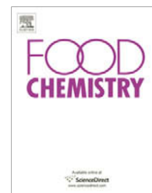
Food Chemistry 119 (2010) 279–285

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages

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A R T I C L E I N F O

Article history:
Received 27 February 2009
Received in revised form 11 April 2009
Accepted 15 June 2009

Keywords:
Fermented sausage
Starter culture
Amino acid
Proteolysis

A B S T R A C T

The main objective of this work was to examine the effects of using five types of commercial starter cultures in fermented sausages. During the fermentation stage, changes in proteolytic characteristics were observed in fermented sausages. Proteolytic activity was high in Ls_b + *Sc*:(*Lactobacillus sakei* + *Staphylococcus carnosus*) and *Pp* + *Sx*:(*Pediococcus pentosaceus* + *Staphylococcus xylosus*) starter-inoculated sausages during processing. Moreover, a slight increase in proteolytic activity was detected during storage in both these sausages. Sarcoplasmic and myofibrillar proteins were also affected by this starter culture addition, during the fermentation, ripening and intense proteolysis were observed in both the fermented sausages. The content of free amino acids was similar at the beginning of the fermentation stage for all the studied batches. However, the high differences in the content of free amino acids at the end of the process could be attributed to the starter culture activity.

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1. Introduction

The fermented meat products are traditional products that have a long history and an inestimable gastronomic value. The products are found in most parts of the world, although Europe is the major producer and consumer of these products. Preservation is usually achieved by a combination of fermentations using water activity-lowering techniques including dehydration and addition of salt. These techniques have been the basis of traditional technologies used before the scientific techniques were understood (Campbell-Platt, 1995). All these transformations are influenced by ripening conditions, raw meat and ingredients and have a considerable effect on the organoleptic quality of fermented meat products. Breakdown products of lipolysis and proteolysis, i.e. peptides, amino acids, carbonyls and volatile flavour compounds contribute to the characteristic flavour and texture of fermented meats (Demeyer et al., 1995; Diaz, Fernández, García de Fernando, De la Hoz, & Ordóñez, 1997). The pattern of the proteolysis in fermented sausages is influenced by several variables such as product formulation, processing condition and starter culture (Hughes et al., 2002).

Lactic acid bacteria with important industrial functionality are being developed to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, and vitamins, or those having probiotic properties (Leroy & De Vuyst, 2004). However, other works have indicated that the use of starter cultures reduces the production of some amines, but not that of others (Ayhan, Kolsarici, & Oscan, 1999; Maijala, Eerola, Lievonen, Hill, & Hirvi, 1995). This fact could be related to the lack of effectiveness of the starter culture depending on the hygienic quality of the raw material used (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2000). It is well known that free amino acids released by starter or endogenous proteases during ripening directly contribute to the basic taste of dry fermented sausages, and indirectly contribute to the development of their typical aroma since they are precursors of many volatile compounds. In particular, the degradation of valine, leucine and isoleucine into methyl-branched aldehydes, acids and alcohols has been linked to the ripened aroma of fermented foods (Stahnke, Holck, Jensen, Nilsen, & Zanardi, 2000). On this basis, the addition of exogenous proteases has been the most studied method for enhancing the proteolytic phenomena in dry fermented sausages and for accelerating ripening and flavour development (Fernández, Ordóñez, Bruna, Herranz, & Hoz, 2000). However, different studies have shown that an increase in the amount of free amino acids is not enough to significantly increase aroma compounds, since the mechanisms of amino acid degradation must also be favoured in order to yield higher amounts of flavour compounds (Diaz et al., 1997). For these reasons, in recent years, atten-

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.06.025

tion has been paid to amino acid catabolism in fermented foods by microorganisms, especially lactic acid bacteria (LAB), but also by other groups, such as staphylococci and fungi in dry sausages (Bruna, Fernández, Hierro, Ordóñez, & De la Hoz, 2000). Transamination and deamination reactions are among the primary phenomena through which typical ripened aroma compounds are formed, and the addition of a commercial amino acid oxidase to sausages has been shown to significantly increase both amino acid breakdown and aroma development of sausages.

It has been suggested that commercial starter cultures, mainly produced in Northern European countries, are not always able to compete well with the house flora that colonise Southern European meat plants, so that their use often results in losses of desirable sensory characteristics (Samelis, Metaxopoulos, Vlassi, & Pappa, 1998). The fitness of commercial meat starter cultures when applied to a particular type of salami is questionable since a culture that performs well in one type of fermented sausage is not necessarily efficient in another type (Garriga et al., 1996).

