THE ACIDOGENIC METABOLISM OF *LACTOBACILLUS PLANTARUM* CRL 681 IMPROVES SARCOPLASMIC PROTEIN HYDROLYSIS DURING MEAT FERMENTATION

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ABSTRACT

The hydrolysis of sarcoplasmic proteins during the growth of Lactobacillus plantarum *CRL 681 isolated from fermented sausage was evaluated. Fermentation was carried out in a meat model system under controlled pH conditions (pH 4.0 and 6.0) at 30C for 96 h. The ability of this strain to grow and degrade soluble meat proteins at both assayed pHs was demonstrated. Results showed that sarcoplasmic protein hydrolysis at pH 4.0, as occurring during sausage fermentation, can be attributed to the combined action of acidic meat endogenous proteases, L. plantarum CRL 681 proteolytic activity and acid-induced changes arising from the bacterial fermentative metabolism. On the other hand, bacterial proteolysis was mainly involved on the hydrolytic changes observed at pH 6.0.*

PRACTICAL APPLICATIONS

Lactobacillus plantarum CRL 681 isolated from Argentinean sausages showed to display technological metabolic traits such as high acidification rate, proteolytic and peptidase activity, biogenic amines degradation and absence of amino acid decarboxylase activity which can beneficially affect the quality and safety of fermented sausages. In addition, from the results of this study, *L. plantarum* CRL 681 acidogenic metabolism not only would assure hygienic quality of fermented sausages but would increase free amino acids and peptides release from meat proteins. As a whole, this strain has the potential to be successfully used as starter culture to improve final product quality.

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INTRODUCTION

The enzymology of dry fermented sausages is a complex process due to the coexistence of enzymes from both endogenous and microbial origin (Sanz *et al.* 2002). Studies carried out over the last two decades have revealed that muscle proteinases are primarily responsible for proteolysis, while bacterial enzymes become important during the latter stages of ripening (Verplaetse *et al.* 1992; Toldrá 1998). These microbial enzymes contribute to the degradation of oligopeptides into small peptides and free amino acids, leading to an increase in their concentration during sausage ripening (Kato *et al.* 1989; Demeyer *et al.* 1992; Johansson *et al.* 1994; Molly *et al.* 1997).

Lactic acid bacteria (LAB) are essential agents in meat fermentation that contribute to the hygienic and sensory properties of meat products (Hammes *et al.* 1990; Lücke 2000). *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* are the most competitive LAB isolated from fermented sausages and are also usually involved in starter cultures (Cocolin *et al.* 2004; Fontana *et al.* 2005; Aymerich *et al.* 2006). All these species are endowed with a certain proteolytic activity that may partly contribute to the degradation of muscle proteins (Fadda *et al.* 1999; Ordoñez *et al.* 1999). *L. plantarum* CRL 681, a bacterium isolated from Argentinean sausages, has been studied in-depth in order to demonstrate its potential as a starter culture. It displays remarkable metabolic features such as good acidification rate, proteolytic and amino peptidase activity, degradation of biogenic amines *in vitro* and absence of amino acid decarboxylase activity (Vignolo *et al.* 1988; Fadda *et al.* 1999, 2001, 2002). These technological metabolic capacities can beneficially affect the quality and safety of fermented sausages while preserving their typicity (Stahnke 2002; Talon *et al.* 2002; Leroy *et al.* 2006).

Some bacterial peptidases that have been purified denote high variability in their biochemical characteristics (Montel *et al.* 1995; Sanz and Toldrá 1997). The effect of technological processing parameters on peptidases expression such as pH, water activity, sodium chloride and nitrite has been reported previously by Sanz *et al.* (2002). Since the acid production from carbohydrate metabolism is the most important feature of LAB during fermentation, the optimal activity of *Lactobacillus* proteolytic enzymes would be affected by a decrease in pH during this process (Sanz *et al.* 2002). In this sense, controlled acidification of meat proteins *in vitro* has been shown to be effective in producing gels, indicating that acid has a clear physicochemical effect on proteins (Fretheim *et al.* 1985; Hermansson and Langton 1988). On the basis of previous findings, it could be assumed that the acid itself has a role in meat protein degradation overlapping bacterial proteolytic activity. The aim of the present study was to discriminate the contribution of *L. plantarum* CRL 681 acid production on sarcoplasmic hydrolysis by using a meat model system

under controlled pH conditions (6.0 and 4.0) mimicking pH changes during sausage fermentation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

