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Effect of *Debaryomyces* spp. on the proteolysis of dry-fermented sausages

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Abstract

The effect of the addition of *Debaryomyces* spp. on the microbial, chemical properties and degradation of meat proteins in dry fermented sausages was investigated. The manufacture of dry fermented sausages with *Debaryomyces* spp. produced a slow decline in pH during early drying stage. However, the final product had lower ammonia, and higher acetic and D-lactic acids without producing any effect on the final pH. Sarcoplasmic proteins were not affected by *Debaryomyces* spp. but the degradation of myofibrillar proteins was accelerated at the beginning of the drying stage even though the final sausage, inoculated with *Debaryomyces* spp., had lower myofibrillar proteolysis. The content of free amino acids was similar at the beginning of the drying 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 *Debaryomyces* spp. activity. The addition of a higher amount of *Debaryomyces* spp. did not contribute to a major proteolysis degree.

Keywords: Fermented sausage; Yeast; Amino acid; Myofibrillar and sarcoplasmic proteins; Proteolysis

1. Introduction

The use of yeasts contributes to the development of the typical sausage flavour through its high lipolytic activity (Sørensen, 1997; Sørensen & Samuelsen, 1996) but there is a poor knowledge on the contribution of its proteolytic activity. The yeast microbiota mainly found in sausages are *Debaryomyces*, *Rhodotorula*, *Hansenula*, and *Torulopsis* (Comi & Cantoni, 1980). Generally, these microorganism are frequently found on the surface of the sausage (Jessen, 1995). In Spanish dry-fermented sausages (salchichón), the most abundant genus is *Debaryomyces*, followed by *Rhodotorula*, *Candida*, *Pichia*, *Yarrowia* and *Trichosporon* (Santos Mendoça, 2000). However, the composition and development of the mycoflora are dependent on the nature of the product, the processing time and the ripening conditions (Ordóñez, Hierro, Bruna, & de la Hoz, 1999; Toldrá, Sanz, & Flores, 2001).

Encinas, López Díaz, García López, Otero, and Moreno (2000) found *Debaryomyces hansenii* as the dominant strain in Spanish fermented products, being also present in all the stages of processing. However, it has been reported that *D. hansenii* population is generally reduced during processing (Gehlen, Meisel, Fischer, & Hammes, 1991; Olesen & Stahnke, 2000). Large-diameter sausages have experienced a reduction in sensory quality due to its noticeable sour taste that it is developed during processing (Flores & Bermell, 1996). Yeasts have been reported to be able to increase the ammonium content and reduce the amounts of lactic and acetic acids, with the concomitant suppression of the acid taste (Gehlen et al., 1991; Miteva, Kirova, Gadjeva, & Radeva, 1986). Better sensory characteristics have been observed when *Debaryomyces* spp. is used in combination with *Lactobacillus curvatus* and *Kocuria varians* (Gehlen et al., 1991). It must be taken into account that yeasts can inhibit the indigenous staphylococci and therefore, it is necessary to use them together with a microorganism exhibiting nitrate reductase activity in order to avoid colour defects.

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Endogenous proteinases, such as cathepsin D, have been reported as responsible for proteolysis during the fermentation stage while bacterial proteinases contribute to proteolysis during drying stages (Verplaetse, Demeyer, Gerard, & Buys, 1992). However, the contribution of *Debaryomyces* spp. to the proteolysis of fermented sausages and its effect on sensory characteristics has been scarcely studied (Olesen & Stahnke, 2000) even though *Debaryomyces* has been proved to be active against sarcoplasmic muscle proteins (Santos et al., 2001) and some of its exopeptidases have been recently purified and characterised (Bolumar, Sanz, Aristoy, & Toldrá, 2003a, 2003b).

The objective of this work was to study the effect of *Debaryomyces* spp., used with starter cultures (lactic acid bacteria and *staphylococci*), in the manufacture of dry fermented sausage and its effect on meat proteolysis in order to better understand its role on flavour generation during the processing of dry-fermented sausages.

2. Materials and methods

2.1. Preparation of Debaryomyces spp.

Debaryomyces spp. CECT 11815 isolated from Spanish fermented sausages (Santos Mendoça, 2000) was used. It was grown at 27 °C during 3 days in Erlenmeyer flasks (250 mL) containing 100 mL of an static medium composed of meat extract (Scharlau Chemie S.A., Barcelona, Spain) (10 g/L), glucose (10 g/L), DL-lactic acid (10 g/L) and adjusted to pH 6.7. The cells were obtained by centrifugation (8000*g*, 10 min at 4 °C), washed with water and diluted in an equal volume of water per gram of wet cells. This suspension was stored at −80 °C until used. The suspension was thawed at 25 °C and the yeast population was calculated by comparison with the absorbance at 650 nm in a spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Uppsala, Sweden).

2.2. Preparation of dry fermented sausages and sampling

Three different batches of fermented sausage were produced. A reference batch and two batches of similar composition to the reference, but with the addition of different amounts of *Debaryomyces* spp. plus the starter culture containing *Lactobacillus sakei*, *Pediococcus pentosaceus*, *Staphylococcus xylosus* and *St. carnosus* (Rhodia Iberia, groupe Rhône-Poulenc).

