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Contribution of starter cultures to the proteolytic process of a fermented non-dried whole muscle ham product

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Abstract

Porcine *longissimus dorsi* muscles were cured by brine injection. Curing brine containing 15% (w/v) NaCl, 1.33% (w/v) glucose, 750 ppm sodium nitrite, and appropriate levels of either *Lactobacillus sakei* LAD, *L. sakei* LAD plus *Kocuria varians* FT4 (formally *Micrococcus varians*), *L. sakei LAD* plus papain and GDL (glucono-delta-lactone) plus *K. varians* FT4, was injected to the muscle at a pumping rate 15% w/v. The effect of these treatments on the proteolysis in the ham system was compared to a control ham, produced without starter culture and containing GDL acidulant to control pH and antibiotics to reduce the contribution of background microflora. Hydrolysis of sarcoplasmic and myofibrillar protein fractions was evaluated by SDS-PAGE and reverse phase-HPLC. Hams with different treatments were also investigated for differences in amino acid profile, protein and non-protein nitrogen level, colour, pH, water activity and moisture and microbiological evolution. There was no significant difference in the gross compositional analysis of any of the treatments compared to the control. There was no significant difference ($p>0.05$) in the protein content, non-protein nitrogen level, SDS-PAGE and free amino acid analysis between the control ham and ham inoculated with proteolytic starter culture. However, it was observed that hams containing starter cultures exhibited decreases in certain peptide fractions and corresponding increases in some free amino acids compared to the uninoculated control. It can be concluded that, while the principle mechanisms resulting in the proteolysis of this non-dried ham product involve the activity of endogeneous cathepsins, the addition of proteolytic starter cultures influence the amino acid profile thereby potentially enhancing the sensorial attributes of the ham.

Keywords: Starter cultures; Proteolytic process; Whole muscle ham product

1. Introduction

The use of starter cultures in meat fermentations has become well established in recent times as a means to increase processing rates and product consistency. While current applications are primarily confined to fermented dried sausages (Jessen, 1995), starter culture preparations have been also been investigated in Norwegian dry-cured ham (Lücke and Hechelmann, 1987) and non-dried fermented ham products (Scannell et al., 2002). Typical starter cultures for cured ham production comprise of a strain of Lactic Acid Bacteria (LAB), to lower the pH, together

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with a nitrite/nitrate reducing strain of *Micrococcaceae* to aid colour development, e.g. commercial starter culture LS-25 produced by Gewürzmüller, Stuttgart, Germany), which is used for fermented sausage production and contains *Lactococcus sakei* and *Staphylococcus carnosus*. The use of starter cultures has been reported to improve the sensory characteristics and microbiological quality of raw hams (Lücke, 1986) but the precise mechanisms by which these improvements are brought about is not widely understood.

In addition to the effects of cure ingredients and processing, enzymatic and chemical processes which occur within the muscle have a considerable influence on the organoleptic quality of fermented meat products. Breakdown products of lipolysis and proteolysis i.e. amino acids, carbonyls and volatile flavour compounds contribute to the characteristic flavour of fermented meats (Fadda et al.,1998). Understanding the enzymological processes from which these compounds arise is, however, complicated by the concurrent action of endogenous muscle enzymes and those derived from microbial metabolism.

Proteolysis in muscle meats occurs primarily as a result of the action of cathepsins, which breakdown sarcoplasmic and myofibrillar proteins (Molly et al., 1997). Proteolytic activity on meat proteins and peptides has also been described for strains of LAB, *Staphylococci*, yeast and moulds (Fadda et al., 1998; Rodriguez et al., 1998), leading to the hypothesis that both endogeneous and microbial peptidases are required for complete hydrolysis of oligopeptides (Toldrá and Verplaetse, 1995; Kenneally, 1999). Recently, there has been considerable interest in determining the effect of proteolytic starter cultures on meat proteins (Fadda et al., 1999a,b; Sanz and Toldrá, 1997a,b). However, most applications are directed towards dry-cured hams and fermented sausage. In earlier research it was found that, even in the absence of a drying step, the fermentation of cured ham with *L. sakei* and *S. carnosus* prior to cooking resulted in a finished product with superior flavour and increased microbiological stability than conventionally produced cooked ham products (Scannell et al., 2002). The objective of the present study is to examine the effects of *Lactobacillus sakei* LAD and *Kocuria varians* FT4 on the proteolytic processes occurring in a non-dried fermented whole muscle ham product.

2. Materials and methods

2.1. Muscle selection

Porcine *longissimus dorsi* muscles with a post-rigor pH of 5.5–5.6, and not exhibiting pale soft exudative (PSE) appearance were selected. The pH was measured at five different locations along the longitudinal axis of the muscle using a pH 320 meter fitted with an Ingold Lot 406-M6-DXK-S7/25 pH probe(WTW, 82362 Weilheim, Germany).

2.2. Starter culture selection

A large selection of *Micrococcus* strains originally isolated from dry cure hams in France and Norway and obtained from the microbiological collection, University College Cork, Ireland, were grown at 30 °C for 24 h in Peptone Medium (PM) broth containing, 1% tryptone (Difco Laboratories, Detroit, MI, USA; 0.5% yeast extract (Difco); 0.5% NaCl (Merck, Darmstadt, Germany); 0.1% glucose (BDH Laboratory Supplies, Poole, England); 1.5% Agar (for solid media: Difco) and adjusted to pH 7.2. Proteolytic activity was determined using the agar well diffusion assay described by Fransen et al. (1997) which incorporates meat protein in the agar composition. Each well was inoculated with 20 μl cell free supernatant and plates were incubated at 30 °C for 48 h. The agar was then stained for 5 min in a mixture containing 0.05% (w/v) coomassie brilliant blue R-250 (BDH), 50% (v/v) methanol (BDH), 9.2% (v/v) acetic acid (BDH), in distilled water and destained overnight in 25% (v/v) ethanol and 5% (v/v) acetic acid in distilled water. Proteolytic activity was determined by the presence of a clear zone around the inoculated wells. Each strain was evaluated in two independent tests and the most proteolytic strain, in this case *K. varians* FT4, was chosen as the proteolytic component of the starter culture combination for this study.

