ELSEVIER

*Meat Science*, Vol. 49, No. 1, 11–18, 1998

PII: S0309-1740(97)00097-1 0309-1740/98 $19.00+0.00

# Proteolytic Activity of *Lactobacillus* Strains Isolated from Dry-fermented Sausages on Muscle Sarcoplasmic Proteins

Silvina Fadda,* Graciela Vignolo, Aída P. R. Holgado & Guillermo Oliver

Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000 Tucumán, Argentina

(Received 07 May 1997; revised version received 07 August 1997; accepted 07 August 1997)

*ABSTRACT*

*The proteolytic activity of seven strains of* Lactobacillus *from two species isolated from dry cured sausages was assayed using a soluble muscle extract as a source of proteins, at a temperature of 30°C. The results indicated that the strains of* Lactobacillus plantarum *tested had the more active proteolytic system, showing the highest amino acid release in the medium after 72 hr of incubation (*L. plantarum *CRL 681) when the microorganism was in the stationary phase of growth. The strains of* L. casei *showed a continued hydrolytic activity with a lower amino acids concentration along the studied period. The SDS–PAGE profiles showed that the major changes in sarcoplasmic proteins were produced in the 13 kDa and 36–40 kDa molecular weights region.* © *1998 Published by Elsevier Science Ltd. All rights reserved*

## INTRODUCTION

Many technological parameters that affect the nature and functional properties of proteins are involved in the preparation of meat products. Changes in colour, consistency and odour occurring during the curing of fermented sausages are due to biochemical reactions which are influenced directly by temperature, relative humidity, curing time, muscle composition, concentration of salt and nitrifying agents. These changes are also dependent on the starter culture previously added (De Masi *et al.*, 1990; Incze, 1992; Zeuthen, 1995; Hugas *et al.*, 1997). Most published works on microbial contributions to the quality of dry sausages deal with the role of microbial fermentation on the acidification, which is essential in the inhibition of the pathogenic flora as well as in the cohesion and colour of the product mass (Liepe, 1983; Bacus, 1984; Lücke, 1985). Different authors have shown that the degradation products derived from proteins and lipids increase during the ripening of several types of dry-cured hams such as American country-style ham (McCain *et al.*, 1968), Parma hams (Bellatti *et al.*, 1983) and Spanish Serrano ham (Aristoy and

*Author to whom correspondence should be addressed. Fax: 54-81-310465; e-mail: sfadda@cerela.edu.ar

Toldrá, 1991). In all cases a progressive increase in the level of non-protein nitrogen and, more specifically, in the free amino acids was observed. Proteolysis in fermented sausages is caused by enzymes from the starter bacteria as well as by the indigenous meat enzymes (Johansson *et al.*, 1996). In fermented dry-sausages, variations in free amino acids patterns have been found without establishing their origin (Dierick *et al.*, 1974). The breakdown of meat proteins has been attributed to the microbial flora involved in the fermentation and it seems that the action of naturally occurring muscle enzymes does not affect the proteolytic picture (Klement *et al.*, 1974; Toldrá and Etherington, 1988). Some studies have been reported on the proteolysis in lactic-fermented meat such as fermented sausages, but the contribution of these microorganisms has not been well studied. These reports also attribute proteolytic activity to *Micrococcus sp.* and to *Staphylococcus sp.*, which contribute to the flavour of fermented meat products (Lücke, 1994).

In contrast with the paucity of information on lactic acid bacteria proteinases in meat and vegetable foods, their significance in dairy products is well documented. The function of proteinases and peptidases in yogurt and cheese fermentation has already been cited in relation to the growth of the starter culture (Bouton *et al.*, 1993; El Soda *et al.*, 1983; Prost and Chamba, 1994; Exterkate and Alting, 1995; Farkye *et al.*, 1995). Although the lactic acid bacteria are weakly proteolytic compared with many other groups of bacteria (e.g. *Bacillus*, *Proteus Pseudomonas*, coliforms) (Kröckel, 1996) the detailed studies of the last decade have provided an insight into the complexity of their proteolytic systems (Ezzat *et al.*, 1988; Naes *et al.*, 1991; Holck and Naes, 1992; Naes and Nissen-Mayer, 1992). As regards this point, however, lactobacilli have not received the same attention in this respect as did lactococci (Exterkate, 1976; Coolbear *et al.*, 1992; Meijer *et al.*, 1996).