L. plantarum CRL 681 was isolated from artisanal fermented sausages produced in Argentina (Vignolo *et al.* 1986) and belongs to CERELA-CONICET culture collection. Cells in the logarithmic growth phase were harvested by centrifugation at 7,000 rpm for 10 min at 4C (IEC MULTI RF centrifuge, Thermo Electron Corporation, Altrincham, Cheshire, UK), washed twice with 20 mM phosphate buffer, pH 6.0, and resuspended in the same buffer (10% of the initial volume). Cells were inoculated in the sterile soluble meat extract (sarcoplasmic extract, see below) to yield approximately 10^7 cfu/mL and incubated at 30C for 96 h in a BIOFLO C22 fermentor (New Brunswick Scientific Co. Inc., Edison, NJ) under controlled pH conditions. Lactic acid (1 M) or NaOH (1 N) were used to adjust and maintain pH values at 4.0 or 6.0, respectively. Non-inoculated sarcoplasmic extracts at pH 4.0 and 6.0 were incubated under the same conditions (controls). Samples were taken at 0, 4, 8, 12, 24, 48, 72 and 96 h for bacterial enumeration, amino acid and gel electrophoresis (sodium dodecyl sulphate–polyacrylamide gel electrophoresis [SDS-PAGE]) analyses. For bacterial enumeration, decimal dilutions were plated on de Man–Rogosa–Sharpe agar (Merck, Darmstadt, Germany) and incubated for 48 h at 30C. For control samples, additional plating was performed on plate count agar medium (PCA) (Merck) (48 h, 37C). Bacterial growth was also estimated by turbidimetry at 680 nm. Two independent experiments were carried out.

Sarcoplasmic Extract

Semimembranosus muscles were obtained from a local beef abattoir and processed as previously described by Fadda *et al.* (1998). Ten grams of muscle were diluted 1:l0 (w/v) with distilled water, homogenized in a Stomacher 400 blender (London, UK) and centrifuged. The supernatant was filter sterilized through 0.22 µm (Millipore, Bedford, MA) and supplemented with 0.1% of sterile Tween 80 and sterile glucose solution. The absence of bacterial growth was confirmed by plating on PCA.

Free Amino Acids and Trichloroacetic Acid-Soluble Peptides Analysis

The pool of free amino acids and trichloroacetic acid (TCA)-soluble peptides (free amino acids) were measured according to the o-phtadialdehyde

(OPA) spectrophotometric assay (Church *et al.* 1983) and modified by Fadda *et al.* (2000). Results were expressed as absorbance at 340 nm and were the mean of at least three replicate assays.

Electrophoretic Analysis (SDS-PAGE)

To analyze sarcoplasmic proteins hydrolysis, samples from inoculated and control batches were taken at 0, 4, 8, 12, 24, 48, 72 and 96 h of incubation at both pHs and were subjected to electrophoresis (SDS-PAGE) (Fritz *et al.* 1989). A Mini Protean 3 Gel Unit (Biorad, Richmond, CA) and 12% polyacrylamide (Biorad) gels were used. SDS-PAGE was carried out during 4 h at 4C as described by Fadda *et al.* (1998). Midrange protein markers (from 66 to 14 kDa) were used as standards (Biorad). Proteins were visualized by Coomassie blue staining (Sigma, St. Louis, MO.).

Statistical Analyses

A completely randomized 2×2 design, with two independent replications was carried out. The factors were *Condition* (sterile condition and inoculated condition) and *pH* (6.0 and 4.0). The effect of two factors on the experimental response (cell growth, OPA and SDS-PAGE analyses during the time) was determined. The analysis of variance was applied for a model with main effects and interaction. The statistical analysis was performed using MINITAB software (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Bacterial Growth and Survival

Growth and survival of *L. plantarum* CRL 681 was evaluated using a meat experimental system under controlled pH conditions. Figure 1 shows that this strain grew at pH 6.0 from 4.6×10^7 to 4.9×10^9 cfu/mL, while a decrease in cell counts being observed after 48 h, reached final counts of 8.0×10^8 cfu/mL at 96 h. This result confirms *L. plantarum* ability to grow on the meat model, as was previously reported in a free pH fermentation system (Fadda *et al.* 1998). When pH 4.0 was assayed, *Lactobacillus* population remained stable and viable cells reached maximum counts (9.0×10^7 cfu/mL) at 72 h of incubation (Fig. 1). The lower growth observed at pH 4.0 may be explained by the acidic conditions existing in the culture media, in which undissociated lactic acid may cause intracellular pH decrease as well as reduction of amino acids and nutrients uptake with the consequent growth suppression (Freese *et al.* 1973; McDonald *et al.* 1990; Piard and Desmazeaud 1991).