A non-proteolytic commercial starter culture preparation of *L. sakei* LAD (Gewürzmüller, Stuttgart, Germany) was used as the acidifying component of the starter culture combination and functioned to reduce the pH of the hams during the fermentation period. Antibiotics selected to repress background microflora were Penicillin (20,000 I.U.) and streptomycin (20 mg/kg muscle).

2.3. Ham processing

In each experiment five different treatments were tested (Table 1). The *longissimus dorsi* muscles were injected (15% pump rate) with a brine solution containing 15% NaCl, 1.33% glucose, 750 ppm sodium nitrite, to which the appropriate addition of starter cultures/additives had been made (Table 1), to give a residual level of 2.25% NaCl, 0.2% glucose and 112.5 ppm sodium nitrite and an initial starter level of 10^7 CFU/g. Sufficient glucono-delta-lactone (GDL) (BDH), was added to the brine to give a residual level of 1% in the muscle. Similarily, enough papain crude powder (P-3375, Sigma) was added to give a residual level of 20 units of proteolytic activity/kg muscle. After injection with the appropriate brine, the muscles were tumbled continuously for 1 h at 14 rpm, individually vacuum packaged and incubated at 12 °C. For the first trial, a fermentation period of 10 days was used, after which the hams were cold smoked (25 °C) for 1 h in a Sümann oven (Sümann Korz and OHG, Walzbachtal, Germany) and returned to the incubator at 12 °C for a further 4 days. Samples were then cooked in the Sümann oven (oven temperature 80 °C/ core temperature 72 °C) and cooled to 4 °C before sensory evaluation was undertaken. For the second experiment, samples were treated as above but incubated at 12 °C for up to 21 days to allow proteolysis proceed undisturbed.

Table 1
Inoculation details of the different ham treatments

Treatment	Code	Starter culture added	GDL[a]	Ab[b]	Papain[c]
1	LO	*Lactobacillus sakei* LAD	–	–	–
2	LP	*Lactobacillus sakei* LAD	–	–	+
3	LK	*Lactobacillus sakei* LAD plus *Kocuria varians*	–	–	–
4	GK	*Kocuria varians*	+	–	–
5	C Control	None	+	+	–

+ = added, – = not added.

[a] GDL = glucono-delta-lactone, added to a residual level of 1% in the muscle.

[b] Ab=antibiotics added (20,000 I.U. Penicillin and 20 mg Streptomycin/kg muscle).

[c] Twenty units of proteolytic activity/kg muscle residual level.

2.4. Experimental design

Two separate experiments were performed. Experiment 1 involved the examination of microbiological growth, pH, and colour properties of hams produced using each treatment and was carried out twice. Experiment 2 was performed to analyse protein, non-protein nitrogen level, moisture level, SDS-PAGE electrophoretograms (myofibrillar and water-soluble proteins) for each ham treatment. For this experiment, one muscle (divided in two portions) was used for each sample for the duration of trial, and samples were removed at the appropriate sample times from alternating portions of the muscle. This experiment was also duplicated.

2.5. Microbiological analyses

Slices of approximately 50 g were homogenised in a blender (Type 708A, Krups, Ireland) which had previously been swabbed with 70% alcohol. A portion of 10 g was aseptically weighed into a sterile stomacher bag (Seward Medical, London, UK) and diluted in 90 ml of 2% sterile buffered peptone water (Oxoid). The samples were then homogenised in a Stomacher 400 Laboratory blender (Seward Medical) for 2 min. Tenfold dilution series were prepared using 1/4 strength ringers (Merck). Appropriate dilutions were spread plated on Peptone Medium Agar [PM broth+1.5 g/l (w/v) technical agar (Oxoid)] to determine *Micrococcaceae* counts. They were differentiated from other strains on the basis of colony morphology. Appropriate dilutions were also spread-plated on deMan Rogosa and Sharpe (MRS) agar (Oxoid) to determine lactobacilli counts. To determine mesophilic aerobic viable counts, appropriate dilutions were pour-plated with Plate Count Agar (PCA) (Oxoid). All plates were incubated aerobically at 30 °C, for 48 h (PM and MRS) or 72 h (PCA). All tests were carried out in duplicate on two different samples.

2.6. Sensory evaluation

Sensory evaluation of the cooked ham was carried out according to the method of Kramer et al. (1974) to determine whether addition of a proteolytic starter culture would affect the sensorial quality of the ham.

Sensory parameters determined were, taste, appearance, texture, overall acceptability, acidity, intensity of smoked flavour and aroma, saltiness, taste or smell of ammonia, sweet taste or aroma, and putrefactive/off odours or flavours. Ham treatments LO, LK and GK (Table 1) were investigated using a rank preference design and an untrained taste panel.

Samples of ham were sliced to 4 mm thickness (diameter 50 mm) and placed on a paper plate, Subjects ranked samples in the order of their preference on the basis of taste, appearance, texture and overall acceptability, in order of preference on a scale of 1–3 (1=best, 3=worst). Panelists were also asked to rate the samples on a scale of 0–4 (0=none, 1=weak, 4=very strong) for the remaining parameters. Water was provided for subjects to rinse their mouths between samples.

Data were examined for significance using Friedmans two-way analysis of variance (in the case of taste, appearance and overall acceptability) or Repeated measures ANOVA (for all other parameters).

2.7. Colour, pH, water activity and moisture level

Colour measurements were carried out using a Chroma-meter CR 300 (Minolta, Osaka, Japan) to measure CIE L^* a^* b^* values (L^*=whiteness, a^*=redness, b^*=yellowness). Colour was analysed at five randomly selected points on the muscle. The pH was measured, as described above, at three randomly selected points, corresponding to those selected for colour analysis. Triplicate 5 g samples of homogenised whole muscle was analysed for water activity using an Aqua Lab CX-2 water activity meter (Decagon Devices, Pullman, WA, USA). Moisture content was also determined on triplicate samples in the CEM AVC 80 machine (CEM Corporation, NC, USA) using AOAC methods (AOAC, 1995a).