The objective of the present work was to study the proteolytic activity of lactic acid bacteria isolated from dry-fermented sausages and also to confirm the capacity of these microorganisms to degrade meat proteins during their growth in a soluble muscle extract.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

*Lactobacillus casei*, strains CRL678, CRL686, CRL704 and CRL705 and *Lactobacillus plantarum*, strains CRL681, CRL682 and CRL685 were isolated from commercial dry cured sausages and characterizated as previously reported by Vignolo *et al.* (1986). Cells were first activated in MRS broth, washed with distilled water and inoculated in the sterile soluble muscle extract, used as culture medium, to yield an initial number of bacteria of approximately $10^5$ CFU $ml^{-1}$ corresponding to an $OD_{680}$nm: 0·15 and incubated at 30°C. Samples were taken at 0 hr, 24 hr, 48 hr, 72 hr and 96 hr and $OD_{680}$nm, pH and proteolytic activity were determined.

### Soluble muscle extract

Muscle *Semimembranosus*, obtained from a commercial beef processor (10 g), was diluted 1:10 (w/v) with distilled water, homogenized in a Stomacher 400 blender (London, UK) for 3 min and centrifuged at 20,000×**g** for 20 min at 4°C. The supernatant was filtered through Whatman paper. The pH was adjusted to 6·0 with NaOH 0·1N and 1% of glucose was added. Finally the extract was filter sterilized through 0·22 $\mu$m (Millipore, Bedford, MA) and 0·1% of sterile Tween 80 was added. The absence of bacterial growth was confirmed by plating in PCA.

**Proteolytic activity**

Each microorganism was tested for proteolytic activity, by measuring the release of soluble amino acids in TCA (trichloroacetic acid) according to the OPA spectrophotometric assay (Church *et al.*, 1983). One ml of 12% TCA was added to 0·5 ml of the bacterial culture. After protein precipitation the extract was centrifuged and a supernatant aliquot containing free amino acids and small peptides was treated with o-phtaldialdehyde. Results are expressed as absorbance at 340 nm. All results are the means of two replicate assays.

The hydrolysis of soluble muscle proteins was detected by SDS-PAGE as described by Laemmli (1970), using a vertical gel electrophoresis unit (Mini-Protean II; Bio-Rad Laboratories, Richmond, CA) and a running gel containing 12% acrylamide, combined with a 5% of stacking gel. Samples were taken every 24 hr and mixed with an equal volume of a sample buffer containing 2% of SDS, 5% of $\beta$-mercaptoethanol, 10% of glycerol, buffer Tris-HCl pH 6·8 and bromophenol blue as front marker and heated for 5 min at 100°C. Twelve $\mu$l of samples containing 16 $\mu$g of proteins and 5 $\mu$l of molecular weight markers per lane were applied onto the gel. The electrophoresis was carried out at 68 mV until the bromophenol blue marker reached the bottom of the gel. Gels were stained with 0·1% (w/v) Coomasie blue R-250, 50% (v/v) ethanol and 10% (v/v) acetic acid at room temperature, shaken for 2 hr and destained until clear background was obtained. The molecular weight markers (Sigma) used as standard were: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), $\alpha$-lactoglobulin (14·2 kDa) and aprotinin (6·5 kDa).

## RESULTS

The proteolytic activity of seven *Lactobacillus* strains from two species were tested in a sterile meat extract. The results obtained showed an active cell metabolism with a pH decreasing from 6·0 to 3·0–3·5 at 96 hr and a fairly good growth capacity in the studied medium. Different extents of proteolysis were observed in the various strains. *L. plantarum* CRL 681 showed high levels of amino acids release with a maximal optical density ($OD_{340}$nm: 0·35) at 72 hr (Fig. 1) while a low hydrolytic activity was observed in *L. plantarum* CRL 682 and CRL 685. The release of amino acids was produced all along the growth cycle in the strain CRL 682 even though a cellular lysis occurred after 48 hr of incubation. Likewise, *L. plantarum* CRL 685 had a significant increase in amino acids concentration after 24 hr, reaching a maximum at 48 hr ($OD_{340}$nm: 0·25).

