glucose-phosphate isomerase	gi|73587307_*Bos taurus*	62.831	7.33	52	Affected by LAB (+)
D	B-enolase; 2-phospho-D-glycerate hydro-lyase; muscle specific enolase; skeletal muscle enolase; enolase 3	gi|20141354_*Oryctolagus cuniculus*	46.90	7.60	32	Affected by LAB (++++)
E	Glyceraldehyde-3-phosphate dehydrogenase	gi|77404273_*Bos taurus*	35.845	8.5	37	Affected in all samples (++++)
Contractile apparatus						
F	Troponin I; TnI	gi|409009_*Oryctolagus cuniculus*	21.201	8.86	34	Recovered on sarcoplasmic extracts by LAB action

[a] Annotation ascribed on the gel, Fig. 2.

3.5. MALDI-TOF MS analyses

Protein bands of interest were cut-off from the gels and submitted to MALDI-TOF MS analyses. Table 3 shows the identity, origin and effect of the assayed conditions on each identified protein. As it can be observed all of them constitute metabolic enzymes with exception of troponin I. It should be noted that troponin I is a myofibrillar protein which was recovered on the sarcoplasmic extracts after 15 days only by effect of LAB strains.

4. Discussion

The proteolytic changes resulting from the growth of *Lactobacillus* strains in meat discs were analyzed. The three strains experienced a good adaptation to the meat environment, these data being in agreement with previous works (Fadda et al., 1998; Champomier-Vergès, Zuñiga, Morel-Deville, Pérez-Martínez, & Zagorec, 1999; Castellano et al., 2004). *L. curvatus* CRL705 and *L. sakei* 23K showed the highest competitiveness on meat as substrate. The pH

decrease observed in this study evidenced the efficient fermentative metabolism of these strains, although this variation was more pronounced than expected in real meat technological conditions due to the high initial bacterial inoculum selected for this study in order to evidence bacterial proteolytic activity.

Muscle conversion into meat involves biochemical and structural changes occurring during 24 h *postmortem*, these events playing a great role in the ultimate quality and palatability of meat. In this report, the release of soluble amino acids and peptides was evaluated in order to check *in situ* muscle and bacterial aminopeptidase activities. As previously demonstrated (Flores, Aristoy, & Toldrá, 1998; Nishimura, Okitani, Rhue, & Kato, 1990; Toldrá et al., 1992; Sentandreu & Toldrá, 2001) the presence of muscle aminopeptidase activity was evidenced by a net augmentation of amino acids at the end of storage time. In this study, the highest amino acid accumulation was observed on meat discs inoculated with *Lactobacillus* indicating the presence of bacterial aminopeptidases in the assayed conditions. Kato et al. (1994) also reported that increases in free amino acids were more remarkable in conditioned pork meat inoculated with lactic acid bacteria than in non-inoculated fresh meat. Some components of the proteolytic system of *L. sakei* and *L. curvatus*, the most prevalent species found in refrigerated vacuum-packaged meat, have been purified and characterized (Montel, Seronine, Talon, & Hebraud, 1995; Sanz & Toldrá, 2001, 2002). Since optimal pH of these enzymes are in the range of 5–8, the higher amino acid release observed in the presence of *L. plantarum* CRL681 could be attributed to a lesser pH decrease (pH: 5.12) which could prevent the inactivation of *L. plantarum* aminopeptidases. These peptidases may contribute to the release of small peptides and free amino acids during *postmortem* conditioning of meat, which will positively impact on meat taste and aroma. These results are in accordance with those of Nishimura (2002) who observed that storage of beef at low temperature improves flavor by increases in free amino acids, peptides and inosine 5′-monophosphate. Moreover, Claeys, De Smet, Balcaen, Raes, and Demeyer (2004) reported a substantial increase in peptide concentration in the 3–17 kDa MW range in fresh meat with increasing *postmortem* time, Argentine and Irish beef contained the higher amount of peptides (<10 kDa) related to taste intensity when compared with other beef sources.