2.8. Determination of protein and non-protein nitrogen level

Total nitrogen content was determined in triplicate at regular intervals during incubation using the Kjeltec System 1026 Tecator distilling unit (Mason's Technology, Dublin, Ireland) as directed by method 981.1 (AOAC, 1995b). The distillate was titrated against 0.1 M HCl (Convol, BDH), the percentage of nitrogen was determined and a conversion factor of 6.25 was used to calculate percentage of protein.

To determine non-protein nitrogen level, homogenised ham samples (~30 g) were blended at low speed in a Waring Commercial Blendor® (Waring Products Division, CT, USA) with 20% trichloro acetic acid for 1 min. The resulting mixture was then allowed to stand at room temperature for 90 min and filtered through Whatman No. 1 filter paper. The protein content of 10 ml of the filtrate was determined in triplicate as described above and the nitrogen content calculated.

2.9. Extraction of myofibrillar and water soluble proteins

Myofibrils were extracted according to the method of Fransen et al. (1997) and were then freeze dried and stored in air tight bottles in a dessicator. Water-soluble proteins were extracted according to the method of Gallardo et al. (1995).

2.10. SDS-PAGE electrophoresis

Myofibrils were diluted 13 mg in 3 ml of a sample buffer consisting of 0.0625 M Tris–HCl (Sigma) pH 6.8, 2% SDS (BDH), 10% glycerol (Merck), 5% 2-mercaptoethanol and 0.1% bromophenol blue, to give a final concentration of approximately 4 mg/ml. Water soluble proteins were similarly diluted to give a protein concentration of 4 mg/ml. The resultant mixtures of protein and sample buffer, were boiled for two min, vortexed and boiled for a further 2 min. Proteins were identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using acrylamide concentrations of 3% for stacking and 10% for resolving gels (Laemmli, 1970). The gels were subsequently stained for 1 h in a 0.125% Coomassie brilliant blue R250 staining solution followed by destaining in a solution of 25% methanol, 10% acetic acid and 65% water. The molecular weights of the proteins were estimated from relative mobilities compared to standard molecular weight markers (Sigma SDS-6H and SDS 7) under identical electrophoretic conditions (Weber and Osborne, 1969).

2.11. Analysis of peptides by RP-HPLC

Preparation of samples for peptide analysis was carried out according to the method of Rodriguez-Nuñez et al. (1995). Peptides were examined at day 0 and after 14 days of fermentation, using a Shimadzu liquid chromatograph consisting of a model LC-9A pump, Sil-9A autosampler and a SPD-6A spectrophotometric detector (Shimadzu, Kyoto, Japan). The column used was a Symmetry 300® C_{18} column [300 Å, 5 μm, 250×4.6 mm] (Waters Chromatography Irl., Dublin, Ireland). Solvents used were deionised water with 0.1% trifluoroacetic acid (A), and acetonitrile with 0.1% trifluoroacetic acid (B). Flow rate was set at 0.8 ml/min, and detection was at 214 nm.

2.12. Analysis of free amino acids

Amino acid analyses were performed on days 0, 7, 14 and 21 by the method of Aristoy and Toldrá (1991). A portion of 8 g of homogenised muscle was diluted 1:5 with 0.1 N HCl (BDH) and homogenised at 4 °C with a stomacher (Seward) for 8 min at medium speed. Subsequently, samples were centrifuged at 10000 rpm for 20 min [J2-21, JA14 rotor] (Beckman Instruments, High Wycombe, UK). The supernatant was filtered through glass wool (Merck) and samples were frozen at −20 °C for further analysis. Samples were thawed when required and deproteinised by mixing with equal volumes of 24% TCA (12% in final volume) and allowed to stand for 10 min. Samples were then centrifuged at 14400×*g* (MSE Microcentaur microfuge; Beckman Instruments) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer (pH 2.2), to give approximately 25 nmol of each amino acid residue per 50 μl of injection volume, and analysed on a 120×4 mm cation exchange column (Na^+ form) using a Beckman 6300 amino acid analyser (Beckman Instruments, High Wycombe, UK).

3. Results and discussion

3.1. Microbiological analyses

Hams inoculated with *K. varians* had an initial *Micrococcaceae* count of approximately 10^7 CFU/g, as determined on PM agar, compared to levels of 10^4–10^5 in all other samples. In keeping with trends observed in other fermented meats (Coventry and Hickey, 1991; Garcia et al., 1992), these levels remained relatively constant throughout the fermentation process (Fig. 1), except where GDL was combined with *K. varians*, where numbers decreased considerably, a phenomenon also reported by Holley et al. (1988). Initial levels of lactobacilli, as determined on MRS agar, approached 10^8 CFU/g in all samples inoculated with *L. sakei*, these levels remained constant for the duration of the fermentation process (Fig. 2). Hams treated with GDL and *K. varians* (LK) contained approximately 10^5 CFU/g lactobacilli initially, but levels increased steadily by 2 log after 10 days fermentation. Lactobacilli counts decreased to undetectable levels after 14 days in control hams containing penicillin and streptomycin. Mesophilic aerobic plate counts mirrored trends observed on both PM and MRS agar, the control maintained levels of 10^4 CFU g^{-1} throughout the process, while treatments LO, LP, LK, inoculated with *L. sakei* remained at $\sim 10^8$ CFU g^{-1} (Fig. 3).