When *L. casei* were studied (Fig. 2) several clear differences were observed among strains. CRL 686, CRL 704 and CRL 705 showed a release of amino acids during the incubation time (96 hr) although a decrease at 48 hr in the first strain and at 72 hr in the second was observed. When *L. casei* CRL 678 was studied, a continuous release of amino acids was found between 24 and 96 hr, but in the first 24 hr a consumption of these metabolites from the soluble meat extract paralleling a fast growth rate was also observed. Although, *L. casei* CRL 705 showed a continuous amino acid increase during the incubation time.

SDS-polyacrylamide gel electrophoretograms of the products of hydrolysis of the soluble meat proteins are shown for five *Lactobacillus* strains in Fig. 3. The bands were identified by their molecular weights, determined by comparison with the standard proteins. *Lactobacillus plantarum* CRL 681 showed to be the more hydrolytic by OPA assay, exhibiting the higher rate of disappearance of several water-soluble protein bands. A 13 kDa band had almost disappeared at 24 hr while the same phenomenon occurred only at

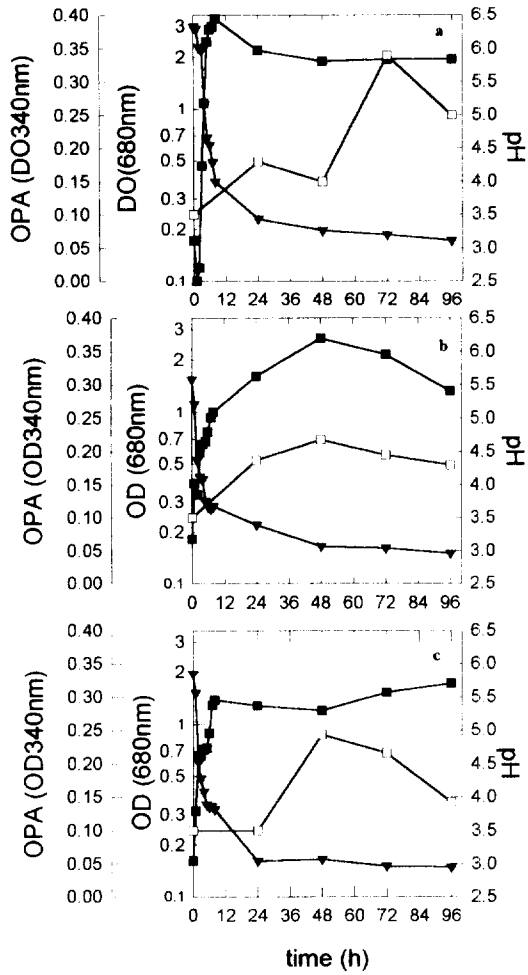


**Fig. 1.** Growth curve (■), pH changes (▲) and free amino acids concentration (□) in *Lactobacillus plantarum* strains, incubated at 30°C in a soluble muscle extract:(a) CRL 681; (b) CRL 682 and (c) CRL 685.

72 hr in the other studied strains. After 24 hr of incubation electophoretic profiles of CRL 681 also showed decrease in staining intensity in the 43 kDa and 36 kDa bands this effect being similar in all studied strains. The most significant level of hydrolysis was observed to take place in the lower molecular weight region (13 kDa). Moreover, Fig. 3 shows that after 96 hr of incubation in the soluble meat medium, all protein bands were affected by the growth of *Lactobacillus* strains.

