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Microbial and physico-chemical changes during the ripening of dry fermented sausages superficially inoculated with or having added an intracellular cell-free extract of *Penicillium aurantiogriseum*

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Abstract

The effect of inoculation of the "salchichón" (dry fermented sausage) surface with an atoxygenic, proteolytic and lipolytic strain of *Penicillium aurantiogriseum* and/or the addition of an intracellular cell-free-extract (ICFE) of the same mould on the ripening process was studied. Four batches of salchichón were manufactured: control, superficially inoculated, added with the intracellular cell free extract and combination of both treatments. Superficial mould modified *Micrococcaceae* counts, pH, free amino acids, lipid fractions, TBARS and some organic acids. The ICFE degraded free amino acids, producing a rise in ammonia. Both treatments combined accelerate the proteolysis and lipolysis of the sausages and also the further amino acid degradation.

Keywords: Dry fermented sausages; Moulds; *Penicillium aurantiogriseum*; Ripening; Proteolysis; Lipolysis; Oxidation; Intracellular cell free extract

1. Introduction

Moulds play an important role in the ripening of some dry fermented sausages. Among the beneficial effects of the fungal microbiota in fermented products moulds provide a pleasant appearance (highly appreciated in southern Europe), and they take part in the ripening phenomena through their extracellular proteases and lipases (Larsen, 1998; Larsen & Jensen, 1999; Toledo, Selgas, Casas, Ordóñez, & García, 1997; Trigueros, García, Casas, Ordóñez, & Selgas, 1995). Lipid and protein hydrolysis have a limited direct effect on the development of the aromatic and sapid compounds of sausages (Ordóñez, Hierro, Bruna, & Hoz, 1999). Although these enzymes produce a rise in the concentration of flavour compounds precursors (free amino acids and fatty acids) they do not accelerate the transformation of these molecules into volatile compounds. In this way, some mould strains also show deaminative activity, which can contribute to amino acid breakdown and the development of some important volatile compounds such as 2-methylpropanal, 2- and 3-methylbutanal from valine, isoleucine and leucine, respectively (Bruna, Fernández, Hierro, Hoz, & Ordóñez, 1999; Bruna, Fernández, Hierro, Ordóñez, & Hoz, 2000a,b).

Penicillium is the most widespread genus in meat and meat products due to its capability to grow at lower temperatures than other genera (Northold & Soentoro, 1988). It is normally present on dry cured ham (more than 60% of the strains isolated) and dry fermented sausages (more than 50%; Cook, 1995). One of the most frequently isolated species is *Penicillium aurantiogriseum* (Cook, 1995) and it is well known that it has proteolytic and lipolytic activity (Núñez, 1995).

Accordingly, it is reasonable to assume that the combined extracellular proteolytic and lipolytic activity of moulds could produce a greater accumulation of free amino acids and fatty acids (volatile compound precursors) than in conventional dry fermented sausages. These precursors can be latter transformed into volatile compounds by the addition of the intracellular

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cell-free-extract (ICFE). The objective of this work was, therefore, to combine the proteolytic and lipolytic effect of a *Penicillium aurantiogriseum* strain isolated from fermented sausages (Trigueros et al., 1995) with the deaminative activity of the same strain (Bruna et al., 2000a), in an attempt to accelerate the breakdown of the precursors of volatile compounds.

2. Material and methods

2.1. Preparation of the spore suspension and the ICFE of P. aurantiogriseum

An atoxigenic and proteolytic and lipolytic strain of *Penicillium* isolated from Spanish fermented sausages (Trigueros et al., 1995) was used. It was identified as *P. aurantiogriseum* by the International Mycological Institute (Egham, UK). To obtain the spores, *P. aurantiogriseum* was grown at 22°C for 7 days in Roux flasks on Sabouraud agar (Oxoid, Basingstoke, UK). Spores were harvested by washing the cultures with saline solution and glass beads, which were added to help dislodge the spores from the mycelium. To remove the mycelial debris and to clarify the turbid suspension the spore suspension was filtered through a sterile gauze, centrifuged at $4800\times g$ for 10 min and resuspended in saline solution. Finally, the spore concentration was adjusted to 10^6 spores/ml using a Thoma chamber (0.1 mm depth $\times$ 0.0025 mm^2 surface).

For the preparation of the ICFE, *P. aurantiogriseum* was grown in Roux flasks in Sabouraud liquid medium (Oxoid) at 22°C and pH 5.5 for 15 days. From the mycelium obtained by centrifugation ($1000\times g$, 15 min), 4 g aliquots were taken and mixed with 10 ml of ground glass and 14.5 mL of 0.2 M, pH 5.5 phosphate buffer. Then, they were treated in a cellular disrupter (Braun MSK, Melsugen, Germany) for 2 min. The final extract was filtered through Whatman No. 4 filter paper and the treatment procedure was repeated for the remaining mycelia. The filtrates obtained were combined and the resulting mixture was passed through a 0.45-μm filter (Millipore Corporation, Bedford, USA) connected to a vacuum pump to eliminate the spores present in the extract. The filtered extracts were collected and the protein content, estimated by Lowry's method (Lowry, Rosenbrough, Farr, & Randall, 1951), was 1.97 mg protein/ml.