The dry fermented sausage consisted of lean pork (80%) and pork back fat (20%) and the following additives (in g/kg of meat mixture) were added: Sodium chloride (28), lactose (10), dextrin (15), sodium caseinate (20), glucose (7), sodium ascorbate (0.5), sodium nitrite (0.15) and potassium nitrate (0.20). The meat was ground through a plate of 6 mm hole diameter, vacuum minced with the remaining ingredients and inoculated with a starter culture (Rhodia Iberia, groupe Rhône-Poulenc) containing *L. sakei*, *P. pentosaceus*, *S. xylosus* and *St. carnosus* being the reference batch. For the yeast added batches, appropriate volumes of *Debaryomyces* spp. CECT 11815 suspensions were added to a final population of 5×10^6 cfu/g for batch L1 and 15×10^6 cfu/g for batch L2 and mixed with the starter culture mentioned above.

The mixture of each batch was stuffed into collagen casings (75–80 mm diameter), being 500 g the final mass of each sausage. The sausages were kept at 3–5 °C during 24 h in a refrigeration chamber. The fermentation stage was done at 24 °C and 90–80% Relative Humidity (RH) for 12 h and then, the temperature was lowered to 20 °C and 90–80% RH for an additional 12 h. Finally, the sausages were dried at 10 °C and 90–75% RH until the end of the ripening process. The total time of the process was 35 days with weight losses of about 45%.

Six sausages were collected at day 0, 6, 21 and 35 (finished sausage) from each batch for microbial, chemical, and proteolysis analyses. All the results were expressed as means of the six replicates per 100 g of dry matter at each processing time and batch.

2.3. Microbial analysis

Sausage samples (20 g) were homogenized with 180 ml of peptone water in a Stomacher Lab-Blender 400 (Seward Medical, London, UK) for 1 min and decimal dilutions prepared.

The yeast concentration was determined by plate counting onto Rose Bengal agar (Scharlau Chemie S.A., Barcelona, Spain) after incubation at 27 °C for 3 days. Lactic acid bacteria counts were determined by the overlay technique using MRS agar (Scharlau Chemie S.A., Barcelona, Spain) and colonies counted after incubation at 30 °C for 3 days. The number of *staphylococci* were determined on Mannitol Salt agar (Scharlau Chemie S.A., Barcelona, Spain) after incubation at 30 °C for 3 days, and colonies were counted.

2.4. Chemical analysis

Moisture content of fermented sausages was determined after dehydration at 100 °C to a constant mass (ISO, 1973). The pH was measured introducing a pH meter FC200B (Hanna Instruments Inc., Woonsocket, USA) in the meat mixture before stuffing and in the centre of the sausages at each processing time.

Ammonia, acetic acid, D- and L-lactic acid concentrations were determined using the same extraction procedure. Five grams of the frozen sausage mixture were homogenized in 20 mL of 1 M perchloric acid by 4

strokes of 30 s in a polytron PTA 10-35 (Kinematica GmbH, Luzern, Switzerland) while cooling in ice. The mixture was adjusted to pH 7–7.5 using KOH, rinsed to 100 mL with water and left at 4 °C during 20 min for potassium perclorate precipitation (Aristoy & Toldrá, 1991). After filtering through glass wool, the sample was kept at 4 °C for ammonia and acetic acid determinations. For DL-lactic acid determination, 25 mL of the filtrate were adjusted to pH 10–11 with KOH and rinsed to 50 mL with water. After 20 min at 4 °C, the mixture was filtered through glass wool and the filtrate used for D- and L-lactic acid determinations.

Ammonia concentration was determined using the method of Bergmeyer and Beutler (1985) as described by Durá, Flores, and Toldrá (2002). Acetic acid concentration was performed by the method of Beutler (1984). D- and L-lactic acids were determined by the method of Gawehn (1984) for D-lactic acid and Noll (1984) for L-lactic acid, both combined and with slight modifications. The reaction mixture consisted of reaction buffer 250 mM glycolglycine, 40 mM glutamate, pH 10 containing 4 mM NAD+ and 14 U/mL of glutamate–piruvate transaminase and a solution of sample. 50 U/mL of D-lactate dehydrogenase were added to this mixture to determine the D-lactate that is equivalent to the concentration of NADH generated, measured by the increment in absorbance at 340 nm in a spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Uppsala, Sweden). Once the reaction was finished, 50 U/mL of L-lactate dehydrogenase were added to the mixture to measure the concentration of L-lactate in the same way as for the D-lactate.

2.5. Protein analysis

The extraction of sarcoplasmic and myofibrillar proteins was done by the procedure described by Molina and Toldrá (1992). Ten grams of the frozen sausage mixture were homogenised in a stomacher Lab-blender 400 (Seward Medical, London, UK) with 100 mL of 30 mM phosphate buffer, pH 7.4 during 4 min. The mixture was centrifuged (10,000*g* during 20 min at 4 °C) and the supernatant used for analysis of sarcoplasmic proteins. The procedure was repeated twice and the pellet was finally resuspended in 9 vol of 100 mM phosphate buffer, pH 7.4 containing 0.7 M potassium iodide. The mixture was homogenised in a stomacher for 8 min and then centrifuged at 10,000*g* for 20 min at 4 °C. The protein concentration of both supernatants containing sarcoplasmic and myofibrillar proteins was determined by the method of Smith et al. (1985) using bichinchoninic acid as reagent and bovine serum albumin as standard.