## DISCUSSION

The results obtained indicate that the studied strains of *Lactobacillus casei* and *plantarum* grow and utilize the diverse nitrogen sources of the soluble meat extract. Bermell *et al.*

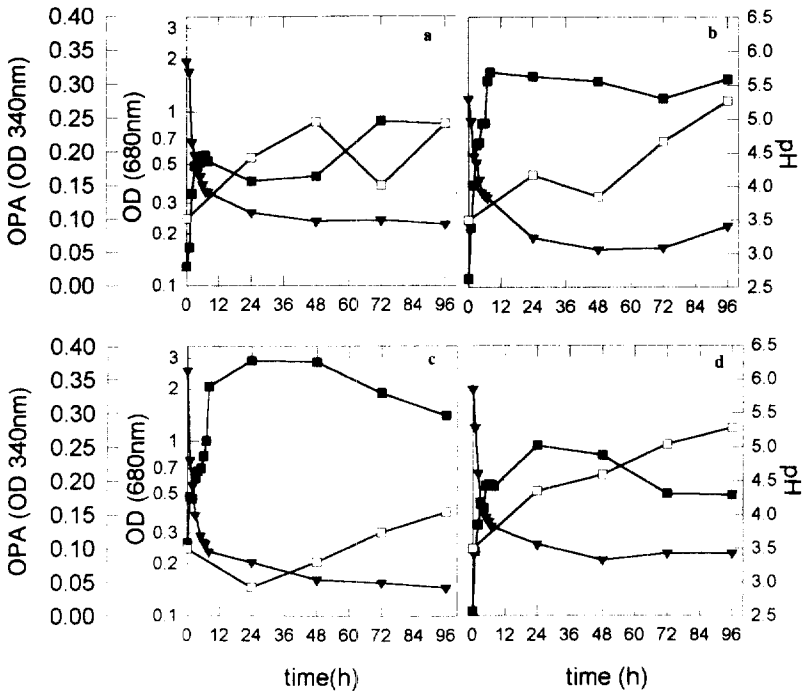


**Fig. 2.** Growth curve (■), pH changes (▲) and free amino acids concentration (□) in *Lactobacillus casei* strains, incubated at 30°C in a soluble muscle extract: (a) CRL 704; (b) CRL 686; (c) CRL 678 and (d) CRL 705.

(1992), have attributed the proteolytic activity found in ham to yeast rather than to the lactic acid bacteria. On the other hand, Kato *et al.* (1994) concluded that the protein degradation in the lactic fermented pork muscle was caused by proteases originated in meats, the lactic acid fermentation acting as the enhancing effector. Likewise, Molly *et al.* (1997) underlay the responsibility of both, meat and bacterial proteinases, in the ripening and flavour generation in dry fermented sausages. In the assayed conditions, our findings are in agreement also with those of Klement *et al.* (1974), Garcia de Fernando and Fox (1991) and Johansson *et al.* (1994) whom concluded that *Pediococcus cerevisiae* could play a role in producing proteolytic enzymes which are able to attack meat proteins.

The concentration of free amino acids released by the studied strains agree with the disappearance of several protein bands in the electrophoretograms. Throughout the incubation (96 hr) the most noticeable difference between *Lactobacillus* strains was the different rate of disappearance of 13 kDa band, which would correspond to soluble myoglobin protein. A cell-wall-associated proteinase activity could be responsible for such protein degradation as was clearly shown by El Soda *et al.* (1986) in *Lactobacillus casei* and *plantarum* grown in milk. Hagen *et al.* (1996) also confirm the effect of the reduction in the maturation time of dry fermented sausages due to the addition of a *Lactobacillus* proteinase. It is likely that a dipeptidase and an aminopeptidase intracellular activity produces a degradation into peptides and amino acids (Requena *et al.*, 1993; Habibi-Najafi and Lee, 1994; Kojic *et al.*, 1995; Tan *et al.*, 1995).

An understanding of the type and extent of proteolysis can help to achieve better quality sausages because the nature and concentration of protein degradating products,