3.2. Colour, pH, water activity and moisture

All hams produced had a_w values between 0.975 and 0.985 and moisture content of approximately 75%

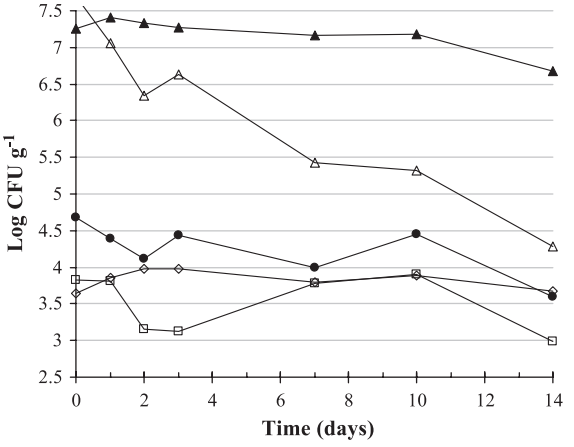

Fig. 1. Changes in the population levels of *Micrococcaceae* in ham fermented with *L. sakei* LAD (●), *L. sakei*+*K. varians* (▲), *L. sakei*+papain (□), GDL+K. *varians* (△) or without added cultures (◇) for 14 days at 12 °C. (Each data point represents the mean of two independent trials).

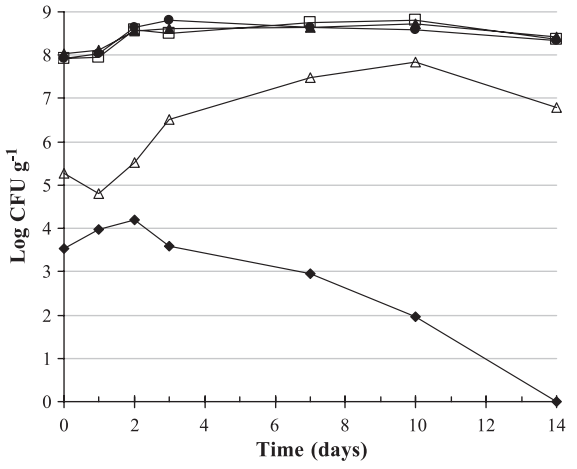

Fig. 2. Changes in the population levels of lactic acid bacteria in ham fermented with *L. sakei* LAD (●), *L. sakei* + *K. varians* (▲), *L. sakei* + papain (□), GDL + *K. varians* (△) or without added cultures (◇) for 14 days at 12 °C. (Each data point represents the mean of two independent trials).

at the end of processing. There was no significant difference ($p>0.05$) between treatments (results not shown).

The initial pH of all the muscles selected was between 5.5 and 5.6. The addition of GDL to the control and treatment GK reduced the pH to 4.8 and 5.2, respectively, on day 0. This was significantly lower ($p<0.05$) than remaining treatments and remained so until day 7 of fermentation. The pH of treatments LO, LP and LK began to decline after day 3, reaching pH 4.8 by day 7 (results not shown). There was no significant difference in pH between treatments LO, LP and LK for the duration of the fermentation, neither was there significant difference between these treatments and those containing GDL after day 7 (results not shown). The final pH (day 14) of the fermented ham was ~5 for treatments LO, LP and LK, and ~4.8 for GK, and the control.

In general, as pH decreases the colour of meat lightens, due to reduced water holding capacity and a corresponding increase in light scattering properties in the muscle fiber (Warriss, 2000). In this study it was observed that lightness (CIE L^* values) increased in all treatments over time. Treatments GM and the control, which contained GDL and had lower starting pH, had significantly higher ($p<0.05$) lightness values than the remaining treatments up to day 3. Treatments LO, LK, and LP showed increasing lightness from day 3, corresponding to acid production in the ham which concurs with earlier results (Scannell et al., 2002). The development of redness in the hams was also related to pH, increasing as pH decreased. CIE a^* values increased more rapidly and maintained significantly higher ($p<0.05$) redness throughout the fermentation process in hams where GDL was employed. Treatments LO, LP and LK increased in redness after day 7, corresponding to a decrease in pH in these treatments, and, while differences between the treatments were not significant, trends indicated that of these treatments LK, containing *K. varians* had higher a^* values (data not shown).

3.3. Protein content and non-protein nitrogen

The protein content of fermented hams produced in this study ranged from 19% to 24% (results not shown). Trends indicated a slight decrease in protein content for all treatments up to day 7, which was concurrent with pH reduction and may be due to loss of salt soluble proteins in the exudates. After day 7, protein content remained constant and there was no significant difference between treatments (results not shown).

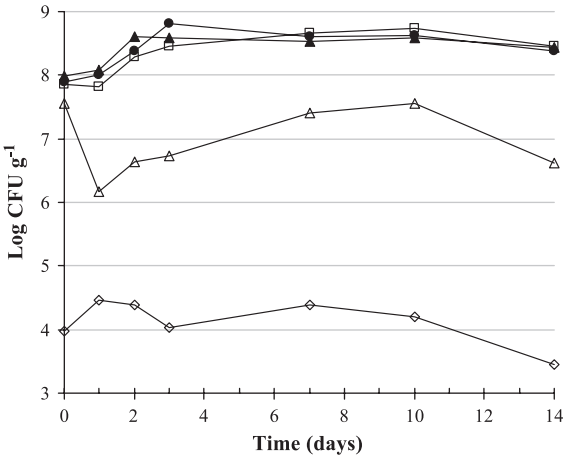

Fig. 3. Changes in the mesophilic aerobic plate counts in ham fermented with *L. sake* LAD (●), *L. sakei* + *K. varians* (▲), *L. sakei* + papain (□), GDL + *K. varians* (△) or without added cultures (◇) for 14 days at 12 °C. (Each data point represents the mean of two independent trials).

Non-protein nitrogen (NPN) levels of between 8.5% and 9.5% on a protein basis were observed for all treatments at the outset of the experiment. In general, NPN increased slightly after 21 days at 12 °C for all treatments (Fig. 4). Treatment LP, contained a commercial preparation of papain (used in the meat industry to improve meat texture and flavour), which was used as a positive proteolytic control, against which the proteolytic activity of the *K. varians* could be compared. LP caused a continuous increase in NPN reaching >12% at the end of processing. This increase in NPN was consistently higher than all other treatments after day 7 and significantly higher ($p<0.05$) than GK, on day 7, than the control and treatment LK on days 11 and 14 and than the control and treatment GK on day 21. There was no significant difference ($p>0.05$) between hams produced using the proteolytic starter, *K. varians*, and the control for the duration of the fermentation, indicating perhaps an more sensitive protocol is necessary to determine the effect of starter culture bacteria to protein breakdown.