The analysis of proteins was done by SDS–PAGE using the method described by Toldrá, Miralles, and Flores (1992). The sarcoplasmic and myofibrillar extracts were mixed in a ratio 1:1 with 50 mM Tris–buffer, pH 6.8, containing 8 M urea, 2 M thiourea, 75 mM dithiothreitol, 3% (w/v) SDS and 0.05% bromophenol blue. The mixture was heated at 100 °C for 4 min and used for electrophoresis. The amount of protein injected into the electrophoresis gels was 12 μg in each lane. Ten percent SDS–PAGE gels were prepared and stained with coomassie brillant blue R-250 (Laemmli, 1970). Standards proteins from BioRad (Hercules, CA, USA) were run simultaneously for molecular mass identification.

The stained gels were analysed by using an image analyser LAS-1000 plus (Fuji Photo Film Co., Tokyo, Japan) and the intensity and relative retention of the bands were determined using the software Multi-Gauge V1.01. For each stage of ripening and batch, three samples were analysed and the results were expressed as a percentage of the total intensity of the proteins present in a lane.

2.6. Analysis of free amino acids

Samples for free amino acid analysis were extracted and deproteinized following the method described by Aristoy and Toldrá (1991). Thus, frozen sausage samples were homogenised with 0.01 M HCl (dilution 1:5) in a Stomacher for 8 min at 4 °C and centrifuged in cold at 10,000*g* for 20 min. Supernatant was filtered through glass wool and stored at −20 °C until use. Thawed samples (300 μl) plus 50 μl of an internal standard (10 mM norleucine) were deproteinised with 750 μl of acetonitrile. The supernatant was derivatised to its phenylthiocarbamyl derivatives according to the method of Bidlingmeyer, Cohen, Tarvin, and Frost (1987). Derivatised samples were analysed in a 1050 Hewlett Packard (Palo Alto, CA, USA) HPLC system with a variable UV detector at 254 nm in a Nova-Pack C-18 column (3.9×300 mm) (Waters Co, MA, USA). The separation was achieved in 65 min at 52 °C, using a gradient between two solvents: 70 mM sodium acetate pH 6.55 containing 2.5% acetonitrile (solvent A) and water:acetonitrile:methanol, 45:40:15, v/v (solvent B) as described by Flores, Aristoy, Spanier, and Toldrá (1997).

2.7. Statistical analysis

The effect of processing time and the addition of different amounts of yeast on the variables studied (microbial, chemical, proteins, peptides and free amino acids) was done by a two factor analysis of variance using the statistic software Statgraphics plus (v 2.0). In those cases where the effect of the factors or their interactions was significant, the means were compared using Fisher's least significant difference (LSD) procedure.

3. Results and discussion

3.1. Microbiological analysis

The evolution of microflora during the process in presented in Fig. 1. In yeast inoculated batches, a reduction of the yeast population was observed during the whole process however this reduction was most pronounced during the first 6 days of processing as also reported by Encinas et al. (2000). The number of lactic acid bacteria and staphylococci were within the range of what could be expected in dry fermented sausages (Santos Mendoça, 2000). A greater inhibition of the staphylococci growth was observed in yeast inoculated batches (Fig. 1(c)) as also suggested Gehlen et al. (1991). In the control batch, the number of staphylococci experienced a slight decrease during the first 6 days of processing while a reduction was observed in L1 and most dramatically in L2.

3.2. pH, moisture and compositional analysis

Results of pH and moisture analyses are shown in Fig. 2. The pH showed a reduction in all three batches during processing although the control sausages showed a stronger decrease, that was statistically different, in the first 6 days of processing (Fig. 2(a)), probably due to lactic acid consumption by the inoculated yeast. However, the pH declined until half of the processing stage (21 days) and its values were maintained in all batches until the end of the processing. The moisture content decreased from initial values of 66–68% to 38–40% along the ripening process (Fig. 2(b)).

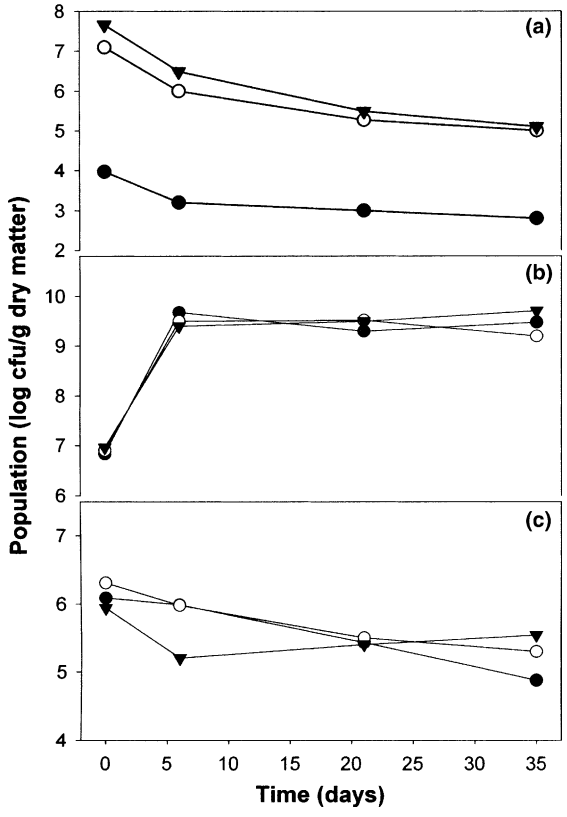

Fig. 1. Changes in microbial populations during the processing of dry fermented sausages. Yeast (a), lactic acid bacteria (b) and *staphylococci* (c). In Control batch C (●), batch L1 with *Debaryomyces* spp. (○) and batch L2 with *Debaryomyces* spp. (▼).