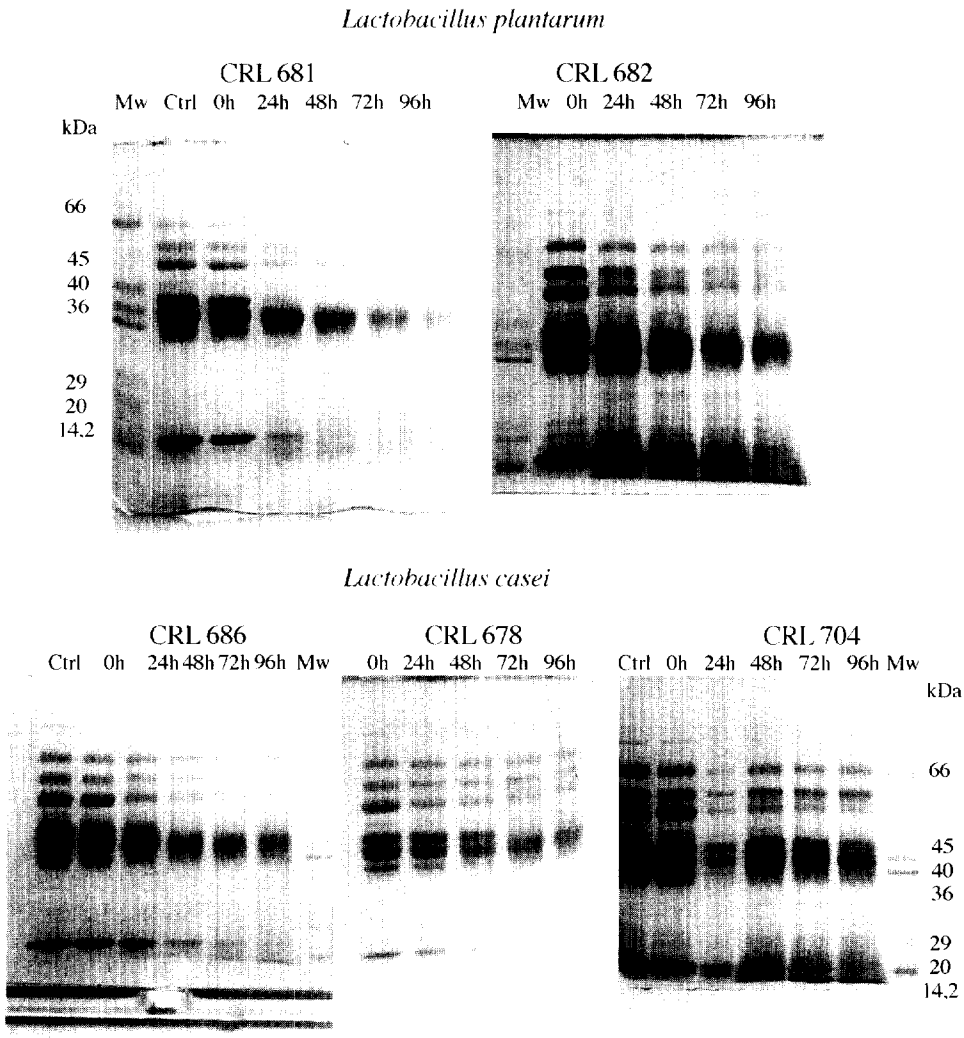


**Fig. 3.** Patterns of hydrolysis of soluble muscle extract by *L. plantarum* and *L. casei* by SDS–PAGE. Conditions—see Materials and Methods.

ranging from peptides to the products of amino acid catabolism can determine, together with other factors, the flavour and texture of fermented sausages. In any event, further study is necessary to elucidate the proteolytic pathway in fermented meats with lactic acid bacteria.

## ACKNOWLEDGEMENT

This work was partially supported by BID-CONICET No. 314 from Consejo Nacional de Investigaciones Cientcfhficas y Técnicas (CONICET), Argentina.

## REFERENCES

Aristoy, M. C. and Toldrá, F. (1991) Deproteinization techniques for HPLC aminoacids analysis in fresh pork muscle and dry-cured ham. *Journal of Agriculture Food Chemistry* **39**, 1792–1795.

Bacus, J. N. (1984) Utilization of microorganisms in meat processing. *Food Technology* **38**(6), 59.

Bellatti, M., Dazzi, G., Chizzolini, R., Palmia, F. and Parolari, G. (1983) pHysical and chemical changes occuring in proteins during the maturation of Parma ham. I. Biochemical and functional changes. *Industri della Conserve* **58**, 143–146.

Bermell, S., Molina, I., Miralles, C. and Flores, J. (1992) Study of the microbial flora in dry-cured-ham. 6. Proteolytic activity. *Fleischwirtschaf* **72**(12), 1684–1685.

