Protein Degradation by *Lactobacillus plantarum* and *Lactobacillus casei* in a Sausage Model System

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ABSTRACT: The proteolytic activity of a starter culture involving *Lactobacillus plantarum* and *Lactobacillus casei* towards meat sarcoplasmic and myofibrillar proteins during the fermentation of a sausage-like system was studied. After 96 h of incubation the proteolytic system of *L. plantarum* CRL681 caused a degradation of both sarcoplasmic and myofibrillar proteins, whereas *L. casei* CRL705 showed a different affinity to meat proteins. The inoculation of both strains showed a higher activity toward sarcoplasmic fraction. These results correlated with a high rate of sarcoplasmic protein degradation observed in SDS-PAGE analysis. The generation of free amino acids as well as the pH drop at the end of the incubation period was maximal in presence of the mixed starter culture, thereby demonstrating the suitability of these strains to be used in the fermentation of meat products.

Keywords: proteolytic activity, meat proteins, lactobacilli, dry fermented sausages

Introduction

DRY-CURING IS A VERY COMPLEX PROCESS DURING WHICH biochemical reactions take place. Lactic acid bacteria are essential agents of the meat fermentation process that contribute to the hygienic and sensory qualities of meat products. This quality is achieved mainly by the metabolic activities of these bacteria on carbohydrates and proteins, resulting in sugar depletion, pH reduction, and the generation of flavor compounds (Hammes and others 1990; Roca and Incze 1990; Brink and Huis In't Veld 1992; Montel and others 1998). Moreover, the low pH attained promotes the inhibition of the contaminating or pathogenic microflora naturally present as well as contribute to the cohesion and color development of the product (Lücke and Hechelmann 1987; Nychas and Arkoudelos 1990; Leistner 1992).

Most attention has been focused on the development of starter cultures with adequate fermentation characteristics, although the number of studies on proteolytic activities of lactic acid bacteria is limited. The protein breakdown that takes place during the ripening of dry fermented sausages leads to an increase in the concentration of peptides and amino acids (Dierick and others 1974; De Masi and others 1990; Johansson and others 1994; Molly and others 1997). This increase is the result of the proteolytic activities of both endogenous and microbial enzymes, although the main role of microorganisms seems to be confined to the secondary hydrolysis of oligopeptides and small proteins (Verpaletse 1994; Molly and others 1997). As opposed to the situation in dry-cured ham manufacture, the microbial flora that develop during ripening markedly influences dry-cured sausage fermentation. As the muscle enzyme system is permanently present in sausages, the action of microbial enzymes would overlap with that of the muscle enzymes (Toldrá 1992).

Lactobacillus species are the most prevalent microorganisms in dry fermented sausages, and their use as starter cultures is widespread (Hammes and others 1990). Even though lactic acid bacteria are known as weak lipolytic and proteolytic organisms (Johansson and others 1994), the potential contribution of *Lactobacillus casei* CRL705 and *Lactobacillus plantarum* CRL681 to the breakdown of sarcoplasmic and myofibrillar proteins has been demonstrated, showing that a pronounced protein degradation requires available enzyme activities (Fadda and others 1998, 1999b). Nevertheless, the coexistence of endogenous enzymes makes it difficult to establish the proteolytic pathway in these systems. These different enzyme systems modulate the dried sausage flavor, whereas the final organoleptic characteristics of the product are determined also by the spices and natural flavoring ingredients used. Moreover, it was also suggested that most of the compounds carrying the characteristic flavor of fermented meat are derived from the protein fraction of the sausage (Demeyer and others 2000).

The extent of proteolysis during ripening of dry fermented sausages varies with factors such as the nature of meat microflora and conditions during processing (Astiasarán and others 1990). The effects of different levels of curing ingredients and process parameters on muscle proteases and peptidases involved in the dry curing process have also been reported (Sanz and Toldrá 1997a; Flores and others 1997; Fadda and others 2001). Temperature and pH during the fermentation were reported to be the most decisive parameters affecting the activity of muscle and bacterial aminopeptidases (Toldrá 1992; Martin and others 1998).