2.2. Determination of the L-amino oxidase activity of the ICFE

L-amino oxidase activity of the ICFE was measured following the method described by Wellner and Lichtemberg (1971), using a commercial L-amino oxidase (Sigma, St. Louis, USA) as a standard. This test is based in the oxidation of phenylalanine by L-amino oxidase, to yield ammonia and phenylpiruvate, whose enol form reacts with borate to form a complex that presents a maximum absorbance at 300 nm. Catalase is added to prevent the keto acid from being destroyed by hydrogen peroxide.

A reaction mixture containing 0.2 ml of a tris-HCl buffer (0.4 M, pH 7.8), 50 μl of a catalase solution (1200 units/ml) and 0.7 ml of the ICFE or 0.7 ml of the commercial L-amino oxidase was placed in a test tube. The reaction was started by the addition of 0.1 ml of a L-phenylalanine solution (0.04 M) and the test tube was immediately placed in a water bath at 37°C with reciprocal shaking. After exactly 15 min the reaction was stopped by the addition of 0.2 ml of a trichloroacetic solution (25%). The reaction mixture was transferred to a conical centrifuge tube and centrifuged (5000 *g*, 20 min, 15°C). A 0.5-ml aliquot of the supernatant was then transferred to a test tube containing 2.5 ml of a borate-arsenate solution (1 M boric acid, 1 M sodium arsenate, pH 6.5) and mixed well. The solution was allowed to stand at least 30 min at room temperature (22–25°C) and then the absorbance was measured at 300 nm, using a blank in which the L-amino oxidase was omitted.

One unit of activity was defined as the amount of enzyme required to give an absorbance of 0.030 at 300 nm under the above conditions.

2.3. Preparation of the fermented sausages

The mixture for salchichón-type dry fermented sausages was prepared using the following formula: (% w/w): pork (55), beef (13.49), pork fat (25), NaCl (2.5), dextrin (1.8), lactose (1.0), glucose (0.8), monosodium glutamate (0.25), sodium ascorbate (0.046), $NaNO_3$ (0.0095), $NaNO_2$ (0.0065), and equal amounts of whole grain and ground black pepper (0.14). The ingredients were processed at 2°C in a mincer equipped with an adjustable plate set at a hole diameter of 5 mm and inoculated with a starter culture of *Lactobacillus plantarum* 4045, *Staphylococcus carnosus* and *Staphylococcus xylosus*. The total mixture was divided into four parts which were used to prepare four separate batches of fermented sausages that were manufactured in the following order: batch C (control) consisted of the initial mixture alone; batch S which was like batch C but it was superficially inoculated with the spore suspension immediately after stuffing; batch E, which was added with ICFE (101 mg protein/kg) and batch E + S, which was like batch E but superficially inoculated with the spore suspension immediately after stuffing. To adjust the moisture content of the different batches, the corresponding amount of phosphate buffer was added to batches C and S. Potassium sorbate (25%) was sprayed on the surface of batches C and E to avoid the

growing of moulds. After sufficient mixing, the mixtures were introduced into 40 mm in diameter collagen sausage casings (Julio Criado, Madrid, Spain) and left to ripen in an Ibercex ripening cabinet, model G-28 (A.S.L., San Fernando de Henares, Spain) after inoculation with the spore suspension when necessary (batches S and E + S). All the equipment was cleaned after the manufacture of each batch to avoid contamination. The sausages were fermented at 22°C and 90% relative humidity (RH) for 12 h. After this, the temperature and RH were slowly reduced to 18°C and 80%, respectively, in 60 h. Finally, the sausages were dried at 12°C and 80% RH until the end of the ripening process (a total of 22 days).

The results recorded here are the mean data obtained with samples from three different processes in which the four batches mentioned before were manufactured with different ingredients but same formulation and technology at different periods of the year. Each of the following analysis were done by triplicate, therefore, each value presented in this work is the mean of nine data.

2.4. Microbial analysis

The total viable micro-organism count was done in Plate Count Agar (PCA; Oxoid) and the *Micrococcaceae* in Manitol Sal Agar (MSA; Oxoid), both incubated at 32°C for 2 days. Lactic acid bacteria (LAB) were grown in MRS agar (Oxoid) at pH 5.6 in a double layer at 32°C for 2 days. Moulds and yeasts were grown in malt extract agar (Oxoid) with oxytetracycline (0.01%) at 25°C for 5 days.

Catalase activity was determined as described by Whittenbury (1964). After growing the mould in nutrient agar (22°C, 1 week), 0.5 ml of a 3% H_2O_2 dilution was spreaded on the plate surface. The test was considered positive if effervescence was observed due to the production of O_2 from H_2O_2.

2.5. Chemical analysis

Dry matter (DM) was determined by drying the sample at 110°C to constant weight. Water activity (a_w) was determined using a Decagon CX1 hygrometer (Decagon Devices, Pullman, USA) at 25°C. The pH was measured in a homogenate of the sample with distilled water (1:10, w/v) using a Crison Digit-501 pH meter (Crison Instruments, Barcelona, Spain).