Total protein contents obtained by the Bradford method highly correlated with SDS-PAGE protein profiles. A decrease in sarcoplasmic protein content in control samples was observed in this study, which is in coincidence with Okumura, Inuzuka, Nishimura, and Arai (1996) and Nishimura (2002)'s reports in which sarcoplasmic proteins were degraded during *postmortem* conditioning. Conversely, in previous works, Fadda et al. (1999a, 1999b) and Sanz et al. (1999) found protein profiles to remain unchanged when sterile sarcoplasmic extracts were analyzed. This disagreement could be explained by the used experimental model systems (whole meat, in the present work vs. liquid sarcoplasmic extract) as well as by the differences in animal species, muscle types and meat conditioning processes that may account for different activities of muscles enzymes. In addition, since non-inoculated meat discs have increased bacterial counts in 3 log cycles after 15 days of storage, the detected sarcoplasmic protein content decline could be attributed to both, endogenous muscle and indigenous bacterial enzymes. Nevertheless, the presence of *Lactobacillus* strains produced larger decreases in total protein contents, being *L. curvatus* CRL705 and *L. sakei* 23K the more active strains. In general, hydrolytic effects of *Lactobacillus* strains were markedly higher in sarcoplasmic than in myofibrillar protein extracts.

Electrophoretic analysis of sarcoplasmic proteins resulted in the disappearance of three protein bands and the generation of a new one as a consequence of *Lactobacillus* growth in meat discs. In spite of *L. plantarum* CRL681 accounted for the maximal amino acid release, *L. curvatus* CRL705 and *L. sakei* 23K were found to be more hydrolytic on soluble meat proteins. Even when the acidification produced by LAB metabolism could play a role on the protein bands disappearance observed on the gels, an enhancing effect of *Lactobacillus* on meat conditioning could be assumed as it was also observed by Kato et al. (1994) on fresh and conditioned pork meat. Moreover, when sterile sarcoplasmic extracts were incubated at pH 4.0 and 6.0, protein band degradation was observed to be more remarkable at pH 4.0. This result suggests a higher endogen enzymatic activity as well as a partial protein denaturalization by the acid effect. Although when sarcoplasmic extract at controlled pH (4.0) was inoculated by LAB, the obtained proteolytic pattern indicated an enhanced degradation confirming the role of LAB on protein hydrolysis (unpublished data).

Analysis of the complete nucleotide sequence of the *L. sakei* 23K and *L. plantarum* WCFS1 genomes (Kleerebezem et al., 2003; Chaillou et al., 2005) reveals that these strains do not contain any known LAB gene for an extracellular protease (Prt) activity, the enzyme required for large polypeptide utilization. Thus, the observed proteolytic ability of the *Lactobacillus* strains used in this study could be also assigned either to the presence of a not yet characterized protease (in the *L. sakei* 23K genome) or to differences in the genome structure among lactobacilli strains. Another possible explanation is that the growth of LAB in meat may propitiate somewhat favorable conditions for the protein degradation by muscle enzymes. On the other hand, the presence of intracellular proteases, as it has been described in *L. casei* (Shin, Jeon, Kim, & Lee, 2004) may additionally account for the observed proteolytic events if the intracellular content is released under specific conditions. Finally, it is noteworthy the appearance of troponin I, a myofibrillar protein, on sarcoplasmic extracts after 15 days in presence of *Lactobacillus* strains. This fact could be explained by a change on the protein conforma-

tion, facilitating its solubilization on meat extracts of low ionic strength.

5. Conclusions

The use of the studied *Lactobacillus* strains inoculated on meat during vacuum-packaged storage at chill temperatures could be of great importance due to their contribution during meat ageing through small peptides and free amino acids release. In particular, the application of *L. curvatus* CRL705, a bacteriocin producer and free amino acids generator, would positively influence the final safety and quality of meat during its storage under chill and vacuum conditions. In addition, protein analyses by MALDI-TOF MS constitute an useful tool for the identification of potential flavor products resulting from meat proteolysis as well as the putative substrates for LAB enzymes.

Acknowledgements

The authors wish to thank P. Castellano, S. Planchon and I. Chafsey for their valuable help on meat discs preparation and MALDI-TOF MS analyses, respectively. R. Raya and S. Leroy are grateful acknowledged for their fruitful discussion and advice. This work was supported by a grant from SECyT (PICT04 20964) and was a part of ECOS project (A03B02).

References

Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Bredholt, S., Nesbakken, T., & Holck, A. (2001). Industrial application of an antilisterial strain of *Lactobacillus sakei* as a protective culture and its effect on the sensory acceptability of cooked, sliced, vacuum packaged meats. *International Journal of Food Microbiology, 66*, 191–196.