Recently, the proteolytic system of lactobacilli involved in meat fermentation is becoming the focus of an increasing number of studies due to the technological role of these organisms (Montel and others 1995; Sanz and Toldrá, 1997a, 1997b; Fadda and others 1999a, 1999b). *L. plantarum* CRL681 and the bacteriocinogenic strain *L. casei* CRL705, originally isolated from sausages, are being investigated for their potential as starter cultures in sausage production (Vignolo and others 1989). The aim of the present work was to evaluate the effect of *L. casei* CRL705 and *L. plantarum* CRL681 as starter cultures on meat proteins during the fermentation of a sausage-like system to corroborate the proteolytic activity described *in vitro*.

Materials and Methods

Strains and culture conditions

Lactobacillus plantarum CRL681 and *Lactobacillus casei* CRL705, originally isolated from dry cured sausages, were used

as starter cultures. The strains were selected according to their acid-producing capacity, bacteriocin production, and proteolytic activity (Vignolo and others 1988; Fadda and others 1998, 1999a; Sanz and others 1999). The strains were routinely grown in MRS broth (Merck, Darmstadt, Germany) at 30 °C, for 24 h. Cells in logarithmic phase of growth were harvested by centrifugation (10,000 x g for 20 min at 4 °C), washed twice in 20 mM phosphate buffer, pH 7.0, and re-suspended in the same buffer (10% of initial volume).

Sausage preparation

All operations were carried out in a laminar flow hood. Beef and pork muscle, obtained 24 h post-mortem from cooled carcasses, were frozen and later aseptically sampled by superficial burning, followed by removal of surface cuts using sterile knives and minced. The procedure resulted in total counts below 10^3 CFU/g. The sausage formulation included 50% beef; 45.5% pork; 3% NaCl; 0.75% glucose; 0.75% sucrose; 0.02% sodium nitrite, and 0.01% ascorbic acid. The meat components, curing salts and other ingredients were thoroughly mixed and the sausage mix was then divided into four 80 g portions. Each portion was inoculated with 10 ml of buffer solution containing 10^6 to 10^7 CFU/ml of *L. plantarum* CRL 681; *L. casei* CRL 705 and *L. plantarum* CRL681 + *L. casei* CRL705 used as a mixed starter culture. The sausage mixtures were stuffed into synthetic PVC casings (20 cm length x 2.5 cm width) and incubated for 4 d in a ripening chamber at 25 °C with 80% relative humidity. Two samples from each batch were collected at 0, 1, 2, 3, and 4 d for further analyses. A non-inoculated control supplemented with sodium azide (0.02%) was assayed simultaneously.

Bacterial counts and pH measurement

At each sampling time bacterial counts were determined on Plate Count Agar and MRS agar (Merck, Darmstadt, Germany) after incubation at 37 and 30 °C during 48 h for total aerobic and lactic acid bacteria respectively. The pH values were monitored in sausages homogenates (sausage: distilled water, 1:10) using a Crison 2001 pH-meter (Crison Instrument S.A, Barcelona).

Protein and amino acid determinations

Sarcoplasmic and myofibrillar proteins were separately analyzed. They were extracted from the sausage model system according to Fadda and others (1999a). Total sarcoplasmic and myofibrillar protein contents at 0 and 4 d of incubation were measured according to Bradford (1976). Individual strains and the mixed starter culture were tested for proteolytic activity by measuring the release of soluble amino acids during the fermentation period in TCA (trichloroacetic acid), according to the OPA spectrophotometric assay (Church and others 1983). Each sample was subjected to protein precipitation, the extract centrifuged and a supernatant aliquot containing free amino acids and small peptides treated with o-phthaldialdehyde. Results are expressed as free amino acid concentration (mM).