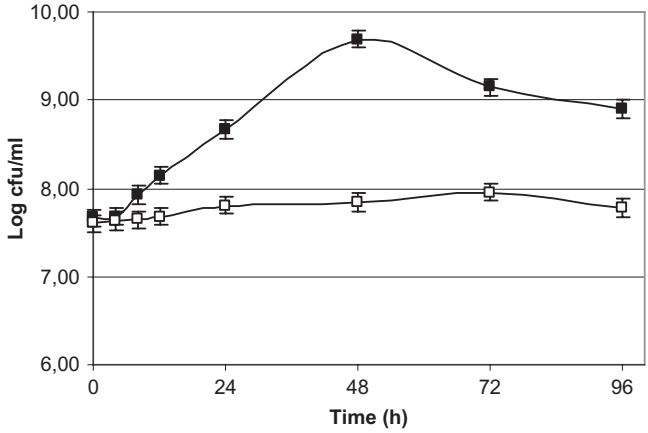

FIG. 1. *LACTOBACILLUS PLANTARUM* CRL 681 GROWTH ON SARCOPLASMIC MEDIUM AT CONTROLLED pH FOR 96 H AT 30C
(■) pH 6.0 and (□) pH 4.0.

In spite of this, *L. plantarum* CRL 681 viability observed at pH 4.0 evidenced a rather high acid resistance, this being in agreement with McDonald *et al.* (1990), who reported high acid tolerance of *L. plantarum*.

Evolution of Free Amino Acids and TCA-Soluble Peptides

In view of the technological importance of amino acids and peptides on flavor development during ripening of meat products (Kato *et al.* 1989; Stahnke 2002; Claeys *et al.* 2004), free amino acids and TCA-soluble peptides (free amino acids) generated by *L. plantarum* CRL 681 in the sarcoplasmic medium at both pHs were analyzed (Fig. 2). In non-inoculated sarcoplasmic medium (control), an initial increase in free amino acids concentration was observed at both pH values, this being attributed to the activity of muscle aminopeptidases (Molly *et al.* 1997; Sentandreu and Toldrá 2001; Ouali and Sentandreu 2002). However, no changes in OD_{340} values were observed after 12 h at pH 6.0, while at pH 4.0, a decrease in OD_{340} values between 12 and 72 h was detected with a negative net amino acid balance at 96 h. Amino acid degradation or chemical modifications could have taken place under sterile and acidic conditions (pH 4.0), as lipid oxidation products present in food systems were reported to be responsible for amino acid degradation by Strecker-type mechanisms as well as for the conversion of the amino acids into their R-keto acids (Zamora *et al.* 2006).

When *L. plantarum* CRL 681 was inoculated, a decrease in free amino acids content occurred during the first 24 and 48 h at pH 6.0 and 4.0, respec-