The total fat of the samples was determined by cold extraction in chloroform and methanol in the presence of antioxidant BHT following the method described by Hanson and Olley (1963) and was quantified gravimetrically. The separation of the different lipid classes was performed according to Fernández, Hoz, Díaz, Cambero, and Ordóñez (1995a) by thin layer chromatography (TLC) on 0.25-mm G-60 silica gel plates (Merck, Darmstadt, Germany), developed with petroleum ether/diethyl ether/acetic acid (80/20/1; v/v/v). Triolein, diolein, monolein, oleic acid and cholesterol (Sigma Chemical, St. Louis, MO) were used as reference standards. A spray of 0.05% of $FeCl_3 \cdot 6H_2O$ solution in a mixture of water/acetic acid/sulfuric acid (90/5/5; v/v/v; Lowry, 1968), followed by heating in an oven at 120°C for 30 min, was used to visualise all lipid fractions. Lipid classes were quantified by densitometry in a Shimadzu CS-9000 densitometer (Shimadzu, Kyoto, Japan) at 390 nm using calibration curves for all the standards used in TLC analysis.

To measure lipid oxidation during ripening, the 2-thiobarbituric acid (TBARS method) described by Salih, Smith, Price, and Dawson (1987) was carried out. For that, 5 g of sausage were homogenised in 15 ml of 0.38 M $HClO_4$ for 3 min in an ice bath. To avoid further oxidation 0.5 ml of a 0.19 M BHT ethanolic solution were added. The homogenate was centrifuged (3000 *g*, 5 min, 5°C) and filtered through Whatman No. 54. An aliquot of 0.7 ml was mixed with 0.7 ml of a 0.02 M TBA solution and heated at 100°C for 30 min. After cooling, the mixture was centrifuged at 3000 *g* for 15 min at 5°C. Finally, the absorbance was measured at 532 nm. Results were expressed as mg malonaldehyde (MDA)/kg sample.

Ammonia content was determined using the Boehringer kit for enzyme analysis (Boehringer Mannheim GmbH, Germany) following the manufacturers instructions for meat products.

Free amino acids were extracted as described by Yang and Sepúlveda (1985) and analysed as described by Bruna et al. (1999). After extraction, amino acids were derivatised with phenylisothiocyanate (PITC). Amines were extracted according to Spinelli, Lakritz, and Wasserman (1974) and analysed after derivatisation with dansyl chloride (Ordóñez, de Pablo, Pérez de Castro, Asensio, & Sanz, 1991). The amino acids and amines derivatives were analysed in a Beckman System Gold *Nouveau* chromatograph (Fullerton, USA) equipped with a Waters column (Milford, USA) Spherisorb S5 ODS2 (25 cm×4.6 mm, 5 μm particle size) maintained at 35°C in a column oven (Jones Chromatography, Mid Glamorgan, UK). Detection was performed at 254 nm in both cases. The different amino acids and amines were identified by comparing the samples with standard solutions (Sigma) analysed in the same conditions.

Organic acids were analysed according to the method described by González de Llano, Rodríguez, and Cuesta (1996). The analysis was done in the same HPLC mentioned above equipped with an Aminex HPX-87H ion exchange column (300×7.8mm) protected by a cation H^+ Microguard cartridge (BioRad Laboratories, Richmond, USA) maintained at 65°C. Detection was done at 210 nm. The different organic acids were identified by comparing the samples with standard solutions (Sigma) analysed in the same conditions.

2.6. Statistical analysis

ANOVA was used to search for significant differences between mean values of the different results. Comparison between batches was performed by the Student Newman–Keul's test ($P<0.05$) using SigmaStat 1.1 (Jandel Corporation, San Rafael, USA).

3. Results and discussion

3.1. L-amino oxidase activity and changes in microbiota

After the ICFE was obtained, the L-amino oxidase activity was measured. The extract showed an activity of 0.37 units/ml.

Lactic acid bacteria (Fig. 1) were the dominant microbiota in all the batches studied and showed the normal behaviour of this microbiota. No significant differences among batches was found. Similar results have been described by many authors (Díaz, Fernández, García de Fernando, Hoz, & Ordóñez, 1996; Fernández et al., 1995a). However, the *Micrococcaceae* deviated from the usual behaviour. A quick increase during the fermentation step to gradually decrease afterwards (Ordóñez, Hierro, Bruna, & Hoz, 1999) was observed. This behaviour was highly influenced by the superficial inoculation of *P. aurantiogriseum* (Fig. 1). Batches S and E + S showed higher counts at the end of the ripening (around 10^6 c.f.u./g) than batches C and E (around 10^3 c.f.u./g). This could be related with the higher pH values registered in batches superficially inoculated with *P. aurantiogriseum* since *Micrococcaceae* are acid-sensitive bacteria (Leuschner & Hammes, 1998; Lücke, 1986). Similar results were found by Bruna, Fernández, Hierro, Ordóñez, and Hoz (2000b) when superficially inoculating *Mucor racemosus*, although differences were less remarkable due to smaller differences in the pH values..

Mould and yeast counts in the interior of the fermented sausages (data not shown) increased from 10^4 c.f.u./g to 10^5 c.f.u./g during the fermentation stage, probably due to the remaining oxygen concentration in the sausage mixture. Afterwards, the counts slowly decreased to reach again 10^4 c.f.u./g at the end of the ripening period. Similar results were described by Zapelena (1997) in Spanish dry fermented sausages.