The hydrolysis of muscle proteins was monitored by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) analysis as described by Laemmli (1970) using a vertical gel electrophoresis unit (Mini-Protean II; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) and a running gel containing 12% and 10% acrylamide for sarcoplasmic and myofibrillar proteins, respectively. Samples were taken every 24 h and mixed with an equal volume of a sample buffer containing 2% of SDS, 5% of β-mercaptoethanol, 10% of glycerol, buffer Tris-HCl pH 6.8, and bromophenol blue as front marker; they were then heated for 5 min at 100 °C. The electrophoresis was carried out at 50 mV until the bromophenol blue marker reached the bottom of the gel. Gels were stained at room temperature, shaken for 2 h and de-stained until clear background was obtained. The molecular weight markers used as standard were: myosin (200.0 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa) from Bio-Rad (Richmond, Calif., U.S.A.). Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

Statistical Analysis

All results presented in this study are the mean of 3 separate batches of meat with 2 replicates each. Data were analyzed by the general linear models procedures of Statistical Analysis System (SAS); $P < 0.05$ values were considered to be significant. Means were compared by LSD.

Results and Discussion

BACTERIAL GROWTH, PH AND NET AMINO ACID CONCENTRATION OF different batches are summarized in Figure 1. In the inoculated batches, the viable counts of *L. casei* CRL705 increased from an initial count of 1 x 0^7 to 5 x 10^8 CFU/g, while *L. plantarum* CRL681 and the mixed culture remained almost constant in the range of 10^8 CFU/g throughout the fermentation time (Figure 1C, 1B and 1D, respectively). Simultaneously the pH dropped from 5.20 to 5.35 down to 4.30 to 4.45 after 96 h in the batches inoculated with individual strains. When both strains were added as a mixed starter culture maximal acidifying capacity was observed in the sausage-like system, the pH decreasing from 5.20 to 3.95. In non-inoculated control batch pH values remained almost constant during the entire fermentation period (Figure 1A).

The net free amino acid generation resulting from the action

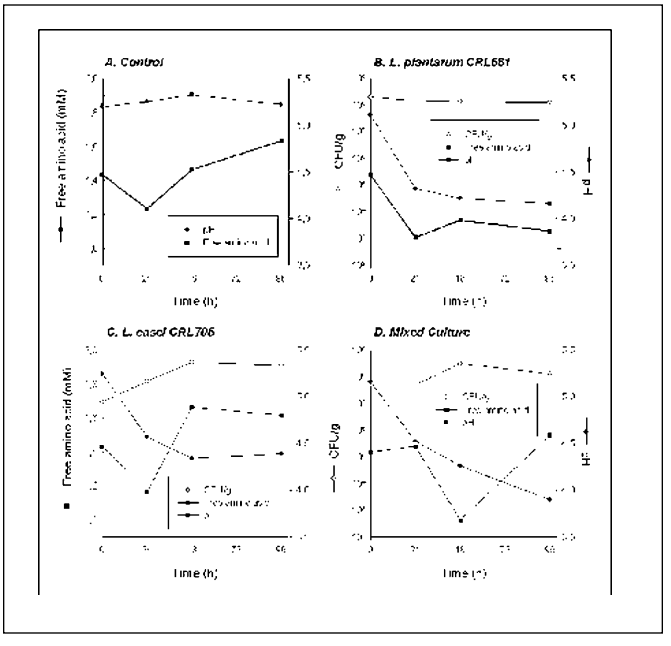

Figure 1—Cell counts (○), pH changes (●) and free amino acids concentration (■) in the sausage model system, incubated at 30 °C during 96 h. (A) Control non-inoculated; (B) *Lactobacillus plantarum* CRL681; (C) *Lactobacillus casei* CRL705 and (D) *L. plantarum* CRL681 + *L. casei* CRL705.