3.4. Gel electrophoresis

Analysis of proteolysis in fermented sausages and dry cured hams often focuses on the electophoretic profiles of the sample measured over time. In the case of both products changes are apparent over time in both the water-soluble and myofibrillar protein fractions. These changes may be a result of proteolysis and protein insolubilisation due to conditions found during processing such as lowering of the pH, heat denaturation, reduced water activity and increased salt content (Klement et al., 1973, 1974; Astiasaran et al., 1990). However, in this study the only cause of protein insolubilisation could be the reduction in pH. The evaluation of the gel electrophoretograms of myofibrillar proteins clearly showed that there was a progressive decrease in myosin heavy chain (MHC) over 21 days, which can be seen by the reduction in the band at approximately 205 kDa (Fig. 5). This reduction was most pronounced in the samples which had papain added (Fig. 5A). The decrease in MHC was also followed by an increase in a protein at ca. 116 kDa over the same time period. The protein band at 45 kDa corresponding to actin (≈40 kDa) was not greatly affected in any of the samples until day 21, except in the sample inoculated with papain where a progressive decrease in actin was observed. An increase in a protein of 29 kDa was also seen in all samples which was more pronounced in samples with papain added. Overall, it was evident that there were no apparent differences in the protein band patterns between samples inoculated with proteolytic starter cultures (Fig. 5B) and the uninoculated control (Fig. 5C). This suggests that proteolytic starter cultures do not play a role in the breakdown of myosin in this fermented whole muscle meat product.

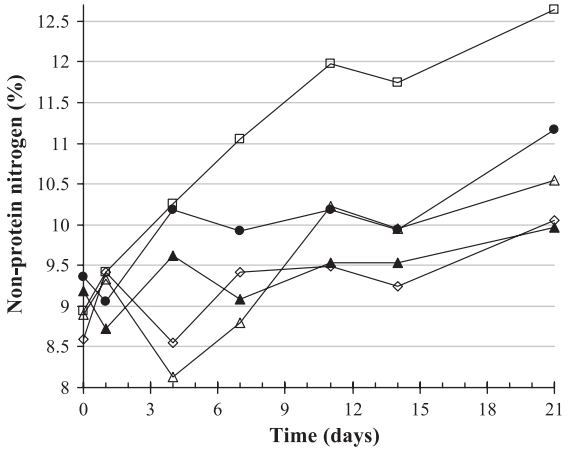

Fig. 4. Changes in the non-protein nitrogen (on a protein basis) of ham fermented with *L. sakei* LAD (●), *L. sakei* + *K. varians* (▲), *L. sakei* + papain (□), GDL + K. *varians* (△) or without added cultures (◇) for 21 days at 12 °C. (Each data point represents the mean of two independent trials).

Analysis of gel electrophoretograms of sarcoplasmic proteins showed differences in the protein band patterns of individual treatments between the two trials. This could have been due to losses of proteins in the exudate, as well as losses of protein solubility, or because samples were taken from different parts of the muscle and the brine ingredients were not uniform, or perhaps the endogenous muscle enzymes were in different states of activity. The only major change common to both trials was a decrease in actin and a protein of 29 kDa over the 21-day period (Fig. 6A). In the second trial it was also evident that a lot of low molecular weight proteins (14.2–24 kDa) appeared from day 7 onwards (Fig. 6B). However, there were no detectable differences in the protein band patterns between

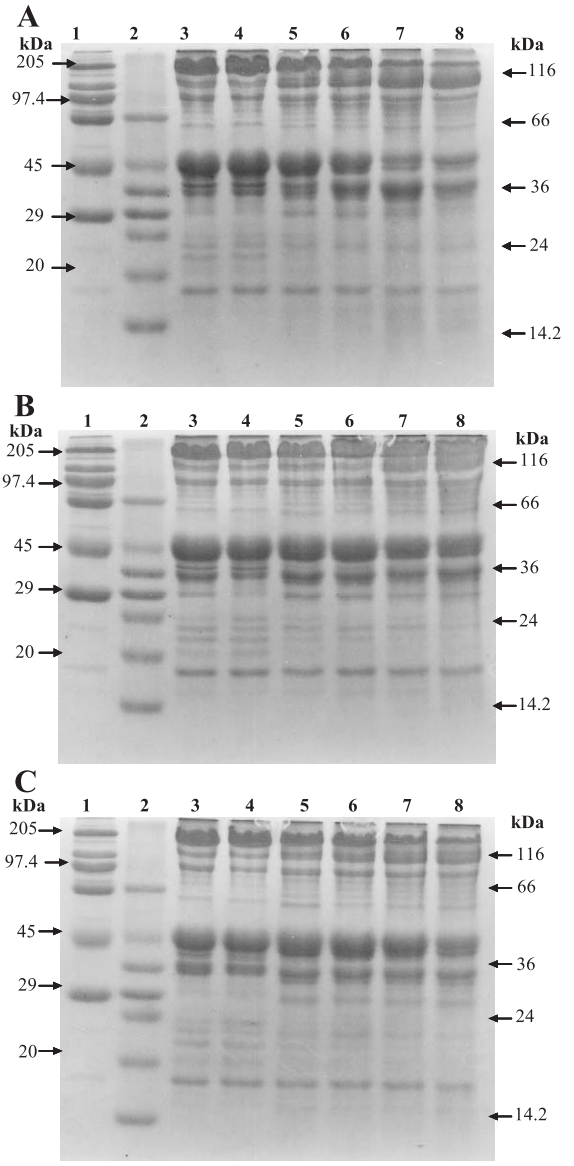

Fig. 5. SDS PAGE profile of myofibrillar proteins for ham fermented with *L. sakei* + papain (A), GDL + *K. varians* (B) or without added cultures (C) for 21 days at 12 °C. (Lanes 1 and two contain standards, Lanes 3, 4, 5, 6, 7 and 8 correspond to sample days 0, 1, 3, 7, 14 and 21, respectively).

samples inoculated with proteolytic starter cultures and the control (data not shown). It was also observed that the action of papain did not have as great an influence on sarcoplasmic proteins as it did on myofibrillar proteins.