3.2. Changes in pH, dry extract and a_w

Fig. 1 also shows changes in pH values during the ripening of the fermented sausages. In superficially inoculated batches (S and E + S), the final pH was significantly higher than in non-inoculated batches. These results coincided with those described by several authors (Grazia, Romano, Bagni, Roggiani, & Guglielmi, 1986; Lücke, 1986; Roncalés, Aguilera, Beltrán, Jaime, & Peiro, 1991). These authors reported that in mould ripened fermented sausages, the pH usually increases at the end of the ripening due to the utilisation of lactate and acetate together with the production of ammonia from amino acid breakdown. In the present work, the corresponding considerations about it will be made in the discussion of the results of organic acids and ammonia.

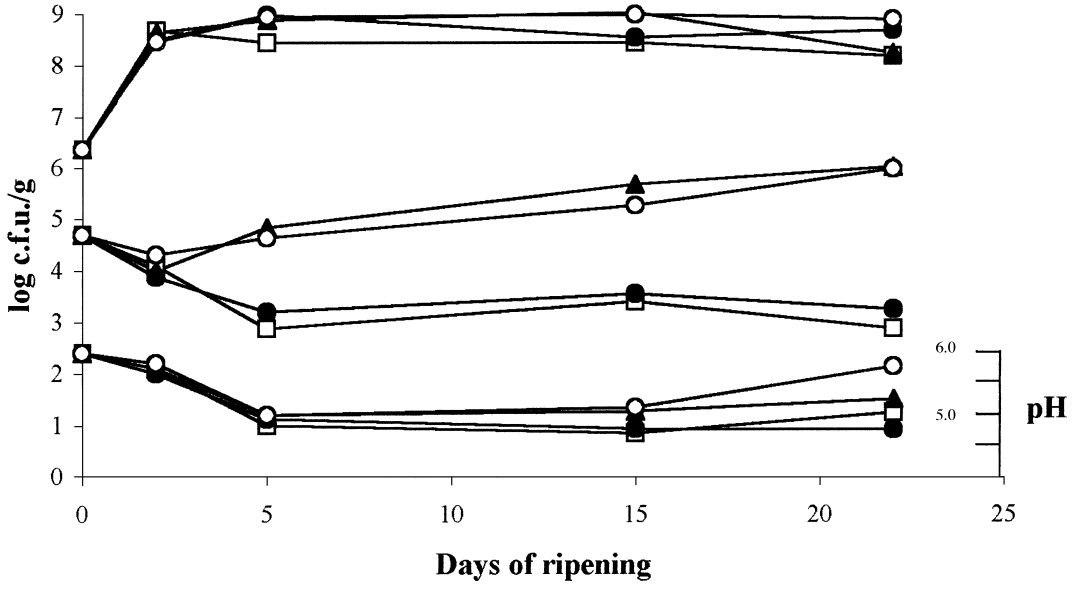

Fig. 1. Changes in lactic acid bacteria (upper curves) and *Micrococcaceae* (middle curves) counts (log c.f.u./g) and pH (lower curves) during the ripening of dry fermented sausages. (□) Control batch; (●) Batch E (Control batch added with an intracellular cell-free-extract of *Penicillium aurantiogriseum*, 101 mg protein/kg); (▲) Batch S (control batch superficially inoculated with a spore suspension of *P. aurantiogriseum*); (○) Batch E + S (Batch E superficially inoculated with the spore suspension extract of *P. aurantiogriseum*).

The dry extract (data not shown) of the different fermented sausages varied over the ripening process from initial values of around 36%, which rose gradually to around 70% at the end of the ripening period. Water shrinkage after fermentation and at the end of the ripening was 11 and 52%, respectively. In parallel, the a_w diminished from initial values of around 0.96 to 0.81–0.82 (data not shown). Changes in both parameters were not significantly affected by the superficial inoculation or the adition of the ICFE and were similar to those described by other authors (Díaz, Fernández, García de Fernanod, Hoz, & Ordóñez, 1997; Fernández et al., 1995a; Lücke, 1998; Nychas & Arkoudelos, 1990). However, Bruna et al. (2000b) found that batches superficially inoculated with *M. racemosus* presented a lower dry extract value and a slightly higher a_w than non-inoculated batches. Several authors (Grazia et al., 1986; Leistner & Ayres, 1968) have attributed to the superficial fungal layer a barrier effect against dehydration. Probably, this effect depends on the mould species grown.

3.3. Changes in organic acids

Table 1 shows the results obtained in the organic acid analysis. The superficial inoculation with the mould gave rise to a decrease in the lactic acid content (16% in mould covered sausages) and an increase in acetic, propionic and n-butyric acid. In cheeses ripened with a surface fungal flora, a drop in the lactic acid content has been observed (Fox, Lucey, & Cogan, 1990) as this acid can be metabolised to CO_2 and H_2O by these organisms. The decrease of lactic acid has also been observed by Grazia et al. (1986) when studying salami inoculated with different mould strains and it has also been reported by Bruna et al. (2000b) when inoculating salchichón with *M. racemosus*. As in cheeses, lactic acid is the main responsible for the pH reduction in sausages.