Several biochemical and physical changes occur during the ripening of fermented sausages that determine the flavour and odour of the end product. These changes are mainly acidification as a result of fermentation, pH decrease, changes in initial microflora, reduction of nitrates to nitrites and formation of nitrosomyoglobin, solubilisation and gelification of myofibrillar and sarcoplasmic proteins, proteolytic, lipolytic and oxidative phenomena, and dehydration (Casaburi et al., 2007; Ordoñez, Hierro, Bruna, & De la Hoz, 1999). Proteolysis is one of the main degradation mechanisms affecting proteins during the ripening of fermented sausages (Diaz et al., 1997). Proteolysis of dry cured meat products has been attributed mainly to either endogenous enzymes (Toldrá, 1998; Verplaetse, 1994) or exogenous enzymes originating from microorganisms (Mauriello, Casaburi, & Villani, 2002; Sanz et al., 1999). Nowadays, consumers pay a lot of attention to the relationship between food and health. Therefore, the market for food with health-promoting properties, the so-called functional foods, has shown a remarkable growth over the last few years.

The aim of this study was to determine the effect of the proteolytic and amino acid changes produced with and without commercial starter cultures in the manufacture of fermented sausages.

2. Materials and methods

2.1. Preparation of fermented sausages

Six separate batches of fermented sausages were prepared including a control (without starter culture), and the fermented sausages were processed: the ingredients used were lean pork, salt (2.0% w/w), glucose (1.0% w/w) and sodium nitrate (0.01% w/w) (Kanto Chemical Co. Inc., Japan). Sausage mixes were inoculated with 0.1% (v/w) of *Lactobacillus sakei* D-1001 (MMF-161; San-ei Sucrochemical Co. Ltd., Japan); 0.025% (w/w) of *Staphylococcus carnosus* (S-B-61 Bactoferm™, Chr. Hansen Inc., Denmark); 0.025% (w/w) of *Staphylococcus xylosus* (S-SX Bactoferm™, Chr. Hansen Inc., Denmark); 0.025% (w/w) of *L. sakei and S. carnosus* (F-SC-111 Bactoferm™, Chr. Hansen Inc., Denmark); and 0.025% (w/w) of *Pediococcus pentosaceus* and *S. xylosus* (F-1 Bactoferm™, Chr. Hansen Inc., Denmark). Each starter culture was used according to the respective manufacturer's instructions.

The mixtures were stuffed into a fibrous casing (40 mm in diameter), at approximately 190 g each. The processing was done at 27 °C and 80% relative humidity (RH) for 2 days; 20 °C and 80% relative humidity (RH) for 3 days; 15 °C and 75% relative humidity (RH) for 4 days, and finally 15 °C and 65% relative humidity (RH) for 12 days. Samples were taken on the 0, 1, 3, 6, 9 and 21 days of ripening for chemical and microbiological analyses.

2.2. Microbiological analysis

Ten grams of fermented sausage samples were aseptically transferred into a sterile plastic bag and were homogenised with 90 ml of sterilised saline (0.85% NaCl). The homogenate was prepared using a Stomacher 400-T (Seward, England), prior to the preparation of 1/10 serial dilutions for microbiological analysis. The following microbial parameters were determined: Total Plate Count (TPC), which was determined using Plate Count Agar (Eiken Chemical Co Ltd.) and by incubating the plates at 37 °C for 48 h; Lactic Acid Bacteria (LAB) count, which was determined by growing LAB in Man Rogosa Sharpe (Oxoid, CM361) and by incubating at 37 °C for 72 h and finally, Coliform count, which was determined using Chromocult® Coliform Agar (Merck 1.10426.0500) and by incubating the plates at 37 °C for 24 h. Tests were carried out in duplicate and the results were expressed as log cfu/g.

2.3. Physical and chemical analysis

pH was determined in slurries made from 10 g of samples in 90 ml of distilled water homogenised in a Stomacher 400-T (Seward, England) using a pH meter model HM-5S (TOA Electronics Ltd., Tokyo, Japan).

2.4. Preparation of sarcoplasmic and myofibrillar proteins

Sarcoplasmic protein extracts were prepared according to the method of Toldrá, Rico, and Flores (1993). Four grams of sausage samples were homogenised (Physcotron NS 51, Microtec Co., Ltd.) with 40 ml of 0.03 M potassium phosphate buffer (pH 7.4) for 2 min at 13,500 rpm. The homogenate was centrifuged for 20 min at 10,000g at 4 °C. The supernatant contained the sarcoplasmic proteins. Myofibrillar proteins were extracted from the resultant pellet by homogenising (Ultra Turrax) with a solution containing 8 M urea and 1% (w/v) β-mercaptoethanol for 2 min. The homogenate was recentrifuged under the same conditions and the supernatant contained the myofibrillar proteins.