3.5. Peptide analysis

Increases in peptidic fractions are common in fermented meat products and have been widely reported by a number of research groups (Dierick et al., 1974; Zapelena et al., 1997a,b; Toldra and Flores, 1998). Some of these peptides are associated with specific tastes such as bitter and savoury tastes. Evaluation of peptides by RP-HPLC showed that ham treatments LO LK and LP a decrease in a peptide which eluted at ca. 12 min, which was not observed for treatment GK, and an increase in three other peaks corresponding to tyrosine, phenylalanine and tryptophan for the four mentioned treatments (data not shown). This phenomenon of decreases in peptidic fractions has also been shown in dry fermented sausages (Zapelena et al., 1997a) and dry cured hams (Rodriguez-Nuñez et al., 1995), and possibly occurs as a result of muscle or microbial

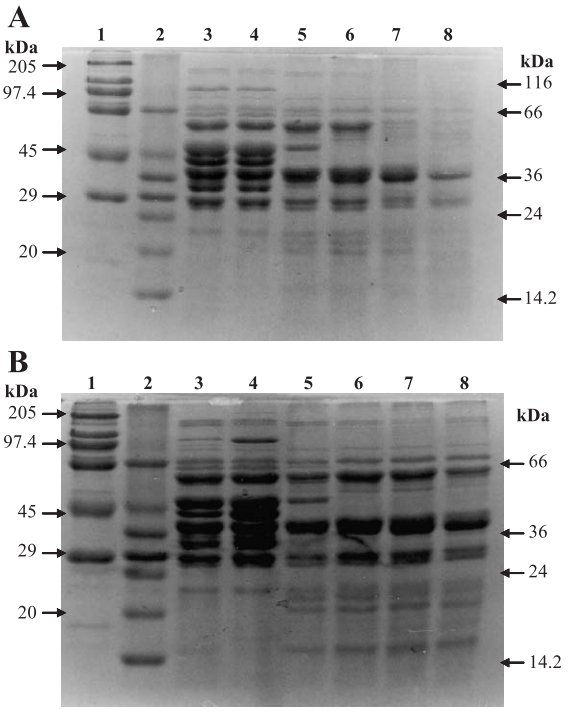

Fig. 6. SDS PAGE profile of sarcoplasmic proteins for ham fermented with *L. sakei* + papain for 21 days at 12 °C from trial 1 (A) and trial 2 (B). (Lanes 1 and two contain standards, Lanes 3, 4, 5, 6, 7 and 8 correspond to sample days 0, 1, 3, 7, 14 and 21, respectively).

exopeptidases, (Molly et al., 1997; Toldra and Flores, 1998). An increase in a peptide that eluted at approximately 9 min was also observed for treatments LK and LP. This contrasts with the fact that the control ham also showed neither decrease in the peak at 12 min nor any increase in the level of the three free amino acids, suggesting that a certain peptidase activity could be attributed to the added starter cultures.

3.5.1. Analysis of free amino acid evolution

During the ripening of fermented sausages and dry cured ham a major increase in FAA is achieved. Some FAA can be responsible for certain flavours in the meat both directly and indirectly as flavour precursors. The increase in FAA concentration can also be beneficial from a health point of view due to the provision of essential amino acids (Toldra and Aristoy, 1993). Evaluation of free amino acids (FAA) indicated an increase in most FAA over the 21-day fermentation period (data not shown). Decreases in certain FAA were observed in some cases, which could be a result of conversion to flavour compounds or biogenic amines. The greatest increase in most instances was in treatment LP. Significant differences ($p<0.05$) were only found for some FAA, and these differences were only found between the samples containing papain and the other samples. No significant differences in individual FAA were observed between samples inoculated with proteolytic starter cultures and the control (Table 2).

3.6. Sensory analysis

The effect of addition of proteolytic and lipolytic enzymes from bacteria on the sensory properties of fermented meats has been examined in detail, with wide ranging results. Arboles and Julia (1992) showed that the addition of a lipase from *Mucor miehei* did not improve the sensory quality of cured products. However, Zalacain et al. (1997 a,b) showed that the addition of lipase from either *Aspergillus* or *Rhizomucor miehei* both improved the sensory properties of fermented sausages. The addition of Pronase E (Diaz et al., 1993) from *Streptomyces griseus*, and a proteinase from *Lactobacillus paracasei* ssp. *paracasei* (Naes et al., 1995) have also both been shown to improve the sensory profile of fermented sausages.

Rank totals from nine subjects, from sensory evaluation of taste, appearance, texture and overall acceptability (Table 3) of cooked fermented hams showed that there were no significant differences between treatments ($p>0.05$) concerning taste, texture and overall acceptability. However, trends indi-

Table 2
Free amino acids (FAA) produced (μg/g protein) on days 0 and 14 from fermented hams inoculated with different starter culture treatments

Treatment[a]	LO		LK		LP		GK		C	
FAA	Time (day)									
	0	14	0	14	0	14	0	14	0	14
Aspartic acid	743±35	1707±212	544±142	1849±97	686±285	3120±19	490±26	2159±14	561±24	1240±491
Cysteic acid	670±93	706±164	691±159	735±43	586±141	787±54	609±144	668±154	555±126	692±70
Threonine	899±62	1769±61	671±218	1857±708	847±398	3132±33	483±37	1869±209	676±59	1130±483
Serine	1350±109	2717±199	1088±445	2902±698	1191±577	4504±456	691±70	2851±54	1011±78	1962±680
Glutamic acid	2826±41	7154±679	3007±1383	7085±231	2867±1242	10079±266	2007±239	7441±550	2622±305	3752±1417
Glycine	1101±29	2121±269	1009±171	2606±645	1083±390	4765±119	835±19	2122±95	948±60	1368±278
Alanine	2537±86	4894±525	1954±375	4890±550	2101±668	7864±50	1716±99	4754±511	1971±50	2617±702
Cysteine	1234±106	2730±224	1015±209	3135±687	1053±282	5937±205	816±5	2623±342	1005±21	1849±356
Valine	884±62	1624±60	728±151	2004±221	810±284	3025±88	573±29	1737±95	710±14	1299±372
Methionine	991±106	2063±111	788±255	2211±23	862±260	3322±22	617±6	2150±173	827±69	1443±61
Isoleucine	1760±148	3766±324	1395±419	4139±89	1506±419	6316±137	1098±55	4100±203	1367±18	3096±765
Leucine	1052±134	2065±121	801±191	2042±277	924±268	3006±96	597±107	1981±70	761±11	1251±458
Tyrosine	910±54	2438±248	983±485	2641±183	808±202	4011±185	593±54	2427±172	750±10	1660±597