Several authors (DeKetelaere, Demeyer, Vandekerckhove, & Vervaeke, 1974; Halvarson, 1973; Hierro, Hoz & Ordóñez, 1997) have also found acetic, propionic and n-butyric acids in dry fermented sausages. Although acetic acid is mainly produced during fermentation of carbohydrates (Kandler, 1983) it can also be formed during the fatty acid oxidation and the catabolism of alanine (Montel, Masson, & Talon, 1998). Since moulds seem to protect against oxidation, the increase of acetic acid observed in the present work in mould covered sausages could be related to the release of alanine due to the proteolytic activity of *P. aurantiogriseum*.

Propionic and n-butyric acids are strongly associated with the aroma. Both of them suffered a very high increase in all batches, at least 116- and 48-fold, respectively. The rise in these organic acids in fermented sausages inoculated with mould could be associated with the lipolytic activity of this strain (Selgas, Casas, Toledo, & García, 1999). However, these compounds can also originate from the fermentation of glucose, from some amino acids or from the oxidation of aldehydes (Gottschalk, 1986; Mateo & Zumalacárregui, 1996). These are the probable sources of this compounds in non-inoculated batches.

The addition of *P. aurantiogriseum* ICFE to sausages determined a 20% increase in the total final content of organic acids. All organic acids analysed increased due to the activity of the extract. In this case, as no lipolytic effect can be attributed to the extract, the increase in acetic, propionic and n-butyric may be related to the degradative activity of the extract on amino acids.

3.4. Changes in lipid fractions and TBARS

In relation to the lipolytic activity of the mould (Table 2), a clear (2.5–3-fold at the end of the ripening) significant ($P < 0.05$) increase in diglyceride content and

Table 1
Changes in organic acids (mg/100 g dry matter) during ripening of dry fermented sausages[a]

Organic acid	Day 0	Day 5				Day 15				Day 22			
	C[a]	C	E[a]	S[a]	E+S[a]	C	E	S	E+S	C	S	E	E+S
Orotic	0.03	n.d.	n.d.	0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Citric	9.21	14.98a	15.01a	13.55a	14.27a	36.87a	34.15a	34.22a	35.55a	38.74a	45.21b	46.27b	49.22c
Pyruvic	0.66	0.96a	3.54b	4.12b	5.00c	3.12a	6.66b	6.31b	7.58c	4.00a	5.58b	7.21c	6.21b,c
Succinic	87.01	0.01a	0.16b	0.19b	0.16b	0.31a	0.29a	0.37a	0.41a	0.04a	0.09a	0.11a	0.12a
Lactic	963.31	1874.15a	1932.14a	1933.21a	1899.89a	2655.84a	2934.58b	2263.01a	2203.31a	2574.14a	2165.71b	2896.33c	2210.01b
Formic+ uric	2.1	23.21a	20.27a	21.47a	19.94a	58.41a	79.21d	74.12c	86.51d	59.37a	68.41b	78.52c	79.32c
Acetic	12.21	54.17a	76.27b	74.15b	77.81b	52.34a	100.00b	101.19b	115.27c	76.21a	112.96b	110.71b	141.25c
Propionic	10.47	248.78a	345.21b	333.25b	412.58c	847.16a	1258.78b	1324.12b	1524.18c	1221.7a	1521.36b	1632.23c	1874.36d
N-butyric	0.06	19.88a	24.1b	21.21a	26.31c	26.31a	35.47b	37.12b	38.41b	29.21a	41.00b	49.98b	42.85b
Total	1085.06	2236.14a	2416.7b	2421.96b	2544.07b	3680.36a	4449.14b	3840.46c	4011.22d	4003.41a	3960.32a	4821.36b	4403.34c

[a] C, Batch C (Control); E, Batch E (Control batch added with an intracellular cell-free-extract of *Penicillium aurantiogriseum*, 101 mg protein/kg); S, Batch S (control batch superficially inoculated with a spore suspension of *P. aurantiogriseum*); E+S, Batch E+S (Batch E superficially inoculated with the spore suspension extract of *P. aurantiogriseum*). Values in a row at the same ripening day with different letters are significantly different ($P < 0.05$) n.d,: not detected.

free fatty acids (FFA), was observed in batches inoculated with *P. aurantiogriseum* on the surface. This effect was clearly noticeable in the earlier stages of ripening, when temperature and RH were more adequate for the mycelium growth. The addition of the ICFE contributed to a slight increase of FFA, which are in agreement with results previously reported (Bruna et al., 2000a). However, the superficial inoculation of *P. aurantiogriseum* trebled the final content of batch C, which demonstrates the intense lipolytic activity of the live mould. These observations coincided with the results reported for this strain (P-3) both in vitro (Trigueros et al., 1995) and in fermented sausages (Selgas et al., 1999). In the present study, the final FFA content at the end of the experiment (22 days) in batches S and E + S was similar to that observed in sausages with a longer ripening period (Nagy, Mihalyi, & Incze, 1989) or when exogenous enzymes (Fernández, Hoz, Díaz, Cambero, & Ordóñez, 1995b).