Bouton, Y., Guyot, P., Dasen, A. and Grappin, R. (1993) Activité protéolytique de souches de lactobacilles thermophiles isoleés de levains at de Comté. I. Validation sur minifromages des techniques de laboratoire. *Lait* **73**, 265–279.

Church, F., Swaisgood, H., Porter, H. E. and Catignani, G. L. (1983) Spectrophotometric assay using o-pHtaldialdehyde for determination of proteolysis in milk. *Journal of Dairy Science* **66**, 1219.

Coolbear, T., Reid, J. R. and Pritchard, G. G. (1992) Stability and specificity of cell-wall-associated proteinase from *Lactococcus lactis* subsp *cremoris* H2 released by treatment with lisozyme in the presence the calcium ions. *Applied and Environmental Microbiology* **58**(10), 3263–3270.

De Masi, T. M., Wardlaw, F. B., Dick, R. L. and Ancton, J. C. (1990) Non protein nitrogen (NPN) and free amino acid contents of dry fermented sausages. *Meat Science* **27**, 1–12.

Dierick, N., Vandekerckhove, P. and Demeyer, D. (1974) Changes in non protein nitrogen compounds during dry-sausage ripening. *Journal of Food Science* **39**, 301–304.

El Soda, M., Said, H., Desmazeaud, M. J., Mashaly, R. and Ismail, A. (1983) The intracellular peptide-hydrolases of *lactobacillus plantarum*. Comparison with *Lactobacillus casei*. *Le Lait* **63**, 1–14.

El Soda, M., Said. H., Desmazeaud, M. J., Le Bars, D. and Zevaco, C. (1986) Cell-wall associated proteinases in *lactobacillus casei* and *Lactobacillus plantarum*. *Journal of Food Protection* **49**(5), 361–365.

Exterkate, F. A. (1976) Comparison of strains of *Streptococcus cremoris* for proteolytic activities associated with the cell wall. *Netherland Milk Dairy Journal* **30**, 95–105.

Exterkate, F. A. and Alting, A. C. (1995) The role of starter peptidases in the initial proteolytic events leading to amino acids in Gouda cheese. *International Dairy Journal* **5**, 15–28.

Ezzat, N., El Soda, M. and El Shafei, H. (1988) The cell-bound proteinase system of *Lactobacillus casei*—purification and characterization. *International Journal of Food Microbiology* **6**, 327–332.

Farkye, N. Y., Madkor, S. A. and Atkins, H. G. (1995) Proteolytic abilities of some lactic acid bacteria in a cheese model system. *International Dairy Journal* **5**, 715–725.

García de Fernando, G. D. and Fox, P. F. (1991) Study of proteolysis during the processing of a dry fermented pork sausage. *Meat Science* **30**, 367–383.

Habibi-Najafi, M. B. and Lee, B. H. (1994) Proline-specific peptidases of *Lactobacillus casei* subspecies. *Journal of Dairy Science* **77**, 385–392.

Hagen, B., Berdagué, J. L., Holck, A. L., Naes, H. and Blom, H. (1996) Bacterial proteinase reduces maturation time of dry fermented sausages. *Journal of Food Science* **61**(5), 1024–1029.

Holck, A. and Naes, H. (1992) Cloning, sequencing and expression of the gene encoding the cell-envelope-associated proteinase from *Lactobacillus paracasei* subsp. *paracasei* NCDO 151. *Journal of General Microbiology* **138**, 1353–1364.

Hugas, M. and Monfort, J. M. (1997) Bacterial starter cultures for meat fermentation. *Food Chemistry* **59**(4), 547–554.

Incze, K. (1992) Raw fermented and dried meat products. *Fleischwirtsch* **72**(1), 58–62.

Johansson, G., Berdagué, J. L., Larsson, M., Tran, N. and Borch, E. (1994) Lipolysis, proteolysis and formation of volatile components during ripening of a fermented sausage with *Pediococcus pentosaceus* and *Staphylococcus xylosus* as starter cultures. *Meat Science* **38**, 203–218.