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

The protein concentrations of the sarcoplasmic and myofibrillar fractions were determined by the Bradford (1976) procedure. The protein concentration was adjusted with deionised water to give a final concentration of 6 mg/ml. Samples were diluted 1:1 with SDS–PAGE sample buffer to give a final concentration of 3 mg/ml and heated at 100 °C for 5 min prior to electrophoresis. SDS–PAGE was done using a vertical gel electrophoresis unit (Mini-Protein 3 Cell. Bio-Rad, Lab. Inc) according to the method of Laemmli (1970). A 12.5% separating gel with 6% stacking gel was used for sarcoplasmic and myofibrillar proteins. Ten microlitres of the sample were injected into each well including standard markers. Electrophoresis was done at 120–150 V. After electrophoresis was complete, the gels were stained with Comassie Brilliant Blue R-250 (0.1%) in fixative (30% methanol and 10% acetic acid). The gels were destained using 5% methanol and 7.5% acetic acid. The molecular weights of the proteins were estimated by running the standard proteins of known weight in the gel. Commercial molecular weight standards (MP-0120) were obtained from Sigma Genosys and these included lysozyme (16.8 kDa); soybean trypsin inhibitor A (25.7 kDa); carbonic anhydrase II (32.7 kDa); ovalbumin (47.8 kDa); bovine serum albumin (84.2 kDa); and phosphorylase B (110 kDa). Quantification of the bands was carried out first by scanning and then by taking a densitometric reading using the ImageQuant TL (Amersham Bioscience Inc., Piscataway, NJ) program. The molecular masses of the protein bands were calculated

from the R_f values by interpolation on the calibration curve constructed using known markers.

2.6. Amino acid analysis

Samples for free amino acid analysis were prepared according to the procedures described by (Mikami, Nagao, Sekikawa, Miura, & Hongo, 1994). Samples (10 g) were homogenised (Physcotron NS 51, Microtec Co., Ltd.) for 1 min with 90 ml of distilled water. The homogenate was centrifuged for 20 min at 10,000g at 0 °C. The supernatant was filtered through Toyo filter No. 5C, (Toyo Roshi Kaisha Ltd., Japan) and the samples were diluted 1:1 with 4% trichloroacetic acid (TCA) to give a final concentration of 2%. They were then incubated at 37 °C for 30 min. The supernatant was filtered again with Toyo filter No. 5C. Thereafter, the solution was ultrafiltered through a Millipore filter having a pore diameter of 0.45 μm (Tosoh W-13-5). Twenty microlitres of each sample were analysed using a fully automated amino acid analyser HITACHI L-8800A (Hitachi Ltd., Japan).

2.7. Statistical analysis

Duncan's multiple range tests were employed to determine any significant difference between treatments (samples without and with starter cultures). Three replicates were performed and the mean values were calculated. Values were considered significantly different when $p < 0.05$. Data were analysed for degree of variation and any significant difference was determined using analysis of variance (ANOVA). All statistical analyses were performed using SPSS statistical program (Version 16 for windows, SPSS Inc., Chicago, USA, 2007).

3. Results and discussion

3.1. Microbial count in fermented sausage

The microbial changes in fermented sausages during fermentation and ripening are shown in Fig. 1. The initial Total Plate Count (TPC) ranges from 3.92 to 7.04 log cfu/g for the control and the starter culture samples, respectively, on 0 day. It increased to a maximum level in the sausages with mixed starter cultures on the 3 day of fermentation at 27 °C, and then a slight decrease was observed from day 9 to day 21 in control and samples with

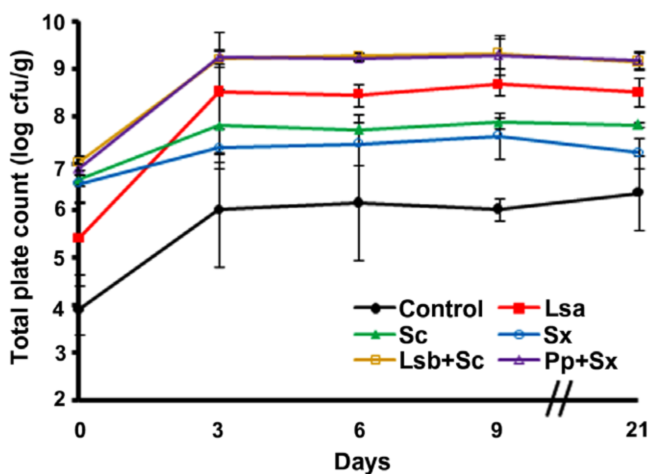

Fig. 1. Total Plate Count (TPC) in control and samples with starter cultures: (●) control (without starter culture); (■) Ls_a: (*L. sakei* D-1001); (▲) *Sc*: (*S. carnosus*); (○) *Sx*: (*S. xylosus*); (□) $Ls_b + Sc$: (*L. sakei–S. carnosus*) and (△) *Pp + Sx*: (*P. pentosaceus–S. xylosus*). The mean values were calculated from three different batches of fermented sausages ($n = 3$).