TBARS values in this work (Table 2) were similar to those described by Meynier, Novelli, Chizzolini, Zanardi, and Gandemer (1999), Zalacain, Zapelena, Astiasarán, and Bello (1995), and Zanardi, Novelli, Campanini, Madarena, and Chizzolini (1998). At the end of the ripening, TBARS values were significantly lower in batches superficially inoculated (S and E + S) than in their respective controls (batches C and E). Therefore, it seems that the superficial mould cover protects against lipoid oxidation. Nevertheless, this effect does not seem to affect the sensory quality of the sausages, as TBARS levels obtained in all batches are among those considered as acceptable for good quality without the appearance of rancid notes (Chizzolini, Novelli, & Zanardi, 1998).

As other authors (Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996; Núñez, Rodríguez, Aranda, Martín, Díaz, & Bermúdez, 1998) have previously observed with some *Penicillium* species, the catalase test was positive. This activity can be important for controlling the concentration of mycotoxins in the sausages since catalase can degrade mycotoxins, as reported by El-Gendy and Marth (1981) when using *Aspergillus parasiticus* myceliar extracts.

The protective effect of moulds against oxidation has been reported by several authors and has been attributed to protection against light, oxygen consumption on the surface and the production of catalase and peroxidase (Bacus, 1986; Cook, 1995). This effect has been clearly described in dry cured ham (Leistner & Ayres, 1968; Núñez, 1995; Núñez et al., 1998) but not in mould ripened sausages. In this way, Berger, Macku, German, and Shibamoto (1990) found a low amount of oxidation products in mould covered salami but they attributed these differences to the manufacture of the sausages or different sample preparation techniques rather than to the mould covering. Also, Viallon et al. (1996) determined low amounts of these oxidation compounds in fermented sausages superficially inoculated with *Debaryomyces hansenii*, but they did not attribute any protective effect to the yeast. However, in a previous work (Bruna et al., 2000b) lower amounts of oxidation derived compounds in salchichón-type sausages superficially inoculated with *M. racemosus* compared to sausages superficially treated with potassium sorbate were found.

3.5. Changes in free amino acids and ammonia

Total free amino acid (FAA) content and the changes of each FAA are shown in Table 3. The mould inoculation on the surface led to an increase in the FAA content of 48% in batch S and 47% in batch E + S compared to their controls (batches C and E, respectively). These results reflect an intense proteolytic activity of the mould as expected from previous experiences *in vitro* on sarcoplasmic and myofibrillar proteins (Trigueros et al., 1995) and in dry fermented sausages (P-3 in Toledo et al., 1997). Grazia et al. (1986) considered that moulds have little importance on the proteolysis in dry fer-

Table 2
Changes in lipid fractions (g/100g D.M.) and TBARS (mgMDA/kg D.M.) during ripening of dry fermented sausages[a]

Fraction	Day 0	Day 5				Day 15				Day 22			
	C	C	E	S	E+S	C	E	S	E+S	C	E	S	E+S
MG+PL	3.22	3.13a	3.37a	3.78b	4.32c	3.53a	3.68a	5.49b	5.84c	3.66a	3.99a	5.94b	6.02b
DG	0.16	0.39a	0.31a	0.53b	0.54b	1.02a	1.10a	1.37b	1.39b	1.28a	1.26a	1.78b	1.98c
FFA	0.05	0.71a	0.72b	1.89c	1.91c	1.16a	1.21b	2.75c	2.20d	1.71a	1.86b	3.52c	3.54c
TG	60.42	60.16a	59.83a	59.31b	58.86b	58.67a	58.04a	56.19b	56.4b	58.16a	57.53a	55.01b	54.50b
TBARS	0.155	0.366a	0.360a	0.277b	0.272b	0.643a	0.640a	0.416b	0.421b	0.756a	0.756a	0.496b	0.504b

[a] C, Batch C (Control); E, Batch E (control batch added with an intracellular cell-free-extract of *Penicillium aurantiogriseum*, 101 mg protein/kg); S, Batch S (control batch superficially inoculated with a spore suspension of *P. aurantiogriseum*); E + S, Batch E + S (Batch E superficially inoculated with the spore suspension extract of *P. aurantiogriseum*). MG + PL, monoglycerides + phospholipids;, DG, diglycerides; FFA, free fatty acids; TG, triglycerides; TBARS, thiobarbituric acid reactive substances. Values in a row at the same ripening day with different letters are significantly different ($P < 0.05$)

mented sausages, but other authors have demonstrated this activity in cheese (Dines-Larsen, Rotvig-Kristiansen, & Kronborg-Hansen, 1998; Fox & Law, 1991) and meat products (Geisen, Lücke, & Kröeckel, 1992; Rodríguez, Núñez, Córdoba, Bermúdez, & Asensio, 1998). In the above mentioned paper, Rodríguez, Núñez, Córdoba, Bermúdez, and Asensio (1998) the proteolytic activity of a *P. aurantiogriseum* strain isolated from dry cured ham was studied and it was concluded that it showed an important proteolytic and aminopeptidasic activity.