Johansson, G. Molly, K. Greenen, I. and Demeyer, D. (1996) Lipolysis and proteolysis in meat fermentation. *Report of the European AIR Project: Optimization of Endogenous and Bacterial*

*Metabolism for the Improvement of Safety and Quality of Fermented Meat Products*, eds M. Raemaekers and D. Demeyer. Lillehammer.

Kato, T., Matsuda, T., Tahara, T., Sugimoto, M., Sato, Y. and Nakamura, R. (1994) Effects of meat-conditioning and lactic fermentation on pork muscle protein degradation. *Bioscience Biotechnology Biochemestry* **58**(2), 408–410.

Klement, J. T., Cassens, R. G. and Fennema, O. R. (1974) The effect of bacterial fermentation on protein solubility in a sausage model system. *Journal of Food Science* **39**, 833–835.

Kojic, M., Fira, D., Bojovic, B., Banina, A. and Topisirovic, L. (1995) Comparative study on cell envelope-associated proteinases in natural isolates of mesophilic lactobacilli. *Journal Applied Bacteriology* **79**, 61–68.

Kröckel, L. (1996) Bacterial fermentation of meats. In *Fermented Meats*, eds G. Campbell-Platt and P. E. Cook, pp. 69–102. Blackie Academic Professional, London.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.

Liepe, H. U. (1983) Starter cultures in meat production. In *Biotechnology*, eds Rehm and Reed. Weinheim Verlag Chemie. Vol. 5 p. 400.

Lücke, F. K. (1985) Fermented sausages. In *Microbiology of Fermented Foods*, ed. B. J. B. Wood, Vol. 2, p. 41. Elsevier Applied Science, London.

Lücke, F. K. (1994) Fermented meat products.. *Food Research International* **27**(3), 299–307.

McCain, R., Blumer, T. N., Craig, H. B. and Steel, R. G. (1968) Free amino acids in ham muscle during successive aging periods and their relation to flavor. *Journal of Food Science* **25**, 142–145.

Meijer, W., Marugg, J. D. and Hugenholtz, J. (1996) Regulation of proteolytic enzyme activity in *Lactococcus lactis*. *Applied and Environmental Microbiology* **62**(1), 156–161.

Molly, K., Demeyer, D., Johanson, G., Raemaekers, M., Ghistelinck, M. and Geenen, I. (1997) The importance of meat enzymes in ripening and flavour generation in dry fermented sausages. First results of a European project. *Food Chemistry* **59**(4), 539–545.

Naes, H., Chrzanowska, J. and Blom, H. (1991) Partial purification of a cell wall bound proteinase from *Lactobacillus casei*. *Food Chemistry* **42**, 65–79.

Naes, H. and Nissen-Meyer, J. (1992) Purification and N-terminal amino acid sequence determination of the cell-wall-bound proteinase from *Lactobacillus paracasei* subsp. *paracasei*. *Journal of General Microbiology* **138**, 313–318.

Prost, F. and Chamba, J. F. (1994) Effect of Aminopeptidase activity of thermophilic lactobacilli on emmental cheese characteristics. *Journal of Dairy Science* **77**, 24–33.

Requena, T., Pelaez, C. and Fox, P. F. (1993) Peptidase and proteinase activity of *Lactococcus lactis*, *Lactobacillus casei* and *Lactobacillus plantarum*. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **196**, 351–355.

Tan, P. S. T., Sasaki, M., Bosman, B. W. and Iwasaki, T. (1995) Purification and characterization of a dipeptidase from *Lactobacillus helveticus* SBT 2171. *Applied and Environmental Microbiology* **61**(9), 3430–3435.

Toldrá, F. and Etherington, D. J. (1988) Examination of cathepsins B, H and L activities in the processing of dry-cured ham. *Journal of Science Food Agriculture* **62**, 157–161.

Vignolo, G. M., Ruiz Holgado, A. P. and Oliver, G. (1986) Isolation and identification of lactic acid bacteria involved in the ripening of cured meat products. *Microbiologie–Aliment–Nutrition* **4**, 87–94.

Zeuthen, P. (1995) Historical aspects of meat fermentations. In *Fermented Meats*, eds G. Campbell-Platt and P. E Cook, pp. 53–68. Blackie Academic and Professional, London.