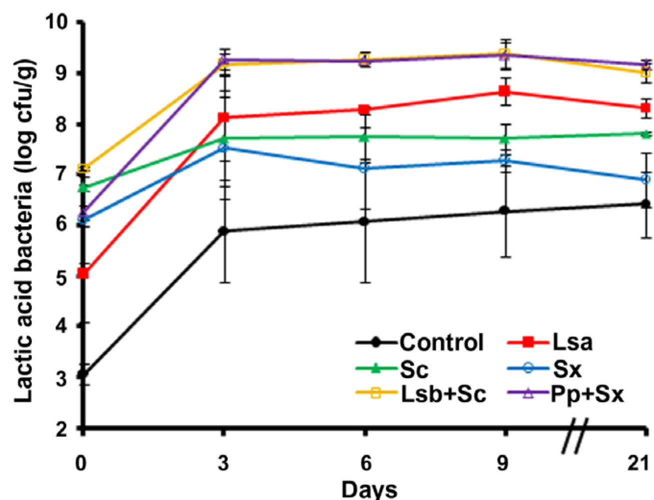

Fig. 2. Lactic Acid Bacteria (LAB) counts in control and samples with starter cultures: (●) control (without starter culture); (■) Ls_a: (*L. sakei* D-1001); (▲) *Sc*: (*S. carnosus*); (○) *Sx*: (*S. xylosus*); (□) $Ls_b + Sc$: (*L. sakei–S. carnosus*) and (△) *Pp + Sx*: (*P. pentosaceus–S. xylosus*). The mean values were calculated from three different batches of fermented sausages ($n = 3$).

starter cultures. The changes in TPC and LAB counts were similar to those reported in other studies on fermented sausages (Gökalp, 1986; Soyer, Ertaş, & Üzümcüoğlu, 2005).

The Lactic Acid Bacteria (LAB) counts are shown in Fig. 2. The initial counts in all samples ranged from 3.07 to 7.11 log cfu/g, on 0 day, and increased from approximately 6.41 to 9.18 log cfu/g at 21 days. The growth rate of the LAB in fermented sausages was consistent with the pH profile. It is well known that high acidification rates are usually accompanied by fast LAB growth rates in the fermented sausages with starter cultures including *L. sakei* D-1001; *L. sakei* + *S. carnosus*; *P. pentosaceus* + *S. xylosus*; *S. carnosus*; and *S. xylosus* and are inverse in control (without starter culture). However, increased lactic acid production by LAB in the presence of glucose has been noticed in spite of an unchanged specific growth rate, suggesting that the additional energy obtained from direct fermentation of glucose is used for functions other than growth (Guyot, Calderon, & Morlon-Guyot, 2000).

As reported by Bover-Cid et al. (2000) in some ripened meat products processed at low temperatures, fermentation is limited

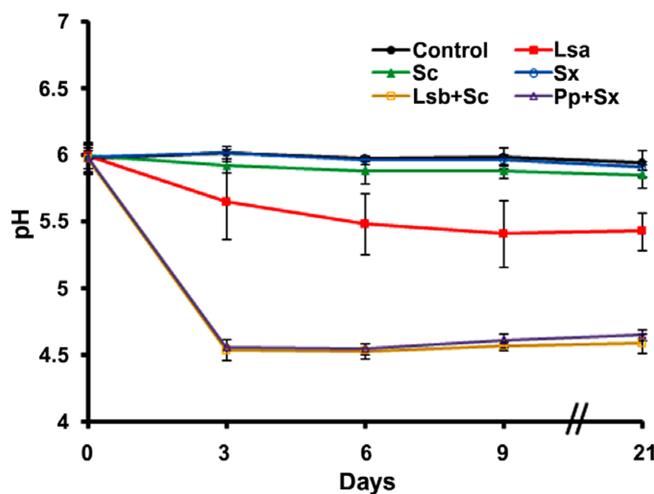

Fig. 3. pH values in control and samples with starter cultures: (●) control (without starter culture); (■) Ls_a: (*L. sakei* D-1001); (▲) *Sc*: (*S. carnosus*); (○) *Sx*: (*S. xylosus*); (□) $Ls_b + Sc$: (*L. sakei–S. carnosus*) and (△) *Pp + Sx*: (*P. pentosaceus–S. xylosus*). The mean values were calculated from three different batches of fermented sausages ($n = 3$). Standard errors ranged from 0.02 to 0.28.