[a] See Table 1 for definition of codes.

cate that hams inoculated with *L. sakei* alone tasted better than the other two samples. In a previous study, panellists found that hams fermented with *L. sakei* and *S. carnosus* had a better taste and texture profile and were more acceptable than delicatessen purchased control (Scannell et al., 2002), however, the contribution of *S. carnosus* to the proteolysis would have been much less in this instance due to the use of a shorter fermentation time.

Analysis of acidity, intensity of smoked flavour and aroma, saltiness, taste or smell of ammonia, sweet taste or aroma and putrefactive/off odours and flavours results (Table 4) showed some significant differences between treatments ($p<0.05$). In the case of acidity all treatments were significantly different from each other ($p<0.05$), GK hams being significantly lower than all treatments tested while, LO hams were significantly more acidic. A similar trend was found in the case of perceived saltiness. Additionally, GK hams were significantly less smoky than the other treatments ($p<0.05$). The combination of an acidification and proteolytic strain in ham produced significantly higher degree of ammonia (taste or smell), as panellists found LO hams to have lowest amounts of detectable ammonia and LK hams to have the greatest ($p<0.05$). There was also a significantly higher ($p<0.05$) detection among panellists of putrefactive/off odours and flavours in GK hams. Overall, however, flavours or aromas detected were not found to be offensive. The hams that contained papain were not analysed for sensorial properties due to excessive softening after cooking, while control sausages were not evaluated due to the presence of antibiotics. This study indicates that the presence of a proteolytic starter culture in the ham results in poorer taste panel acceptability than hams fermented only using an acidifying strain.

Table 3
Rank sums of sensory evaluation for ranking the preference in cooked fermented hams inoculated with either *L. sakei* LAD (LO), LAD + *K. varians* 4 (LK) or GDL + *K. varians* 4 (GK). Values represent mean results for two independent trials

	LO	LK	GK
Taste	12.5	21.25	20.25
Appearance	16.5	20.25	17.25
Texture	13	18.75	22.25
Overall acceptability	11.5	20	22.25

Table 4
Rating of cooked fermented hams inoculated with either *L. sakei* LAD (LO), LAD + *K. varians* 4 (LK) or GDL + *K. varians* 4 (GK). 0 = none, 9 = weak, 36 = extreme

Parameter	LC	MC	GC
Acidity	20	12	10
Smokiness	16	14	12
Saltiness	21	16	13
Ammonia level	1	3	3
Sweetness level	8	6	3
Putrefaction level	1	2	1

4. Conclusion

Results from this study indicate that, while endogeneous meat enzymes are responsible for the key proteolytic processes of a fermented whole muscle product, a certain amount of peptidase activity could be attributed to proteolytic microorganisms. However, since sensory analysis showed no significant improvement to the flavour profile of the hams, further research is necessary to determine how beneficial it will be to add these strains to ham as starter cultures.

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References

AOAC, 1995. AOAC official method 985.14. Moisture in meat and meat products (Rapid microwave drying method). In: Cunniffe, P. (Ed.), Official Methods of Analysis of AOAC International, 16th ed., vol. 39, pp. 1–2. Washington, DC, USA.

AOAC, 1995. AOAC Official method 985.15. Fat in meat and poultry products (Rapid microwave-solvent extraction method). In: Cunniffe, P. (Ed.), Official Methods of Analysis of AOAC International, 16th ed., vol. 39, pp. 1–3. Washington, DC, USA.

Arboles, J., Julia, E., 1992. Aroma in cured meat products. In:

Smulders, F.J.M., Toldra, F., Flores, J., Prieto, M. (Eds.), New Technologies for Meat and Meat Products. Audet Tijdschriften, pp. 109–124.

Aristoy, M.C., Toldrá, F., 1991. Amino acid analysis in fresh pork and dry cured ham by HPLC of phenylisothiocyanate derivatives. Proceedings of the 37th International Congress of Meat Science and Technology, Kulmbach, Germany, vol. 2, pp. 847–850. Session 6.

Astiasaran, I., Villanueva, R., Bello, J., 1990. Analysis of proteolysis and protein insolubility during the manufacture of some varieties of dry sausage. Meat Sci. 28, 111–117.

Coventry, J., Hickey, M.W., 1991. Growth characteristics of meat starter cultures. Meat Sci. 30, 41–48.

Diaz, O., Fernandez, M., Garcia de Fernando, G.D., de La Hoz, L., Ordonez, J.A., 1993. Effect of the addition of pronase E on the proteolysis in dry fermented sausages. Meat Sci. 34, 205–216.

Dierick, N., Vandekerchore, P., Demeyer, D., 1974. Changes in non protein nitrogen compounds during sausage ripening. Journal of Food Science 39, 301–304.

Fadda, S., Vignolo, G., Holgado, A.P.R., Oliver, G., 1998. Proteolytic activity of *Lactobacillus strains* isolated from dry fermented sausages on muscle sarcoplasmic proteins. Meat Sci. 49, 11–18.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.C., Oliver, G., Toldrá, F., 1999a. Hydrolysis of pork muscle scarcplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. Appl. Environ. Microbiol. 65, 578–584.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.C., Oliver, G., Toldrá, F., 1999b. Characterisation of muscle scarcplasmic and myofibrillar protein hydrolysis caused by *Lactobacillus plantarum*. Appl. Environ. Microbiol. 65, 3540–3546.