of endogenous and bacterial proteolytic enzymes on sarcoplasmic and myofibrillar proteins is also shown in Figure 1. A net increase of free amino acids was observed after 96 h at 25 °C in the non-inoculated batch (Figure 1A), while individual *Lactobacillus* strains produced different extents of proteolysis. Even when both strains show a consumption of amino acids during the 1st 24 h, reaching an amino acid concentration of 1.16 and 1.06 mM for *L. casei* and *L. plantarum* respectively, the former produced a maximal accumulation of these metabolites at 48 h of incubation (1.66 mM) with a net increase at 96 h of 0.16 mM (Figure 1C). On the contrary, *L. plantarum* CRL681 showed an almost constant release/consumption balance between 24 and 96 h (Figure 1B). When the mixed culture was studied, a change in cell numbers between 24 and 48 h was observed paralleling a decrease in the amino acid concentration from 1.43 to 1.00 mM (Figure 1D). Nevertheless, a low free amino acid accumulation (7%) during the fermentation period was promoted by the mixed culture.

SDS-polyacrylamide gel electrophoretograms of sarcoplasmic and myofibrillar proteins are shown in Figure 2 and 3. Control samples from sarcoplasmic extract, lacking any bacterial enzyme, reflected the activity of endogenous proteinases responsible for the disappearance and/or decrease in intensity of protein bands at approximately 98, 65, 55, and 45 kDa (Figure 2, lane 3). The inoculation of the sausage model system with *L. plantarum* CRL681 drastically hydrolyzed protein bands with sizes of about 200, 150, 99 to 50, and 45 to 25 kDa, which disappeared or decreased in intensity after 96 h of incubation at 25 °C (Figure 2, lane 5). The proteolytic activity of *L. casei* CRL705, even less pronounced, also caused degradation or reduction in the intensity of bands of about 25 to 35 kDa as well as the hydrolysis of bands in the region between 70 and 200 kDa (Figure 2, lane 7). After 96 h of incubation the proteolytic activity of the mixed culture caused degradation or reduction in the intensity of protein bands in the whole molecular mass range, and some lower molecular mass peptides in the front of the gel were observed (Figure 2, lane 9). The simultaneous action of both strains resulted in a stronger sarcoplasmic protein hydrolysis.

The protein profiles resulting from the hydrolysis of myofibrillar protein are shown in Figure 3. In control samples, the activity of tissue proteinases was responsible for the degradation of protein bands corresponding to 200 kDa (myosin), 66 kDa, and 43 kDa (actin) (Figure 3, lane 3). Individually, bacterial hydrolytic effect did not produce remarkable effects on myofibrillar proteins when comparing to the control (Figure 3, lane 5 and 7). Even though when *L. casei* CRL705 was inoculated, myosin and actin were hydrolyzed, while other, faint bands of intermediate molecular masses (60 to 35 kDa) appeared at the end of the incubation period (lane 7). In contrast, *L. plantarum* CRL681 only caused the degradation of myosin (200 kDa) and some bands in the range between 20 and 10 kDa (lane 5). The inoculation of both strains in the sausage-like system resulted in stronger protein degradation (Figure 3, lane 9); the proteolytic system of both microorganisms were responsible for the degradation of protein bands of 200 kDa and 25 to 35 kDa, and for a partial hydrolysis in the range of 45 and 55 kDa. Nevertheless, after 96 h of incubation, proteinase system from *L. casei* and *L. plantarum* individually or as mixed culture contributed to the hydrolysis of protein bands corresponding to myosin and actin with the appearance of other faint bands of intermediate molecular mass as 122 kDa (heavy meromyosine) and 97 kDa.