The high final contents of FAA determined in batches S and E+S are comparable to those obtained after addition of exogenous proteases to fermented sausages (Bruna et al., 1999; Bruna, 2000a; Díaz et al., 1997; Zapelena, Zalacain, Paz de Peña, Astiasarán, & Bello, 1997). The most affected amino acids by the superficial inoculation of the mould (comparing batch S vs C) were Cis (×2.92), Gln (×2.59), Thr (×2.39), Glu (×2.08), Ser (×1.79), Leu (×1.71), Asn (×1.57) and Gly (×1.48).

On the other hand, addition of ICFE of *P. aurantiogriseum* (batches E and E+S), produced a reduction in the FAA content of around 5% in comparison with their respective controls (batches C and S). These results are in agreement with those obtained in previous works (Bruna et al., 2000a) with an intracellular of this same strain of mould. A drop in the content of most of the free amino acids was noticed due to the ICFE (comparing batches E *vs* C), specially Cys (×0.26), His (×0.53), Asp (×0.60), Tau+GABA (×0.62) and Tyr (×0.66).

Table 3 also shows changes in the ammonia content in the different batches of fermented sausages. As expected, the superficial growth of *P. aurantiogriseum* caused an increase of ammonia of 46% (S vs C) and 38% (E+S vs E) respectively. These results are in total agreement with the proteolytic activity recorded in the mould inoculated sausages, since ammonia is one of the main products of protein breakdown (Montel et al., 1998). The increase in ammonia content may be one of the possible causes of the higher pH observed in batches S and E+S (Table 1). Grazia et al. (1986) also found an increase of this compound in dry fermented sausages superficially inoculated with selected mould strains.

The addition of the ICFE produced an increase in the ammonia content of 17.5% in relation to control sausages (E vs C). These findings, together with the demonstrated L-amino oxidase activity of the ICFE (0.37 units/ml), suggest that deamination is one of the main pathways of amino acid breakdown (Wellner & Lichtemberg, 1971).

Table 3
Changes in free amino acids and ammonia (mg/100g dry matter) during ripening of dry fermented sausages[a]

Amino acid	Day 0	Day 5				Day 15				Day 22			
	C	C	E	S	E+S	C	E	S	E+S	C	E	S	E+S
Asp	20.3	8.6a	6.7b	7.9a	8.8a	15.3a	8.9b	14.9a	23.8c	19.3a	11.5b	20.0a	35.1c
Glu	12.9	57.5a	64.0b	75.4c	56.9a	81.2a	79.7a	120.4b	96.9c	72.2a	95.4b	150.4c	116.2d
Hxp	0.9	n.d.	0.9a	2.1b	n.d.	1.7a	n.d.	2.7b	0.2c	n.d.	0.7a	1.7b	0.2c
Ser	6.7	3.6a	5.6b	4.6c	n.d.	6.0a	8.0b	10.3c	9.4c	7.1a	8.4b	12.7c	12.4c
Asn	n.d.	2.0a	n.d.	3.0b	8.1c	4.6a	4.3a	5.7a	4.4a	4.2a	4.7a	6.6b	4.6a
Gly	10.9	14.2a	18.3b	23.3c	16.8b	19.5a	22.9a	36.1b	27.2c	27.1a	26.2a	40.2b	43.4b
Gln	292.8	47.6a	96.6b	133.4c	117.6d	67.7a	75.8b	118.7c	57.1c	49.7a	88.6b	128.5c	64.1d
His	3.5	11.0a	7.6b	10.9a	13.7a	15.6a	13.3a	19.2b	24.5c	25.1a	13.2b	21.3c	37.1d
Tau+GABA	48.8	67.3a	59.6b	67.4a	95.5c	70.7a	55.4b	73.0a	77.7a	81.1a	50.0b	74.7c	96.8d
Thr	6.2	10.5a	14.3b	17.2c	11.7a	13.0a	18.9b	22.6c	13.6a	11.7a	17.7b	28.0c	1.1d
Ala+Arg	83.2	92.1a	109.4b	85.3c	140.0d	184.9a	69.9b	224.9c	215.7c	226.5a	164.1b	248.4c	273.9d
Pro	5.0	11.5a	13.9a	17.9b	11.5a	85.4a	15.5b	233.3c	131.1d	115.6a	160.2b	161.0b	221.1c
Tyr	6.3	6.2a	7.2a	11.9b	8.6c	9.5a	6.7b	14.6c	9.2a	16.2a	10.7b	14.7c	19.2d
Val	34.6	51.5a	52.5a	63.7b	91.9c	27.2a	54.4b	38.7c	35.3c	34.9a	30.5b	47.5c	50.0c
Met	5.8	14.4a	15.7a	19.6b	14.0a	22.8a	19.3a	35.2b	25.2c	24.1a	27.3a	13.4b	33.2c
Cys	0.5	0.6a	0.8a	1.4b	0.9a	0.3a	0.5b	0.5b	1.7c	5.4a	1.4b	2.4c	8.0d
Ile	0.5	26.0a	25.4a	31.4b	34.5b	31.2a	26.9b	41.0c	39.4c	41.3a	30.4b	46.0c	52.9d
Leu	10.4	26.6a	25.2a	32.7b	26.3a	38.7a	33.5a	54.3b	47.3c	38.8a	41.5a	66.2b	57.0c
Phe	0.6	16.9a	22.5b	32.4c	19.2a	23.3a	20.1a	30.9b	31.3b	24.4a	23.4a	35.8b	37.4b
Trp	0.7	20.6a	7.1b	35.1c	18.3d	31.7a	20.7b	35.3a	43.1c	34.3a	25.1b	38.5a	50.2c
Lys	6.1	17.2a	19.5a	24.5b	19.7a	37.1a	30.1b	50.5c	42.8a	50.1a	38.5b	61.7c	64.3c
Cis	1.6	0.6a	2.6b	4.4c	6.0d	5.5a	1.9b	5.5a	6.9c	1.2a	3.3b	3.5b	3.0b
Total	558.4	506.5a	575.2b	705.3c	719.1c	792.5a	586.4b	1188.1c	963.5d	910.1a	872.8b	1353.2c	1281.4d
NH3	28.5	31.2a	36.5a	51.2b	55.2b	41.8a	56.8b	82.5c	89.7c	55.4a	65.1b	80.2c	89.9d