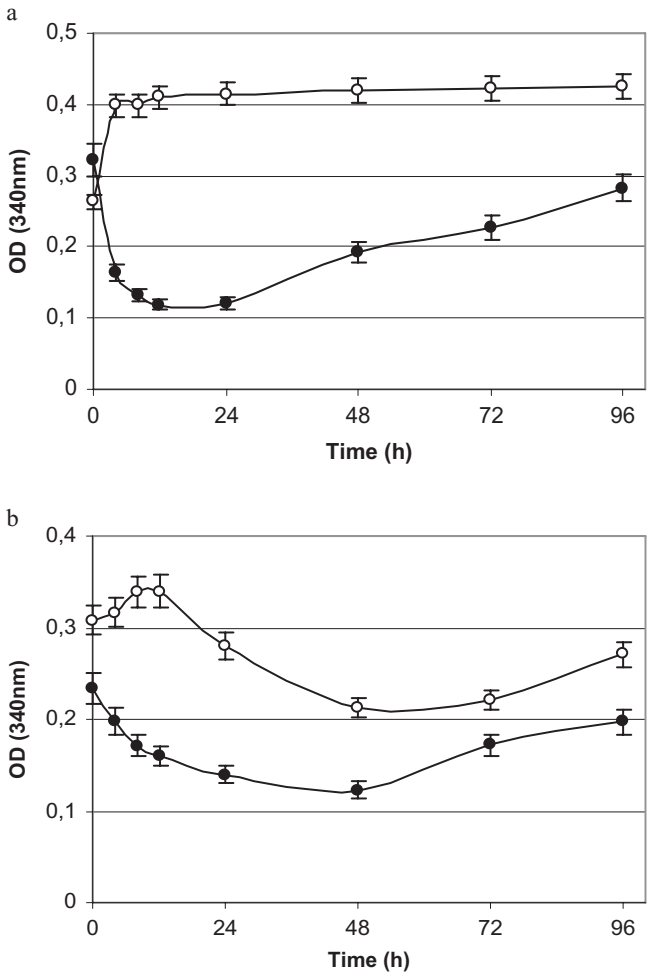

FIG. 2. TRICHLOROACETIC ACID-SOLUBLE PEPTIDE AND FREE AMINO ACID CONTENTS IN SARCOPLASMIC MEDIUM
Non-inoculated (○) and inoculated with *Lactobacillus plantarum* CRL 681 (●) incubated at controlled pH for 96 h at 30C. (a) pH 6.0; (b) pH 4.0.

tively (Fig. 2). The more pronounced amino acid decrease observed at pH 6.0 can be related to bacterial amino acid consumption paralleling the higher growth rate recorded at this pH (Fig. 1). The increase in OD_{340} values after 24 h observed at pH 6.0 (Fig. 2a) is in agreement with the previously reported *L. plantarum* CRL 681 aminopeptidase activity which would contribute to flavor enhancement during sausage ripening (Fadda *et al.* 1999). On the other hand,

the increase in free amino acids at pH 4.0 observed after 48 h (Fig. 2b) may arise from muscle exopeptidases which would be promoted at this low pH, since almost all bacterial aminopeptidases were reported to be inhibited under acidic environment (Sanz and Toldrá 1997). The observed events at pH 4.0 would mimic *Lactobacillus* strains as a starter culture during sausage fermentation in which a dramatic acidification of the meat batter is produced.

Monitoring Sarcoplasmic Protein Degradation by SDS-PAGE

Electrophoretic patterns derived from the hydrolysis of sarcoplasmic proteins by *L. plantarum* CRL 681 under controlled pHs are shown in Fig. 3. Protein degradation at pH 6.0 was evidenced after 24 h (Fig. 3a, lane 8), the disappearance of protein bands in the 97–45 kDa range being the most important change during the incubation period (Fig. 3a, lanes 8–11). The observed protein hydrolysis may be attributed only to bacterial activity since no changes occurred in non-inoculated controls after 96 h (Fig. 3a, lanes 2–3). Even when these results agree with the reported proteolytic ability of *L. plantarum* CRL 681 at free pH (Fadda *et al.* 1998), in the present study, a slighter hydrolytic profile was observed since no acid effect existed at pH-controlled conditions.

The protein patterns resulting from the hydrolytic changes that occurred at pH 4.0 are shown in Fig. 3b. Control samples reflected protein breakdown in the range of 97–66 kDa (Fig. 3b, lanes 2–3). The acid environment would be responsible for bands disappearance at 96 h, causing protein structural changes as well as the activation of acidic muscle proteases (Ouali and Sentandreu 2002). Similarly, an enhancement of muscle protein hydrolysis after acidification, probably due to muscle protease activation, was reported by Saunders (1994) and Syed Ziauddin *et al.* (1995). Acid-induced changes such as denaturation and protein network formation were also observed during Thai-style sausage fermentation due to the effect of acid on meat proteins (Visessanguan *et al.* 2004). When sarcoplasmic medium at pH 4.0 was inoculated with *L. plantarum* CRL 681, a higher protein degradation respect to pH 6.0 was observed; this hydrolysis being particularly remarkable from 48 h up to the end of the incubation period (Fig. 3b, lanes 9–11). The highest hydrolytic changes were observed at pH 4.0 in the 66–40 kDa range as well as in the myoglobin band (17 kDa). The protein profile obtained at pH 4.0 is in agreement with results from Kato *et al.* (1994), who found that the protein degradation in lactic acid-fermented pork meat was caused by meat proteases and that lactic acid fermentation acted as enhancing effector. On the basis of the results obtained from SDS-PAGE and free amino acid analyses, it could be inferred that acidic environment acts as a promoting agent of the sarcoplasmic protein hydrolysis while it is a limiting factor for amino acid release (Fig. 2b).