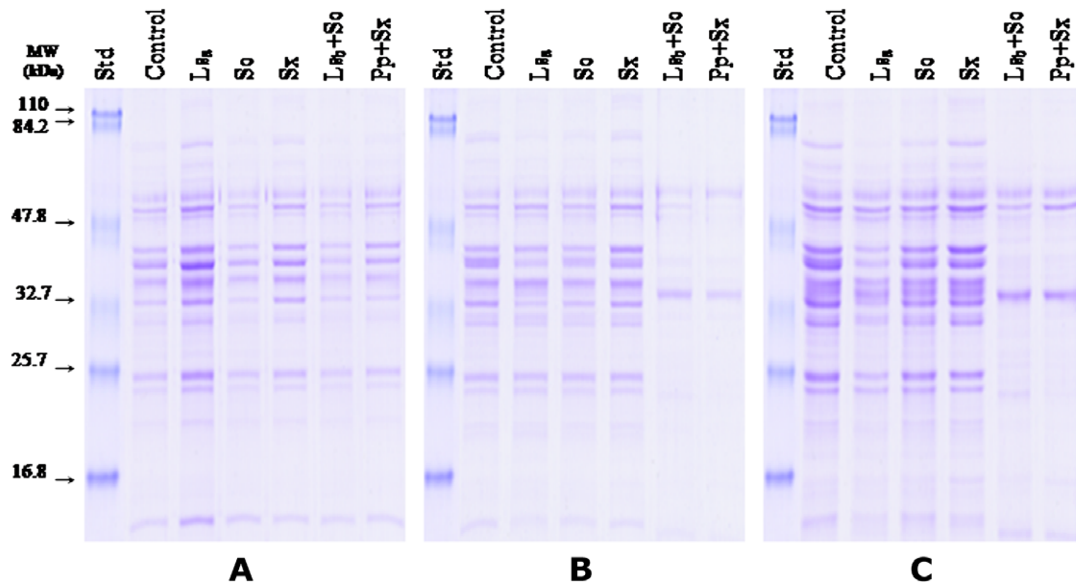

Fig. 4. SDS–PAGE profile of sarcoplasmic proteins throughout the processing and storage of fermented sausages. (A) 0 day; (B) 3 day and (C) 21 day; Std: (protein standard); control: (without starter culture); Ls_a: (*L. sakei* D-1001); *Sc*: (*S. carnosus*); *Sx*: (*S. xylosus*); $Ls_b + Sc$: (*L. sakei–S. carnosus*) and *Pp* + *Sx*: (*P. pentosaceus–S. xylosus*).

and thus the pH does not decrease by more than 0.2–0.4 units. Indeed, during the drying stage, the pH may return to values similar to those of the ripened meat due to the liberation of peptides, amino acids and ammonia from proteolytic reactions. Traditional dry sausages produced in the Massif Central (France) are dried at low temperatures and do not undergo a fermentation period (Rason, Martin, Dufour, & Lebeque, 2007).

Coliform group counts were not detected in all the samples of fermented sausages at 0–21 days, because raw material processing and product processing in this study were done in hygienic conditions.

3.2. pH of fermented sausage

The changes in pH values of the samples are shown in Fig. 3. The initial pH was approximately 6.0 on day 0 and the pH on day 21 varied from 4.59 to 5.94. There was a significant difference in pH between the control and different starter cultures treatments, there was a slight decrease in pH in the sample with starter cultures Ls_a: (*L. sakei* D-1001), $Ls_b + Sc$: (*L. sakei–S. carnosus*), and *Pp* + *Sx*: (*P. pentosaceus–S. xylosus*), the pH of these samples ranged from 6.0 to 5.06 while the pH decrease was most pronounced on the 3 day (pHs 4.54 and 4.56) and was constant on the 21 day (pH 4.60). In the samples with starter culture Ls_a: (*L. sakei* D-1001) the pH decreased after 3 days to pH 5.4 and remained constant up to the 21 day at 5.3. However, the pHs of the control (without starter culture) and samples with *Sc*: (*S. carnosus*) and *Sx*: (*S. xylosus*) remained constant at 5.8 and 5.9, respectively, until 21 days of ripening. The course of acidification differed between samples of fermented sausages but this confirmed the effectiveness of the lactobacilli in the starter cultures for acidification of sausages. Differences in acidification in starter cultures could be