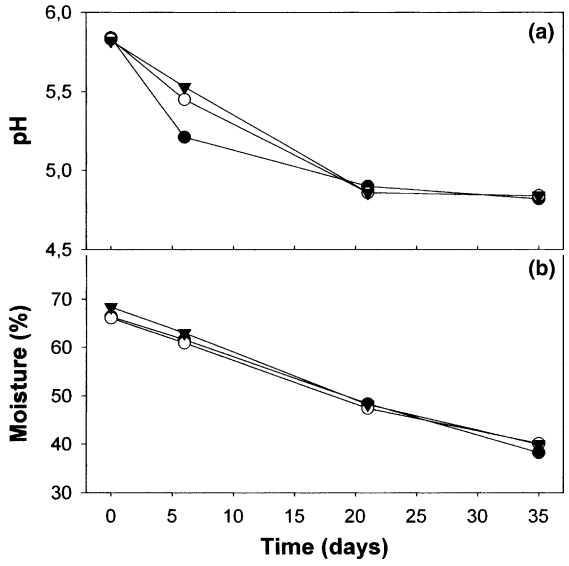

Fig. 2. Changes in pH (a) and moisture (b) during processing of dry fermented sausages. In Control batch C (●), batch L1 with *Debaryomyces* spp. (○) and batch L2 with *Debaryomyces* spp. (▼).

The results of the ammonia, acetic acid and D, L-lactic acids contents are presented in Table 1. All these compounds showed an increase during processing. Ammonia content increased specially during the first 6 days of processing. However, ammonia content showed a significant ($p < 0.05$) higher increase in the control than in the inoculated batches during the drying period (days 21 and 35 of processing). The ammonia content detected in the three batches is higher than those reported by other authors for similar products but using different starters (Bolumar, Nieto, & Flores, 2001a; Bruna, Fernández, Hierro, Ordóñez, & de la Hoz, 2000a, 2000b).

The evolution of acetic acid concentration during processing showed different behaviour depending on the studied batch. All batches showed an increase in acetic acid concentration until half of the drying period (21 days). After this time, in the control and L2 batches acetic acid concentration decreased until the end of the drying process although the L1 batch maintained its concentration. However, at the end of the processing (35 days), the two batches inoculated (L1 and L2) showed significantly ($p < 0.05$) higher acetic acid concentrations than the control batch. The generation of acetic acid can be due to any of the microorganims present in the fermented sausages. The production of acetic acid during

Table 1
Changes in ammonia, acetic acid and D,L-lactic acids during processing of dry fermented sausages

Stage	Batch	Concentration (mg/100 g dry matter)			
		Ammonia	Acetic acid	D-lactic acid	L-lactic acid
0 day	C	18^{1}	$2{,}9^{1}$	89^{1}	888^{1}
	L1	11^{1}	$1{,}3^{1}$	59^{1}	1268^{1}
	L2	14^{1}	$0{,}0^{1}$	88^{1}	1047^{1}
6 days	C	43^{2}	$32{,}3b^{2}$	1235^{2}	1675^{12}
	L1	40^{2}	$23{,}3a^{2}$	1012^{2}	1611^{1}
	L2	42^{2}	$49{,}3c^{2}$	1183^{2}	1185^{1}
21 days	C	$59b^{3}$	$62{,}7a^{4}$	1790^{3}	$2173a^{2}$
	L1	$50a^{3}$	$72{,}0b^{3}$	1825^{3}	$2546ab^{2}$
	L2	$55ab^{3}$	$84{,}3c^{4}$	1807^{3}	$3072b^{2}$
35 days	C	$85b^{4}$	$53{,}2a^{3}$	$1443a^{2}$	3194^{3}
	L1	$73a^{4}$	$72{,}1b^{3}$	$2129b^{4}$	3510^{3}
	L2	$69a^{4}$	$76{,}7b^{3}$	$2023b^{3}$	3211^{2}
P stage × batch		>0.05	<0.001	<0.001	>0.05

a–c, Different letters in the same ripening stage shows significant differences ($p < 0.05$) among batches.
1–4, Different numbers in the superscript shows significant differences ($p < 0.05$) among ripening stages of the same batch.

processing is similar to those reported by other authors (Bolumar, Aristoy, & Toldrá, 2001b; Bruna et al., 2000a, 2000b) and the content in the final products are within the typical range of values for fermented sausages (Montel, Talon, Berdagué, & Cantonnet, 1993; Gehlen et al., 1991).