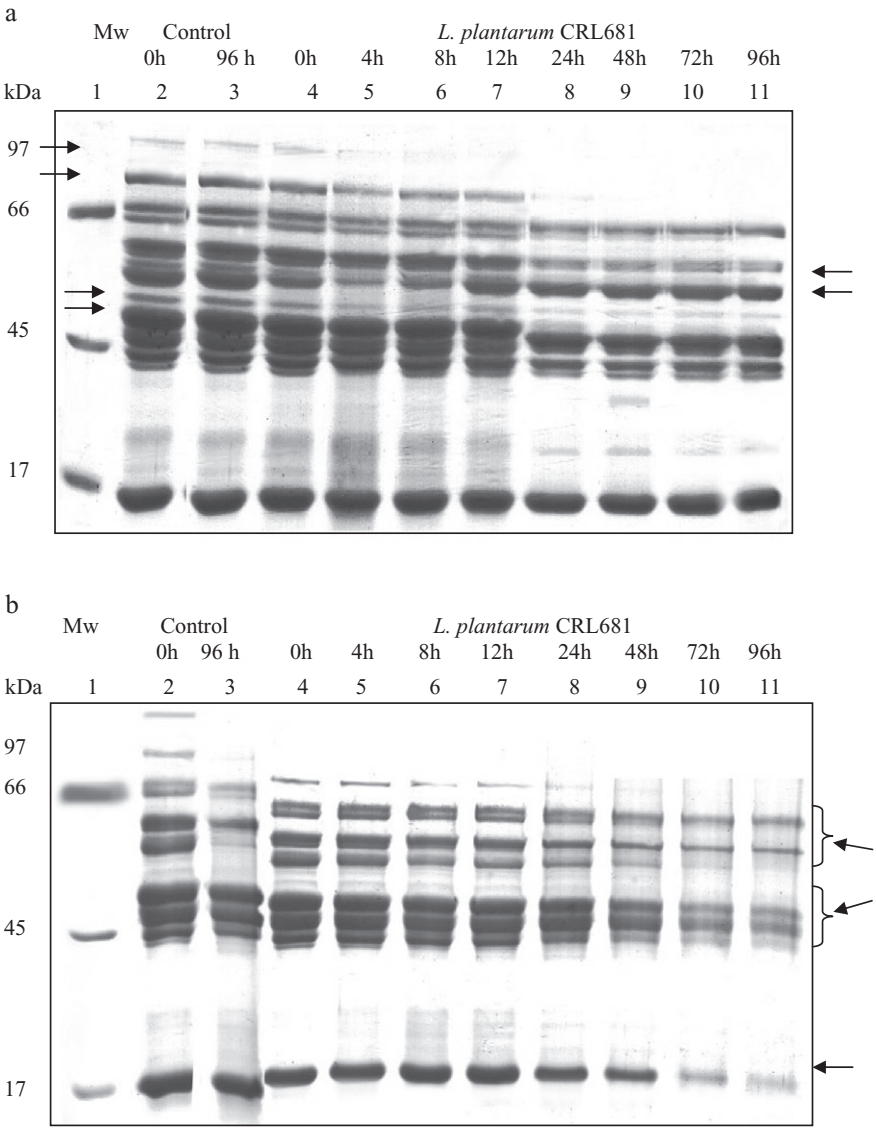

FIG. 3. SODIUM DODECYL SULPHATE–POLYACRYLAMIDE GEL ELECTROPHORESIS OF SARCOPLASMIC PROTEIN HYDROLYSIS BY *LACTOBACILLUS PLANTARUM* CRL 681 (a) Incubated at controlled pH 6.0 for 96 h at 30C. (b) Incubated at controlled pH 4.0 for 96 h at 30C. Lane 1, Molecular weight markers; lane 2, Control, 0 h; lane 3, Control, 96 h; lane 4, *L. plantarum* CRL 681, 0 h; lane 5, *L. plantarum* CRL 681, 4 h; lane 6, *L. plantarum* CRL 681, 8 h; lane 7, *L. plantarum* CRL 681, 12 h; lane 8, *L. plantarum* CRL 681, 24 h; lane 9, *L. plantarum* CRL 681, 48 h; lane 10, *L. plantarum* CRL 681, 72 h; lane 11, *L. plantarum* CRL 681, 96 h.

CONCLUSION

The effect of lactic acid production by *L. plantarum* CRL 681 to sarcoplasmic protein degradation was demonstrated in this work. A pH-controlled model system was used to discriminate the bacterial proteolytic activity from that of the acid produced by the strain itself. At pH 4.0, as occurring during sausage fermentation, the synergic effect of meat proteases, LAB proteolytic system and lactic acid promoted soluble-protein hydrolysis, while *L. plantarum* was exclusively involved on the hydrolytic changes observed at pH 6.0. The bacterial acidogenic metabolism not only would assure hygienic quality of fermented sausages but would increase free amino acids and peptides release from meat proteins improving the sensory quality of the final product.

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