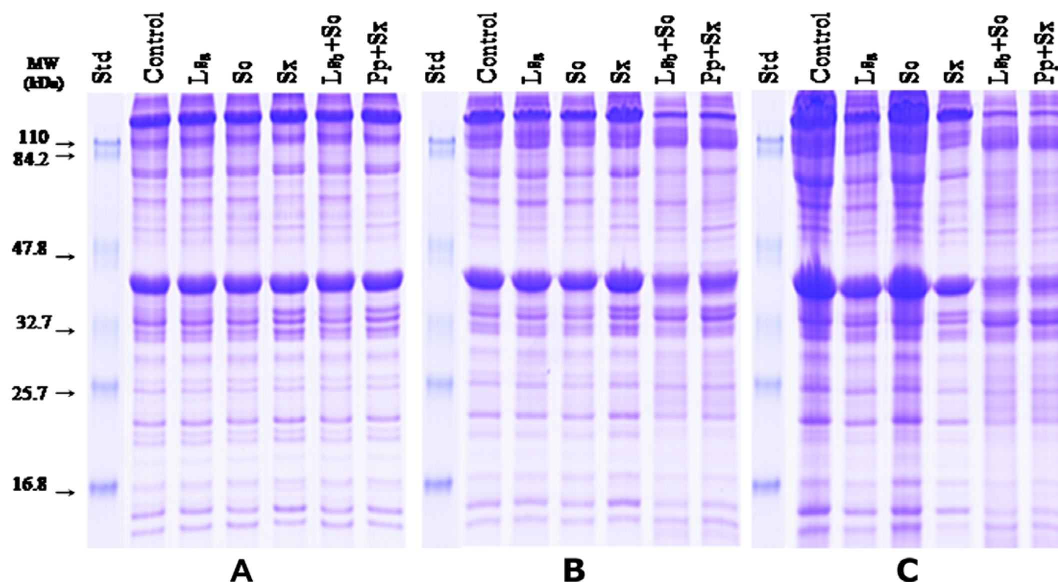

Fig. 5. SDS–PAGE profile of myofibrillar proteins throughout the processing and storage of fermented sausages. (A) 0 day; (B) 3 day and (C) 21 day; Std: (protein standard); control: (without starter culture); Ls_a: (*L. sakei* D-1001); *Sc*: (*S. carnosus*); *Sx*: (*S. xylosus*); $Ls_b + Sc$: (*L. sakei–S. carnosus*) and *Pp* + *Sx*: (*P. pentosaceus–S. xylosus*).

due to the occurrence of lactobacilli with low acidifying power, or due to a consequent loss of acidifying and deaminase activities as reported by Dellaglio, Casiraghi, and Pompei (1996). The rapid growth of LAB at the initial stage of fermentation was beneficial in decreasing the pH of the fermented sausages, which was responsible for reducing or eliminating undesirable bacteria, such as enterobacteriaceae. The reduction of enterobacteriaceae by a decrease in pH is in agreement with the results demonstrated previously (Castano, Fontan, Fresno, Tornadijo, & Carballo, 2002; Garriga et al., 1996; Hughes et al., 2002).

3.3. Proteolysis of sarcoplasmic proteins

The electrophoretic patterns of sarcoplasmic proteins in fermented sausages without and with starter cultures at various stages of fermentation and ripening are shown in Fig. 4. The greatest change in sarcoplasmic protein pattern occurred between processing days 0 (before stuffing) and 3 (during fermentation), while only small differences were observed during fermentation and drying in control and single starter-inoculated sausages. Protein bands at 36, 30, and 25.7 kDa proteins disappeared during the first 3 days in sausage with mixed starter cultures.

The band intensity of samples at 0, 3 and 21 days in the control and single starter culture show changes during fermentation and storage. After the 3 day of processing, the 36, 30, 25.7 and 20 kDa peptides bands decreased. Thereafter, these bands disappeared in samples with mixed starter cultures *L. sakei* + *S. carnosus* and *P. pentosaceus* + *S. xylosus* during fermentation and ripening of the sausages.

The findings of the present study agreed with the available literature, whereas sarcoplasmic proteins were affected by the endogenous muscle and microbiological enzymes, indicating that endogenous proteases and starter culture play a significant role in proteolysis during ripening of fermented sausages (Demeyer et al., 1995; Diaz et al., 1997; Hughes et al., 2002). On the other hand, sausages without starter culture showed a weaker activity of endogenous proteases compared with exogenous proteases reported by Santos et al. (2001) and Casaburi et al. (2007).

3.4. Proteolysis of myofibrillar proteins

SDS–PAGE electrophoretograms of myofibrillar proteins are shown in Fig. 5 for fermented sausages. The intensity of the band decreased gradually after fermentation and drying in the control and single starter culture sausages, but only a few differences were detected during fermentation and ripening in the samples with mixed starter cultures *L. sakei* + *S. carnosus* and *P. pentosaceus* + *S. xylosus*. This was probably due to the pH.

The main differences were observed in the 80, 40, 34, 30, 18 and 16.8 kDa bands between days 0 and 3. Their intensity decreased during fermentation. The 80 kDa band degraded completely in the mixed starter culture *L. sakei* + *S. carnosus* and *P. pentosaceus* + *S. xylosus* samples after the 3 day, but was not completely degraded in control and single starter culture samples of fermented sausages. The 45 kDa (actin) band intensity decreased between days 0 and 3 in the control and starter-inoculated sausages. Degradation of actin (45 kDa) during ripening of fermented sausages was in conflict with the findings of Casaburi et al. (2007), Diaz et al. (1997) and Mauriello et al. (2002), but agreed with the results reported by Garcia de Fernando and Fox (1991), Hughes et al. (2002) and Martin, Córdoba, Rodriguez, Nunez, and Asensio (2001). Tropomyosins (T1 and T2, 38 and 30 kDa) (arrows) decreased during ripening, while tropomyosin T1 and other bands such as 66 and 97 kDa accumulated during storage in control fermented sausages. This accumulation was also detected in single starter culture sausages, but was not as clear as sausages with mixed starter cultures.