The content of D-lactic acid during processing showed an increase until half of the drying process (21 days). After this time, a reduction was observed in the D-lactic acid content in the control bacth. Finally, at the end of the drying process (35 days), the two inoculated batches (L1 and L2) showed a significantly ($p < 0.05$) higher concentration than the control batch, indicating a clear effect of the inoculated yeast on the concentration of D-lactic acid. On the other hand, L-lactic acid concentration also showed an increase in concentration during processing. However, the unique difference observed was at 21 days of processing. The lactic acid content obtained in the final fermented sausages was higher than the values reported by other authors (Bruna et al., 2000a, 2000b; Gehlen et al., 1991; Johansson, Berdagué, Larsson, Tran, & Borch, 1994; Montel et al., 1993). These results are in contrast with those obtained by Gehlen et al. (1991) who reported an increase in ammonia concentration and a reduction in D,L-lactic acids producing an increase in pH values in fermented sausages inoculated with *D. hansenii*. Furthermore, Encinas et al. (2000) observed a higher increase in lactic acid in sausages with lower yeast counts. Our results showed a higher ammonia and lower lactic acid contents in the control batches than in the *Debaryomyces* spp. inoculated batches, although the final pH was not significantly ($p > 0.05$) different among batches. Therefore, the final pH of dry fermented sausages must be affected by other factors additional to the ammonia and lactic acids contents. In this sense, Demeyer (1992) indicated that the rate of pH decline is related to the interaction between sugar and protein metabolism. An increase in pH values during the drying process has been reported as a consequence of the ammonia and amine generation (Hughes et al., 2002), proteolytic activity of endogenous cathepsins (Verplaetse, 1994), extra cellular protease activity of lactic acid bacteria (Cocolin, Manzano, Cantón, & Comi, 2001) and lactic acid consumption by yeast (Encinas et al., 2000).

3.3. Protein degradation

Sarcoplasmic and myofibrillar proteins were followed by SDS–PAGE in the three batches (C, L1 and L2) at various stages of ripening as shown in Figs. 3 and 4, respectively. The hydrolysis of sarcoplasmic proteins in the three batches were very similar and main changes were observed by 21 days due to the decrease of several bands such as those with MW 97, 60, 43, 35 and 18 kDa and the increase of other bands with MW of 39, 25 and 20 kDa (Fig. 3). At the end of the ripening process (35 days), the hydrolysis of the sarcoplasmic proteins was very similar in the three batches with small differences detected by image analysis of the bands (data not shown). However, the addition of a higher amount of *Debaryomyces* spp. in batch L2 did not produce a different proteolytic behaviour as expected and as also reported by Martín et al. (2002).

The hydrolysis of myofibrillar proteins showed differences among batches. The main differences were observed in the bands corresponding to myosin (200 kDa, A in Table 2 and Fig. 4) and actin (45 kDa, and C in Table 2 and Fig. 4) although other differences were observed in myofibrillar proteins as shown in Table 2. The

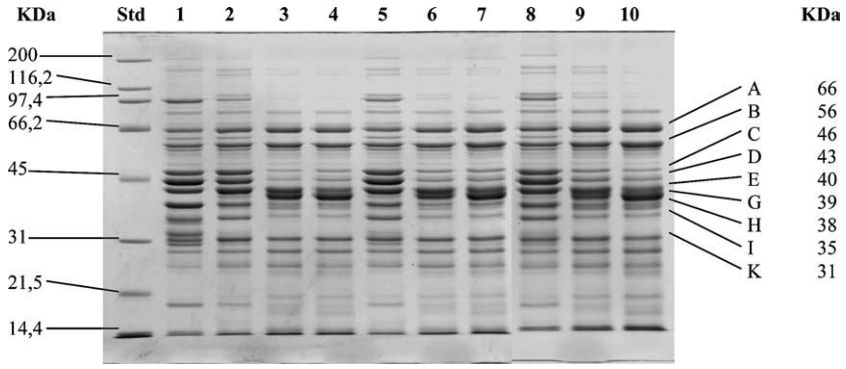

Fig. 3. 10% SDS–PAGE of sarcoplasmic proteins from Control (lanes 2–4), L1 (lanes 5–7) and L2 (lanes 8–10) batches during ripening stages. Std, standards, lanes: (1) Initial (0 days), (2) C-6 days, (3) C-21 days, (4) C-35 days, (5) L1-6 days, (6) L1-21 days, (7) L1-35 days, (8) L2-6 days, (9) L2-21 days, (10) L2-35 days.

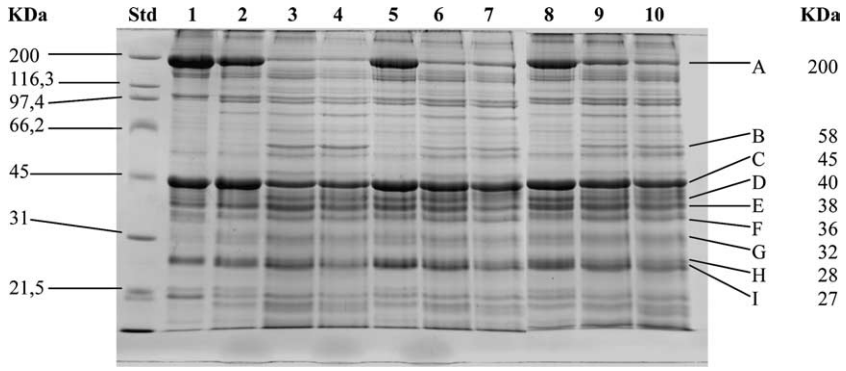

Fig. 4. 10% SDS–PAGE of myofibrillar proteins from Control (lanes 2–4), L1 (lanes 5–7) and L2 (lanes 8–10) batches during ripening stages. Std, standards, lanes: (1) Initial (0 days), (2) C-6 days, (3) C-21 days, (4) C-35 days, (5) L1-6 days, (6) L1-21 days, (7) L1-35 days, (8) L2-6 days, (9) L2-21 days, (10) L2-35 days.