Total protein contents after 96 h of incubation are shown in Table 1. Protein degradation in control samples without inoculation, was observed to be more active in myofibrillar fraction than in sarcoplasmic with 57.6% and 36.3% of protein hydrolysis after 96 h, respectively. When the sausage-like system was inoculated, different affinity of *Lactobacillus* strains for meat proteins was observed. *L. plantarum* CRL681 degraded sarcoplasmic and myofibrillar proteins similarly, with a total protein content after 96 h of 0.191 and 0.120 mg/ml, respectively, which represents 68.7% and 65.2% of protein degradation. *L. casei* CRL705 was slightly more active towards sarcoplasmic fraction reaching 65.8% of protein degradation at the end of the incubation period in contrast with 49.8% of hydrolysis for myofibrillar proteins. When the mixed culture was inoculated in the sausage model system, 81.6% of sarcoplasmic degradation after 96 h was observed (0.110 mg/ml of total protein content), indicating a high activity toward this meat fraction. The total protein content for myofibrillar proteins was 0.093 mg/ml after 96 h indicating a degradation of 72.6% by the mixed culture.

The results obtained indicated active metabolisms of the studied strains of *L. plantarum* CRL681 and *L. casei* CRL705, with

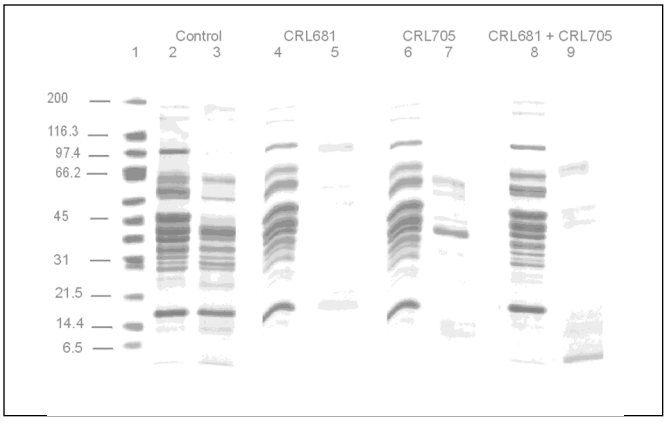

Figure 2—SDS-PAGE of sarcoplasmic protein hydrolysis by *L. plantarum* CRL681 (lanes 4, 5); *L. casei* CRL705 (lanes 6, 7) and the mixed culture (lanes 8, 9) at 0 and 96 h of incubation. Control non-inoculated (lanes 2, 3). Lane 1: molecular weight markers.

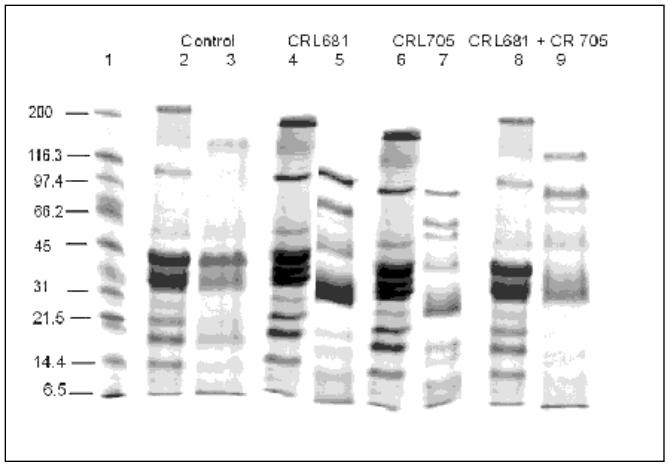

Figure 3—SDS-PAGE of myofibrillar protein hydrolysis by *L. plantarum* CRL681 (lanes 4, 5); *L. casei* CRL705 (lanes 6, 7) and mixed culture (lanes 8, 9) at 0 and 96 h of incubation. Control non-inoculated (lanes 2, 3).