Table 1
Amino acid content of samples of fermented sausages without and with starter cultures during ripening.

	0 day						3 day						21 day					
	Control	Ls_a	*Sc*	*Sx*	$Ls_b + Sc$	$Pp + Sx$	Control	Ls_a	*Sc*	*Sx*	$Ls_b + Sc$	$Pp + Sx$	Control	Ls_a	*Sc*	*Sx*	$Ls_b + Sc$	$Pp + Sx$
Asp	5.1a	3.4a	3.1a	3.4a	3.1a	2.8a	6.5a	9.4a	5.6a	6.3a	5.4a	5.4a	5.1c	16.0b	19.4ab	18.7ab	24.6a	13.1b
Thr	7.6a	5.9a	5.1a	5.3a	5.3a	5.4a	24.2a	24.0a	23.1a	25.0a	10.4b	24.7a	49.0ab	46.1b	49.6ab	51.8a	33.5c	46.0b
Ser	7.8a	6.1ab	5.7ab	5.6ab	4.9b	4.7b	29.8a	29.1a	25.8b	30.1a	17.9c	14.0d	52.3a	51.5a	50.0a	54.6a	43.1b	30.4c
Glu	23.8ab	26.6a	20.0ab	19.7ab	18.8ab	17.8b	60.3 cd	76.0b	57.0d	65.4c	96.5a	101.0a	117.5e	146.3c	127.1d	128.7d	170.1a	160.1b
Gly	14.7ab	17.9a	13.8ab	13.8ab	12.4ab	10.9b	33.0b	36.7a	31.9b	36.9a	27.1c	26.7c	59.1ab	65.2a	61.4ab	62.4ab	53.7bc	44.8c
Ala	32.4a	28.9a	28.3a	27.5a	27.8a	26.5a	75.6a	72.8ab	71.8ab	78.0a	59.3c	65.7bc	135.1a	126.5ab	124.2ab	132.5a	110.2b	106.6b
Cys	1.0a	1.1a	0.7a	1.0a	0.9a	0.8a	1.0a	1.3a	0.8a	1.1a	1.0a	1.1a	1.5a	1.2a	0.9a	1.1a	1.9a	1.3a
Val	7.3a	8.3a	5.7a	6.0a	5.5a	5.4a	26.2a	26.5a	24.4b	27.4a	20.3d	22.8c	47.5ab	50.6a	47.8ab	49.7a	47.8ab	44.0b
Met	3.1a	3.4a	2.1a	2.7a	2.6a	2.2a	12.4ab	12.2ab	11.5bc	13.0a	10.8c	11.8b	21.1b	22.1ab	21.2b	22.4ab	24.3a	23.3ab
Ile	4.1a	4.7a	2.6a	2.9a	2.6a	2.5a	17.4a	17.2a	14.2b	18.3a	12.2c	7.5d	29.2b	34.3a	32.5b	34.1a	30.9ab	20.2c
Leu	8.8a	9.8a	5.6a	5.9a	5.2a	5.2a	33.3ab	33.4ab	31.5ab	34.6a	27.9c	30.0 cd	62.4a	65.1a	57.1a	64.2a	64.5a	65.2a
Tyr	6.7a	6.1a	4.3a	4.5a	4.6a	4.6a	16.7a	16.7a	17.3a	18.7a	12.0b	15.0b	22.8a	33.4a	30.4a	32.0a	24.5a	23.0a
Phe	5.9a	6.5a	4.3a	4.9a	3.9a	4.0a	17.7c	18.1bc	17.1c	19.2bc	20.4b	22.6a	33.1b	34.6b	32.5b	33.6b	42.7a	41.1a
Lys	10.1a	11.5a	7.1a	7.1a	6.5a	6.4a	42.1a	36.5b	39.0b	43.4a	20.5c	24.3c	83.4ab	78.2ab	85.7a	86.4a	69.9bc	60.2c
His	6.6a	7.2a	4.8a	4.9a	4.7a	4.9a	17.0a	15.4b	14.6bc	16.7a	13.3c	13.6c	26.8abc	29.4ab	26.6bc	30.6a	27.8abc	25.5c
Arg	10.3a	8.9a	6.1a	6.2a	5.4a	5.9a	27.6a	21.4b	14.2c	29.0a	1.4d	2.0d	45.7a	27.3b	8.1c	45.7a	7.8c	3.4c
Pro	5.2a	5.4a	5.1a	5.2a	4.8a	5.2a	24.6a	23.2a	22.8a	24.5a	10.6b	13.3b	47.5a	40.5b	45.5a	44.8a	19.3c	17.0c
Total	160.6a	161.7a	124.4a	126.6a	118.9a	115.2a	465.2b	470.0b	422.5c	487.4a	366.9e	401.4d	839.1ab	868.4ab	820.1b	893.2a	796.5b	725.1c

Control (without starter culture), Ls_a: (*L. sakei* D-1001), *Sc*: (*S. carnosus*), *Sx*: (*S. xylosus*), $Ls_b + Sc$: (*L. sakei*–*S. carnosus*) and $Pp + Sx$: (*P. pentosaceus*–*S. xylosus*).
Results are expressed as means of three replicates in mg/100 g of fermented sausage.
a–e, Means with different letters in the same ripening stage are significantly different ($p < 0.05$) among batches.