differences among batches were observed after the first 6 days of processing as observed by a lower intensity of myosin (200 kDa) and actin (45 kDa) bands in the yeast inoculated batches (L1 and L2) than in the control batch. This hydrolysis, especially by cathepsins B and L, can be favoured by the pH that was significantly higher in the inoculated batch than in the control batches. Furthermore, the hydrolysis can be produced by *Debaryomyces* spp. as confirmed Rodríguez, Núñez, Córdoba, Bermúdez, and Asensio (1998) who found an important myosin and actin hydrolysis in several *Debaryomyces* strains. However, at the end of the processing (35 days) the proteolysis was higher in the control than in L1 and L2 batches. The addition of higher amounts of *Debaryomyces* spp. did not contribute to extended proteolysis. The contribution of the yeast in the first 6 days of processing is in contrast to the results obtained by Martín et al. (2002) who reported an absence of proteolysis in the presence of *Debaryomyces* spp.

3.4. Free amino acid generation

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 (DeMasi, Wardlaw, Dick, & Acton, 1990; Hughes et al., 2002; Molly et al., 1997). The contribution of microrganisms to the proteolysis can be related to changes in the sausage flavour. Carboxy and aminopeptidase activities have been detected in *Debaryomyces* and therefore, this proteolytic activity can affect the final flavour of the product (Bolumar et al., 2001b, 2003a; Santos et al., 2001).

Results of free amino acid generation and related compounds during processing of the control and yeast inoculated sausages are presented in Table 3. The increase in the total free amino acid concentration was detected in the three batches as also reported by Hierro, de la Hoz, and Ordóñez (1999), Bruna et al. (2000a, 2000b), Bolumar et al. (2001a) and Hughes et al. (2002). The main differences in the content of total free amino acids among batches were detected in the middle (21 days) and at the end of the processing (35 days), where lower quantities were detected in the L1 batch than in the other batches.

The differences observed for each individual free amino acid during processing were remarkable also at

Table 2
Evolution of myofibrillar proteins during ripening of dry fermented sausages expressed as percentage of the total intensity of the proteins present in the lane

Stage	Batch	Band (kDa)								
		A (200)	B (58)	C (45)	D (40)	E (38)	F (36)	G (32)	H (28)	I (27)
0 day	T	26.0ab^{3}	0.0^{1}	15.6^{2}	2.2^{1}	4.6^{1}	2.3^{1}	0.9	6.9^{2}	0.0^{1}
	L1	26.5b^{4}	0.0^{1}	16.93	2.3^{1}	5.0^{1}	2.3^{1}	0.7^{1}	7.0^{2}	0.0^{1}
	L2	24.4a^{4}	0.0^{1}	16.4^{2}	2.7^{1}	5.4^{1}	3.1^{1}	1.2^{1}	7.4^{3}	0.0^{1}
6 days	T	15.4c^{2}	0.0^{1}	19.4b^{3}	2.1^{1}	6.3^{2}	4.0a^{2}	3.6^{2}	5.2b^{2}	2.8a^{2}
	L1	14.0b^{3}	0.2^{1}	15.5a^{23}	2.2^{1}	6.5^{2}	4.5ab^{2}	3.4^{2}	3.8a^{1}	5.3b^{3}
	L2	11.5a^{3}	0.4^{1}	14.7a^{2}	2.1^{1}	7.0^{2}	4.7b^{2}	4.1^{2}	5.2b^{2}	4.7b^{2}
21 days	T	1.7a^{1}	2.0b^{2}	10.0a^{1}	7.1ab^{2}	7.8a^{3}	4.9a^{3}	4.8^{3}	6.0b^{2}	7.0b^{4}
	L1	2.6a^{1}	1.2a^{2}	13.1b^{1}	6.2a^{2}	9.4b^{3}	5.6b^{3}	4.7^{3}	5.5b^{2}	7.9b^{4}
	L2	5.8b^{2}	1.2a^{2}	14.9c^{2}	7.4b^{3}	8.3a^{3}	4.8a^{2}	4.1^{2}	3.3a^{1}	3.8a^{2}
35 days	T	1.7a^{1}	2.0b^{2}	11.4a^{1}	8.0b^{2}	8.1b^{3}	4.3^{23}	4.5a^{23}	3.3^{1}	4.0^{3}
	L1	3.9b^{2}	1.3a^{2}	13.8b^{12}	6.0a^{2}	5.8a^{12}	4.5^{2}	5.9b^{4}	3.9^{1}	3.9^{2}
	L2	2.8ab^{1}	1.4a^{2}	12.8ab^{1}	5.6a^{2}	5.4a^{1}	4.4^{2}	5.9b^{3}	4.2^{12}	3.7^{2}
P Stage × Batch		<0.001	>0.05	<0.001	<0.01	<0.001	>0.05	<0.05	<0.01	<0.001

a–c, Different letters in the same ripening stage shows significant differences ($p < 0.05$) among batches.
1–4, Different numbers in the superscript shows significant differences ($p < 0.05$) among ripening stages of the same batch.