Table 1—Total protein content in meat protein fractions after incubation during 96 h at 25 °C of the sausage model system

	Meat protein fraction (mg/ml)					
	Sarcoplasmic			Myofibrillar		
	0h	96h	(%)§	0h	96h	(%)§
Control	0.592 ± 0.03*	0.377 ± 0.02	(36.3)	0.330± 0.03	0.140± 0.02	(57.6)
L. plantarum CRL681	0.610 ± 0.04	0.191± 0.03	(68.7)	0.345± 0.03	0.120± 0.02	(65.2)
L. casei CRL705	0.615 ± 0.04	0.210 ± 0.02	(65.8)	0.335± 0.02	0.168± 0.02	(49.8)
Mixed culture	0.598 ± 0.03	0.110 ± 0.03	(81.6)	0.340± 0.03	0.093± 0.02	(72.6)

* Results are means of triplicate determinations.
§Percent of degradation

pH decreasing from 5.35 to 3.95 after 96 h of incubation at 25 °C, indicating a good growth performance in the meat system. These results are in agreement with those of Vignolo and others (1989) and Montel and others (1993), who found the bacterial starter culture to be responsible of the final pH in meat fermentation. Different extents of proteolysis were observed between strains in the assayed conditions. Although some authors (Toldrá and others 1992; Kato and others 1994) attributed proteolysis in fermented meat products mainly to the action of endogenous enzymes, our findings agree with those of García de Fernando and Fox (1991), Johansson and others (1994) and Molly and others (1997), who concluded that lactic acid bacteria could play a role in producing proteolytic enzymes that are able to attack meat proteins. In previous work (Fadda and others 1998) the proteolytic activity of *L. casei* and *L. plantarum* using a soluble muscle extract as a source of proteins was demonstrated.

The concentration of free amino acids released by the studied strains together with the degradation extent of sarcoplasmic and myofibrillar proteins correlate with the disappearance of several protein bands in the electrophoretograms. The proteinase activity of *L. plantarum* CRL681 and *L. casei* CRL705 was able to initiate pronounced hydrolysis of sarcoplasmic proteins. The greater affinity of both *Lactobacillus* strains for sarcoplasmic proteins is in agreement with previous works (Fadda and others 1999b; Sanz and others 1999), in which electrophoretic analyses undoubtedly demonstrated the suitability of sarcoplasmic proteins as substrates for proteinases from whole cells of *L. casei* CRL705 and *L. plantarum* CRL681. The strongest hydrolytic activity seemed to be extra-cellularly located when compared to the degradation caused by the addition of exclusively cell free extracts. A cell-wall-associated proteinase activity could be responsible for such protein degradation as was clearly shown by Kunji and others (1996) in most dairy lactic acid bacteria. Hagen and others (1996) also confirm the effect of the reduction in the maturation time of dry fermented sausages due to the addition of a *Lactobacillus* proteinase. However, the quoted activity did not fully account for the proteolysis observed, and other endopeptidases could contribute to the whole hydrolysis of sarcoplasmic proteins into peptides and amino acids (Kojic and others 1995; Tan and others 1995). On the other hand, muscle aspartic and cysteine proteinases have been regarded as the enzymes mainly implicated in the initial hydrolysis of myofibrillar proteins (myosin and actin) and in the generation of smaller fragments (Molly and others 1997). From our results, residual endogenous activity could not be completely excluded and was responsible for the degradation of myosin and actin, but a minor implication of bacterial proteinases could not be discarded either. These results are in coincidence with the lower degradation percentage obtained for myofibrillar proteins after 96 h of incubation.

As stated above, the high affinity of the 2 studied *Lactobacillus* strains for sarcoplasmic proteins allows us to hypothesize that in meat systems, this protein fraction may contribute to amino acid supply for bacterial growth according to the general decrease in total free amino acid contents observed in the 1st step of incubation (at 24 h and 48 h with individual and mixed culture, respectively). In a 2nd step, when sugars were fairly exhausted and sarcoplasmic proteins almost completely degraded, microbial proteinases may also use myofibrillar proteins to sustain growth with the concomitant generation of hydrolytic compounds.

Conclusion

THE POTENTIAL CONTRIBUTION OF THE MIXED CULTURE INVOLVING *L. casei* CRL705 and *L. plantarum* CRL681 to the hydrolysis of meat proteins was demonstrated in a sausage-like system. Moreover, the good acidogenic capacity together with the production of a bacteriocin by *L. casei* CRL705 would allow to obtain safe fermented meat products with desirable flavor and texture.

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