[a] C, Batch C (Control); E, Batch E (control batch added with an intracellular cell-free-extract of *Penicillium aurantiogriseum*, 101 mg protein/kg); S, Batch S (control batch superficially inoculated with a spore suspension of *P. aurantiogriseum*); E+S, Batch E+S (Batch E superficially inoculated with the spore suspension extract of *P. aurantiogriseum*). Values in a row at the same ripening day with different letters are significantly different ($P < 0.05$) n.d., not detected.

Table 4
Changes in amines (mg/100g dry matter) during ripening of dry fermented sausages[a]

Amine	Day 0	Day 5				Day 15				Day 22			
	C	C	E	S	E+S	C	E	S	E+S	C	E	S	E+S
Tryptamine	2.8	3.1a	1.8b	2.4c	1.9b	7.1a	7.4a	7.3a	6.7a	7.3a	8.5b	8.8b	8.7b
Phenylethylamine	2.0	0.99a	1.2b	0.80c	1.0a	1.8a	1.2b	2.8c	3.1d	1.7a	2.3a	2.1a	3.1b
Putrescine	2.1	5.1a	6.4b	4.7c	4.7c	15.2a	14.3a	12.7b	20.7c	19.3a	20.1a	20.7a	21.0a
Histamine + Cadaverine	2.7	9.3a	10.7b	8.8a	6.2c	18.0a	16.8a	17.2a	14.3b	21.5a	20.3a	25.3b	19.8a
Tyramine	n.d.	n.d.	0.12	n.d.	n.d.	0.47a	0.12b	n.d.	0.02c	2.1a	2.1a	3.3b	3.1b
Spermidine	0.08	0.66a	1.1b	0.87c	0.80c	1.1a	1.0a	1.1a	1.5b	1.1a	0.9a	2.0b	1.0a
Spermine	1.1	2.3a	2.0a	1.8a	1.8a	0.08a	0.16b	0.15b	0.2b	0.28a	0.23a	0.32a	0.28a
Total	10.78	21.51a	23.35c	19.37b	16.41d	43.82a	41.05a	41.34a	46.68b	53.42a	54.6a	62.62b	57.04c

[a] C, Batch C (Control); E, Batch E (control batch added with an intracellular cell-free-extract of *Penicillium aurantiogriseum*, 101 mg protein/kg); S, Batch S (control batch superficially inoculated with a spore suspension of *P. aurantiogriseum*); E + S, Batch E + S (Batch E superficially inoculated with the spore suspension extract of *P. aurantiogriseum*). Values in a row at the same ripening day with different letters are significantly different ($P < 0.05$). n.d., not detected.

The increase in the concentration of ammonia and some organic acids, as well as the modification of the amino acid pattern has, undoubtedly, a deep influence on the sensory attributes of the dry fermented sausages. This aspects will be described in a following paper (Bruna, Hierro, Hoz, Mottram, Fernández, & Ordóñez, 2001).

3.6. Changes in amines

Table 4 shows changes in the content of the eight amines detected in the different batches of fermented sausages. The final amine content was around 4.5–5.5 fold higher than levels determined on day 0. The amine concentration was not affected by the mould layer since batch S and batch E + S showed similar levels and profiles to those of the corresponding control batches (C and E). The amine levels reported in different sausages by several authors vary from 9 mg/100 g dry matter (Hierro, Hoz, & Ordóñez, 1999) to 120 mg/100g dry matter (Díaz et al., 1997). Data in the present work was similar to that reported by Ayhan, Kolsarici, and Özkan (1999) and Bover-Cid, Schoppen, Izquierdo-Pulido, and Vidal-Carou (1999) in Turkish "soudjouck" and Spanish "salchichón", respectively.

In relation to the effect of the ICFE (batch E), a 17% rise in the amine content was observed compared to batch C. Among these compounds, tyramine and spermidine registered the highest increases (57 and 82%, respectively). These results agree with the decrease observed for Tyr and Ala + Arg.

4. Conclusions

In the light of these observations it can be concluded that *P. aurantiogriseum* showed intense proteolytic and lipolytic activities and also played a very important role against lipid oxidation in sausages where it was superficially inoculated. On the other hand, ICFE had an important effect in amino acid degradation. The combination of both treatments suggests that it is possible to enhance or accelerate the biochemical processes that lead to the formation of flavour compounds in sausages.

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