21 and 35 days. After 6 days of processing, higher quantities of Glu and Orn were detected in the control than in yeast inoculated batches ($p < 0.05$). However, many free amino acids showed significant differences among batches at the middle and end of processing. Therefore, the effect of *Debaryomyces* spp. in the generation of free amino acids has to be analysed at the end of processing. In this case, the higher pH of the yeast inoculated batches at 6 days of processing does not affect the enzymes involved in the generation of free amino acids but could affect the generation of their substrates.

Several authors have reported major release of free amino acids at the beginning of the process in coincidence with the fermentation stage (Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1993). This increase has been attributed to the higher temperatures applied during fermentation compared to the low temperature applied during drying (Díaz et al., 1993). In this experiment, the highest increment was also detected after 6 days of processing that is the beginning of the drying stage. After this time, the increase in amino acids concentration depended on the batch and specific amino acid studied but, in general, as deduced from the total amino acid concentration only batch L2 showed a significant increase ($p < 0.05$) at 21 days and in the control batch a significant increase ($p < 0.05$) was detected after 35 days of processing. The significant ($p < 0.05$) reduction in the concentration of free amino acids in batch L2 can be produced by a more intense microrganism metabolism than their production during the latter stages of ripening as suggested by Hughes et al. (2002) and Ordóñez et al. (1999).

The main changes observed in free amino acids at the end of processing showed a higher proportion of Glu and Ser in the control than in yeast inoculated batches and also larger quantities of Asp, Asn, Gly, Gln, Ala, Pro, Tyr, Orn, His, Thr, Val, Met, Lys, β-Ala and Car in control than in L1 batch. The L1 batch did not show a higher quantity of any of the analysed free amino acids. The contents of Arg, Leu, Phe and Ans were similar in the three batches at the end of processing. The addition of *Debaryomyces* spp. produced a limited effect on the free amino acid generation although the effect was different depending on the quantity of yeast inoculated. Many factors can affect the generation of free amino acids such as the presence of different substrates, the pH, the presence of different microorganisms and their evolution during processing. It was remarkable that the large amounts of hydrophobic amino acids, usually associated with bitter taste, generated during processing such as Met, Val, Leu, Ile, Phe and Trp (MacLeod, 1994) were generally higher in control and L2 than in L1 batches. The significant proportions of hydrophobic amino acids during sausage ripening has been reported by numerous authors (Ansorena, Zapelena, Astiasarán, & Bello, 1998; Hughes et al., 2002). Some of these amino acids, especially those branched-chain amino acids, have been proved to be metabolised by *Debaryomyces* spp. generating volatile compounds (Durá, Flores, & Toldrá, 2004) of importance for the typical aroma of dry fermented sausage (Montel, Masson, & Talon, 1998). Also, high quantities of Ala and Glu, contributors of sweet taste (MacLeod, 1994) and umami sensation (Maga, 1998), respectively, were found in the final sausages. Therefore, the balance of these free amino acids will

Table 3
Free amino acid (FFA) concentration in dry-fermented sausages during ripening