Castellano, P., Holzapfel, W., & Vignolo, G. (2004). The control of *Listeria innocua* and *Lactobacillus sakei* in broth and meat slurry with a bacteriocinogenic *Lactobacillus casei* CRL705. *Food Microbiology, 21*, 291–298.

Castellano, P., & Vignolo, G. (2006). Inhibition of *Listeria innocua* and *Brochothrix thermosphacta* in vacuum-packaged meat by addition of bacteriocinogenic *Lactobacillus curvatus* CRL705 and its bacteriocins. *Letters in Applied Microbiology, 43*(2), 194–199.

Chaillou, S., Champomier-Vergès, M. C., Cornet, M., Crutz-Le Coq, A. M., Dudez, A. M., Martin, V., et al. (2005). The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nature Biotechnology, 23*(12), 1527–1533.

Champomier-Vergès, M. C., Zuñiga, M., Morel-Deville, F., Pérez-Martínez, G., & Zagorec, M. (1999). Relationship between arginine degradation, pH and survival in *Lactobacillus sakei*. *FEMS Microbiology Letters, 180*, 297–304.

Church, F. C., Swaisgood, H. E., Porter, H. D., & Catignani, G. L. (1983). Spectrophotometric assay using *o*-phtaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science, 66*, 1219–1227.

Claeys, E., De Smet, S., Balcaen, A., Raes, K., & Demeyer, D. (2004). Quantification of fresh meat peptides by SDS-PAGE in relation to ageing time and taste intensity. *Meat Science, 67*, 281–288.

Díaz, O., Fernández, M., García de Fernando, G., de la Hoz, L., & Ordoñez, J. A. (1997). Proteolysis in dry fermented sausages: Effect of selected exogenous proteases. *Meat Science, 46*, 115–128.

Fadda, S., Sanz, Y., Aristoy, M., Vignolo, G., Oliver, G., & Toldrá, F. (1999a). Characterization of muscle sarcoplasmic and myofibrillar protein hydrolysis caused by *Lactobacillus plantarum*. *Applied and Environmental Microbiology, 65*(8), 3540–3546.

Fadda, S., Sanz, Y., Aristoy, M., Vignolo, G., Oliver, G., & Toldrá, F. (1999b). Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. *Applied and Environmental Microbiology, 65*(2), 578–584.

Fadda, S., Vignolo, G., Ruiz Holgado, A. P., & Oliver, G. (1998). Proteolytic activity of *Lactobacillus* strains isolated from dry-fermented sausages on sarcoplasmic muscle proteins. *Meat Science, 49*, 11–18.

Flores, M., Aristoy, M. C., & Toldrá, F. (1998). Feedback inhibition of porcine muscle alanyl and arginyl aminopeptidases in cured meat products. *Journal of Agriculture and Food Chemistry, 46*, 4982–4986.

Fritz, J., Swartz, D., & Greaser, M. L. (1989). Factors affecting polyacrilamide gel electrophoresis and electroblotting of high molecular weight myofibrillar proteins. *Analytical Biochemistry, 180*, 205–209.

Hamelin, M., Sayd, T., Chambon, C., Bouix, J., Bibé, B., Milenkovic, D., et al. (2006). Proteomic analysis of ovine muscle hypertrophy. *Journal of Animal Science, 84*(12), 3266–3276.

Hansen, K. M., & Bautista, D. A. (2000). Brochothrix. In K. Robinson, C. A. Batt, & P. D. Patel (Eds.). *Encyclopedia of food microbiology* (Vol. 1, pp. 314–318). Bath: Academic Press.

Honikel, K. O. (1992). The biochemical basis of meat conditioning. In J. Smulders, M. Frans, F. Toldrá, J. Flores, & M. Prieto (Eds.), *New technologies for meat and meat products* (pp. 135–161). Ecceamst, Audet: Tijdschriften B.V..

Kato, T., Matsuda, T., Tahara, T., Sugimoto, M., Sato, R., & Nakamura, R. (1994). Effects of meat conditioning and lactic fermentation on pork muscle protein degradation. *Bioscience Biotechnology and Biochemistry, 58*, 408–410.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O. P., & Leer, R. (2003). Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Sciences, 100*(4), 1990–1995.