Myosin light chains (MLC1 and MLC2, 24 and 20 kDa) also degraded and disappeared during the ripening of sausages. As reported by Diaz et al. (1997), during the fermentation and ripening of dry fermented sausages a large number of biochemical reactions associated to the degradation of myofibrillar and sarcoplasmic proteins took place. These biochemical reactions are promoted by muscle endopeptidases (calpains I and II and cathepsins B, D, H and L) as reported by Toldrá (2006. The role of muscle enzymes in dry cured meat products with different drying conditions and microbial proteinases, which are bound either to the cell wall or to the cell membrane, has been reported.

3.5. Free amino acids contents of fermented sausages

The changes in the contents of free amino acids observed in fermented sausages during ripening are given in Table 1. The total free amino acid contents of the sausages constituted 115.2/100–160.6 mg/100 g of samples (before stuffing) on 0 day. An increase in the content of amino acids was observed and ranged between 366.9 and 487.5 mg/100 g during the fermentation on day 3, and a further increase up to the range of 725.1–893.2 mg/100 g of total free amino acids was observed at the ripening stage of fermented sausages (21 days). The highest total free amino acid concentration of 893.2 mg/100 g was observed with a single starter culture *Sx*: (*S. xylosus*), whereas the lowest total free amino acid concentration of 725.1 mg/100 g was observed with a mixed starter culture *Pp* + *Sx*: (*P. pentosaceus*–*S. xylosus*). The hydrolysis of meat proteins generates polypeptides that can be further degraded to smaller peptides and free amino acids. This degradation can be produced by endogenous and microbial enzymes as reported by different authors (De Masi, Wardlaw, Dick, & Acton, 1990; Hughes et al., 2002; Molly et al., 1997). The increase in the total free amino acid concentration was detected in all batches as reported by Hierro, De la Hoz, and Ordóñez (1999), Bruna et al. (2000), Bolumar, Nieto, and Flores (2001) and Hughes et al. (2002). The main differences in the content of total free amino acids among batches were detected on day 3 and at the end of the processing on day 21. The amino acids in which differences, which were primarily responsible for the increase in total free amino acids during ripening, were observed were Glu (glutamic acid), Ala (alanine) and Lys (lysine) in the samples with starter cultures Ls_a: (*L. sakei* D-1001), *Sc*: (*S. carnosus*), Ls_b + *Sc*: (*L. sakei*–*S. carnosus*) and *Pp* + *Sx*: (*P. pentosaceus*–*S. xylosus*). Ala (alanine) was high in samples with *Sx*: (*S. xylosus*) as a starter culture, and control (without starter culture). Mateo, Domínguez, Aguirrezábal, and Zumalacárregui (1996) reported an increase in the total free amino acid content during the ripening of chorizo which was similar to the results reported in the present work. The change occurred during fermentation and ripening process indicating that the highest enzymatic activity took place during these stages (Verplaetse, De Bosschere, & Demeyer 1989). Several authors have reported a major release of free amino acids at the beginning of the process in coincidence with the fermentation stage (Diaz et al., 1997). This increase has been attributed to the higher temperatures applied during fermentation compared to the low temperature applied during drying. The most significant decreases occurred in the content of Arg (arginine) in the sample with *Pp* + *Sx*: (*P. pentosaceus*–*S. xylosus*), as a starter culture. The decrease in the content of amino acids may indicate their metabolism by bacteria (Bover-Cid et al., 2000; Ordoñez et al., 1999; Sekikawa et al., 2003).

4. Conclusion

We examined the effects of five types of commercial starter cultures on the proteolytic profiles of fermented sausages. Two types of starter cultures *L. sakei*–*S. carnosus* and *P. pentosaceus*–*S. xylosus* were responsible for degradation of sarcoplasmic and myofibrillar proteins during fermentation and drying stages of sausages. This degradation was confirmed by reduced intensity of protein band on SDS–PAGE profiles. Quantitative analysis showed that another starter culture *S. xylosus* alone produced significantly higher amino acid content than all other starter cultures, the high differences in the content of free amino acids at the end of the process could be attributed to the starter culture activity. These differences might affect organoleptic characteristics.

Acknowledgement

This study was supported in part by a grant the 21 Century COE Program (A-1), from the Ministry of Education, Culture, Sports, and Technology, Japan.

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