Fransen, N.G., O'Connell, M.B., Arendt, E.K., 1997. A modified agar medium for the screening of proteolytic activity of starter cultures for meat fermentation purposes. Int. J. Food Microbiol. 36, 235–239.

Gallardo, J.M., Sotelo, C.G., Pineiro, C., Perez-Martin, R.I., 1995. Use of capillary zone electrophoresis for fish species identification. Differentiation of flatfish species. J. Agric. Food Chem. 43, 1238–1244.

Garcia, M.L., Selgas, M.D., Fernandez, M., Ordonez, J.A., 1992. Microorganisms and lipolysis in the ripening of dry fermented sausages. Int. J. Food Sci. Technol. 27, 675–682.

Holley, R.A., Jui, P.A., Wittmann, M., Kwan, P., 1988. Survival of *S. aureus* and *S. typhimurium* in raw ripened dry sausages formulated with mechanically separated chicken meat. Fleischwirtschaft 68, 194–201.

Jessen, B., 1995. Starter cultures for meat fermentations. In: Campbell-Platt, G., Cook, P.E. (Eds.), Fermented Meats. Blackie Academic & Professional Publishers, Glasgow, Scotland.

Kenneally, P.M., 1999. Lipolysis and Proteolysis in Fermented Meat Products. PhD Thesis of The National University of Ireland, UCC, Cork Ireland.

Klement, J.T., Cassens, R.G., Fenema, O.R., 1973. The association of protein solubility with physical properties in a fermented sausage. J. Food Sci. 38, 1128–1131.

Klement, J.T., Cassens, R.G., Fenema, O.R., 1974. The effect of bacterial fermentation on protein solubility in a sausage model system. J. Food Sci. 39, 833–835.

Kramer, A., Kahan, G., Cooper, D., Papavasiliou, A., 1974. A nonparametric ranking method for the statistical evaluation of sensory data. In: Jellinek, G. (Ed.), Chemical Senses and Flavour 1. Sensory Evaluation of Food; Theory and Practice (1975). Ellis Horwood Publishers, Chichester, England, pp. 121–133.

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

Lücke, F.K., 1986. Microbiological processes in the manufacture of dry sausage and raw ham. Fleischwirtschaft 66, 1505–1509.

Lücke, F.K., Hechelmann, H., 1987. Starter cultures for dry sausages and raw ham-composition and effect. Fleischwirtschaft 67, 307–314.

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

Naes, H., Holck, A.L., Axelsson, L., Andersen, H.J., Blom, H., 1995. Accelerated ripening of dry fermented sausage by the addition of a *Lactobacillus* proteinase. Int. J. Food Sci. Technol. 29, 651–659.

Rodriguez, M., Nuñez, F., Cordoba, J.J., Bermudez, M.E., Asensio, M.A., 1998. Evaluation of proteolytic activity of microorganisms isolated from dry cured ham. J. Appl. Microbiol. 85, 905–912.

Rodriguez-Nuñez, E., Aristoy, M.C., Toldrá, F., 1995. Peptide generation in the processing of dry cured ham. Food Chem. 53, 187–190.

Sanz, Y., Toldrá, F., 1997a. Purification and characterisation of an aminopeptidase from Lactobacillus sake. J. Agric. Food Chem. 45, 1552–1558.

Sanz, Y., Toldrá, F., 1997b. Aminopeptidases activities from Lactobacillus sake in models of curing ingredients and processing conditions for dry sausage. J. Food Sci. 63, 894–896.

Scannell, A.G.M., Kenneally, P.M., McCarthy, D., Schwarz, G., Arendt, E.K., 2002. Optimisation of fermentation conditions for the production of fermented hams. Eur. J. Food Res. 215, 183–188.

Toldra, F., Aristoy, M.C., 1993. Availability of essential amino acids in dry cured ham. Int. J. Food Sci. Nutr. 44, 215–219.

Toldra, F., Flores, M., 1998. The role of muscle proteases and lipases in flavour development during the processing of dry-cured ham. Crit. Rev. Food Sci. Nutr. 38, 331–352.

Toldrá, F., Verplaetse, A., 1995. Endogenous enzyme activity and quality for raw product processing. In: Lundström, K., Hanson, I., Wicklund, E. (Eds.), Composition of Meat in Elation to Processing, Nutritional and Sensory Quality. ECCEAMST, Utrecht, pp. 41–45.

Warriss, P.D., 2000. Post-mortem changes in muscles and its conversion to meat. Meat Science, An Introductory Text. CABI, London, UK, pp. 93–105.

Weber, K., Osborne, M., 1969. The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. J. Food Biochem. 244, 4406–4412.

Zalacain, I., Zapelena, M.J., De Pena, M.P., Astiasaran, I., Bello, J.,

1997a. Lipid fractions of dry fermented sausages change when starter culture and/or *Aspergillus* lipase are added. J. Food Sci. 62 (5), 1076–1079.

Zalacain, I., Zapelena, M.J., De Pena, M.P., Astiasaran, I., Bello, J., 1997b. Use of lipase from *Rhizomucor miehei* in dry fermented sausages elaboration: Microbial, chemical and sensory analysis. Meat Sci. 45 (1), 99–105.

Zapelena, M.J., Zalacain, I., De Pena, M.P., Astiasaran, I., Bello, J., 1997a. Addition of a neutral proteinase from *Bacillus subtilis* (Neutrase) together with a starter to a dry fermented sausage elaboration and its effect on the amino acid profiles and the flavour development. J. Agric. Food Chem. 45, 472–475.

Zapelena, M.J., Zalacain, I., De Pena, M.P., Astiasaran, I., Bello, J., 1997b. Effect of the addition of a neutral proteinase from *Bacillus subtilis* (Neutrase) on nitrogen fractions and texture of Spanish fermented sausage. J. Agric. Food Chem. 45, 2798–2801.