Koohmaraie, M. (1994). Muscle proteinases and meat aging. *Meat Science, 36*, 93–104.

Lauret, R., Morel-Deville, F., Berthier, F., Champomier-Vergeès, M.-C., Postma, P. W., Ehrlich, S. D., et al. (1996). Carbohydrate utilization in *Lactobacillus sakei*. *Applied and Environmental Microbiology, 62*, 1922–1927.

Leroy, F., Verluyten, J., & De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology, 106*, 270–285.

Montel, M. C., Seronine, M. P., Talon, R., & Hebraud, M. (1995). Purification and characterization of a dipeptidase from *Lactobacillus sake*. *Applied and Environmental Microbiology, 61*, 837–839.

Nishimura, T. (1998). Mechanism involved in the improvement of meat taste during *post mortem* ageing. *Food Science and Technology International, Tokyo, 4*(4), 241–249.

Nishimura, T. (2002). Influence of the peptides produced during *post mortem* conditioning on improvement of meat flavor. In F. Toldrá (Ed.), *Research advances in the quality of meat and meat products* (pp. 65–78). Trivandrum, India: Research Singpost (Chapter 4).

Nishimura, T., Okitani, A., Rhue, M. R., & Kato, H. (1990). Survey of neutral aminopeptidase in bovine, porcine, and chicken skeletal muscles. *Agricultural and Biological Chemistry, 54*, 2769–2775.

Okumura, T., Inuzuka, Y., Nishimura, T., & Arai, S. (1996). Changes in sensory, physical and chemical properties of vacuum-packed pork loins during the prolonged conditioning at 4 °C. *Animal Science and Technology, 67*, 360–367.

Rodríguez, M., Nuñez, F., Córdoba, J. J., Bermúdez, M. E., & Asensio, M. A. (1998). Evaluation of proteolytic activity of microorganisms isolated from dry cured ham. *Journal of Applied Microbiology, 85*, 905–912.

Sakala, R. T., Hayashidani, H., Kato, Y., Hirata, T., Makino, Y., Fukushima, A., et al. (2002). Change in the composition of the microflora on vacuum packaged beef during chiller storage. *International Journal of Food Microbiology, 74*, 87–99.

Sanz, Y., Fadda, S., Aristoy, M., Vignolo, G., Oliver, G., & Toldrá, F. (1999). Hydrolytic action of *Lactobacillus casei* CRL705 on pork muscle sarcoplasmic and myofibrillar proteins. *Journal of Agricultural and Food Chemistry, 47*(8), 3441–3448.

Sanz, Y., & Toldrá, F. (2001). Purification and characterization of an X-prolyl-dipeptidyl peptidase from *Lactobacillus sakei. Applied and Environmental Microbiology, 67*(4), 1815–1820.

Sanz, Y., & Toldrá, F. (2002). Purification and characterization of an arginine aminopeptidase from *Lactobacillus sakei*. *Applied and Environmental Microbiology, 68*(4), 1980–1987.

Sentandreu, M. A., & Toldrá, F. (2001). Dipeptidyl peptidase activities along the processing of Serrano dry-cured ham. *European Food Research and Technology, 213*, 83–87.

Shin, Y., Jeon, W. M., Kim, G. B., & Lee, B. H. (2004). Purification and characterization of intracellular proteinase from *Lactobacillus casei* ssp. *casei* LLG. *Journal of Dairy Science, 87*, 4097–4103.

Toldrá, F., Aristoy, M. C., Part, C., Cerveró, C., Rico, E., Motilva, M. J., et al. (1992). Muscle and adipose tissue aminopeptidase activities in raw and dry cured ham. *Journal of Food Science, 57*, 816–818.

Vermeiren, L., Devlieghere, F., & Debevere, J. (2004). Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. *International Journal of Food Microbiology, 96*, 149–164.

Verplaetse, A. (1994). Influence of raw meat properties and processing technology on aroma quality of raw fermented meat products. In *Proceedings of the 40th international congress on meat science and technology* (pp. 45–65). The Hague.

Vignolo, G., Ruiz Holgado, A. P., & Oliver, G. (1988). Acid production and proteolytic activity of *Lactobacillus* strains isolated from dry sausages. *Journal of Food Protection, 51*(6), 481–484.