Concentration	Day 0			Day 6			Day 21			Day 35			RS × B[A]
	T	L1	L2	T	L1	L2	T	L1	L2	T	L1	L2	
Non essential FAA													
Asp	3.4[1]	3.7[1]	4.0[1]	3.9[1]	4.2[1]	4.3[1]	8.9ab[2]	6.1a[12]	10.3b[2]	13.1b[3]	8.5a[2]	11.7b[2]	>0.05
Glu	23[1]	21[1]	23[1]	107b[2]	67a[2]	77a[2]	125b[23]	86a[2]	160c[4]	134c[3]	79a[2]	105b[3]	<0.001
OHPro	3.2[1]	3.4[12]	3.7[12]	3.8a[1]	3.4a[1]	4.7b[23]	4.5ab[2]	4.3a[2]	5.0b[3]	3.2ab[1]	2.8a[1]	3.8b[1]	>0.05
Ser	8.3[1]	8.1[1]	8.7[1]	28.0b[2]	19.5a[2]	22.6ab[2]	40.2b[3]	29.3a[3]	37.2b[3]	47.7c[4]	32.7a[4]	40.3b[3]	>0.05
Asn	2.9[1]	3.2[1]	3.3[1]	9.1[2]	8.8[2]	9.3[2]	11.9ab[3]	9.6a[23]	14.2b[3]	15.2b[4]	11.9a[3]	14.5b[3]	>0.05
Gly	20.6[1]	20.3[1]	22.7[1]	32.3[2]	28.8[2]	32.9[2]	36.8b[23]	29.5a[2]	42.7c[3]	39.0b[3]	30.2a[2]	37.4b[23]	>0.05
Gln	119[3]	117[3]	122[3]	104a[2]	114ab[3]	114b[3]	72a[1]	66a[2]	89b[2]	66b[1]	54a[1]	58ab[1]	<0.01
Ala	58[1]	61[1]	69[1]	81[2]	81[2]	90[2]	90a[23]	79a[2]	105b[3]	99b[3]	84a[2]	97b[23]	<0.01
Arg	7.1[2]	7.9[12]	9.8[3]	0.0a[1]	11.1b[2]	15.8c[4]	6.4b[2]	5.1b[1]	0.0a[1]	7.2[2]	4.5[1]	4.0[2]	<0.001
Pro	8.2[1]	8.4[1]	9.7[1]	32.7[2]	24.7[2]	28.0[2]	36.5b[2]	26.0a[2]	39.2b[3]	37.6b[2]	28.5a[2]	35.2ab[23]	<0.001
Tyr	5.3[1]	5.2[1]	6.1[1]	19.0[2]	16.0[2]	18.5[2]	28.3b[3]	22.8a[3]	25.2ab[3]	33.8b[4]	29.9a[4]	34.5b[4]	>0.05
Orn	0.9[1]	1.1[1]	1.4[1]	26.4c[2]	8.0a[1]	15.5b[2]	27.4b[2]	20.3a[2]	34.2c[4]	27.8b[2]	18.0a[2]	26.1b[3]	<0.01
Essential FAA													
His	4.2[1]	4.3[1]	4.5[1]	10.3[2]	8.6[2]	9.4[2]	13.7b[3]	10.7a[23]	18.5c[3]	15.6b[3]	11.9a[3]	16.6b[3]	<0.05
Thr	6.7[1]	6.5[1]	6.8[1]	19.1[2]	15.2[2]	17.3[2]	26.5b[3]	19.4a[23]	28.4b[3]	32.1b[4]	23.1a[3]	28.9b[3]	>0.05
Val	8.5[1]	8.7[1]	8.9[1]	33.0[2]	29.1[2]	34.7[2]	51.3b[3]	42.8a[3]	43.7a[3]	60.6b[4]	52.9a[4]	58.1ab[4]	>0.05
Met	3.4[1]	3.6[1]	3.5[1]	13.4[2]	11.1[2]	12.7[2]	24.5b[3]	19.3a[3]	23.4b[3]	28.8b[4]	25.3a[4]	28.6b[4]	>0.05
Ile	4.5[1]	4.4[1]	4.7[1]	14.4[2]	13.7[2]	16.4[2]	27.6[3]	24.8[3]	26.0[3]	36.1ab[4]	34.9a[4]	38.5b[4]	>0.05
Leu	9[1]	9[1]	9[1]	60b[2]	51a[2]	57ab[2]	97b[3]	85a[3]	90ab[3]	107[4]	100[4]	107[4]	>0.05
Phe	5.8[1]	5.6[1]	5.5[1]	32.1[2]	28.3[2]	30.6[2]	52.1b[3]	44.9a[3]	43.7a[3]	56.1[3]	53.6[4]	56.5[4]	>0.05
Trp	2.1[1]	2.2[1]	2.0[1]	5.9ab[2]	5.2a[2]	6.6b[2]	8.0[3]	7.5[3]	8.0[3]	9.3ab[4]	8.9a[4]	9.8b[4]	>0.05
Lys	8[1]	7[1]	8[1]	22[2]	17[12]	23[2]	34a[3]	25a[23]	44b[3]	43b[4]	29a[3]	42b[3]	>0.05
Other FAA													
β-Ala	6.4	6.6[12]	7.1[1]	7.6	7.1[2]	7.4[1]	7.1b	6.1a[1]	8.7c[2]	7.5b	6.1a[1]	7.1b[1]	<0.05
Tau	116[1]	125	146[1]	144[12]	142	160[1]	153a[2]	142a	204b[2]	143a[12]	133a	173b[1]	>0.05
Natural dipeptides													
Car	279[12]	319[3]	330[23]	303[2]	294[3]	319[2]	236a[1]	231a[2]	389b[3]	237b[1]	164a[1]	223b[1]	<0.001
Ans	17.2[123]	17.5[23]	19.4[23]	20.4[3]	18.7[3]	17.5[2]	16.7a[2]	13.7a[2]	23.7b[3]	12.8[1]	9.8[1]	11.8[1]	<0.001
Total	732[1]	781[1]	840[1]	1134[2]	1029[2]	1155[2]	1237b[23]	1056a[2]	1522c[3]	1312b[3]	1036a[2]	1269b[2]	<0.05

Results are expressed as means of six replicates in mg per 100 g of dry matter.
a–c, Means with different letters in the same ripening stage are significantly different ($p < 0.05$) among batches.
1–4, Means with different numbers in the same batch are significantly different ($p < 0.05$) among ripening stages.
[A] Interaction between ripening stage and batch.

affect the sensory characteristics of the product (Ordóñez et al., 1999; Toldrá et al., 2001).

4. Conclusions

The manufacture of dry fermented sausages with *Debaryomyces* spp. produced a slower decline of pH during the beginning of the drying stage. At the end of processing, it also resulted in a lower content of ammonia and a higher content of acetic and D-lactic acids without producing any effect on the final pH. Therefore, the final pH of dry fermented sausages must be affected by other factors not only the content of ammonia and lactic acids. The effect of *Debaryomyces* spp. on proteolysis was followed by an accelerated degradation of myofibrillar proteins at the beginning of the drying stage. The higher pH of yeast inoculated batches at the beginning of the drying stage did not affect the enzymes involved in the generation of free amino acids as seen by the absence of differences among batches in this stage. Important differences in the content of free amino acids, that can be attributed to *Debaryomyces* spp. activity, were detected in the final sausage and therefore, they will have a high impact on the final sensory properties of the fermented sausage.

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