Role of *Penicillium aurantiogriseum* on the ripening: Effect of the superficial inoculation and/or the addition of a intracellular cell free extract on the microbial and physico-chemical parameters of dry fermented sausages.

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Background

Moulds play an important role in the ripening of some dry fermented sausages. Among the beneficial effects of the fungal microbiota in fermented products they give the product an agreeable appearance (highly appreciated in Southern Europe) and they can show an intense enzymatic activity because they can produce extracelular proteases and lipases (Trigueros *et al.*, 1995; Toledo *et al.*, 1997; Larsen, 1998; Larsen and Jensen, 1999). Lipid and protein hydrolysis have a limited direct repercussion on the development of the aromatic and sapid compounds of sausages (Ordóñez *et al.*, 1999). Although these enzymes produce a rise in the concentration of precursors of aromatic and sapid compounds (free amino acids and fatty acids), they do not accelerate the transformation of these 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- and 3-methylbutanal (Bruna *et al.*, 1999a,b; 2000a,b). Therefore, it is reasonable to assume that the combined extracellular proteolytic and lipolytic activity of moulds and their intracellular metabolic activity on amino acids and fatty acids could produce a greater accumulation of volatile compounds than in conventional fermented sausages.

Objective

The objective of this work was to combine the proteolytic and lipolytic effect of a *Penicillium* 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, in order to shorten the ripening period.

Methods

An atoxigenic and proteolytic and lipolytic strain of *Penicillium aurantiogriseum* was used. A spore suspension (10^6 spores/mL) was prepared as described by Toledo et al. (1997). Catalase activity was determined as described by Whittenbury (1964).The intracellular cell free extract (ICFE) was prepared by disrupting the mycellium in pH 5.5, 0.2 M phosphate buffer.

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 minced and inoculated with a starter culture of *Lactobacillus plantarum* 4045 and *Staphylococcus carnosus* and *S. xylosus*. The total mixture was divided into four parts which were used to prepare four separate batches of fermented sausages: batch C (control) consisted of the initial mixture alone; batch S which was like batch C but was superficially inoculated with the spore suspension immediately after stuffing; batch E, which was added with ICFE (100.87 mg protein/kg) and batch E+S, which was like batch E but superficially inoculated with the spore suspension immediately after stuffing. 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 synthetic sausage casings (40 mm in diameter) and left to ripen in an ripening cabinet, after inoculation with the spore suspension when necessary (batches S and E+S). The sausages were fermented at 22 °C and 90% relative humidity (R.H.) for 12 h. After this, the temperature and R.H. were slowly reduced to 18 °C and 80%, respectively, in 60 h. Finally, the sausages were dried at 12 °C and 80% R.H. until the end of the ripening process (a total of 22 days). The results recorded here are the mean values of the results obtained with samples from three different manufacturing processes carried out with different ingredients but the same formulation and technology.

Dry matter (D.M.) was determined by drying the sample at 110 °C to constant weight. pH was measured in a homogenate of the sample with distilled water (1:10, w/v). Ammonia content was determined using the Boehringer kit for enzyme analysis. The separation of the different lipid classes was performed by TLC according to Fernández *et al.* (1995). To measure lipid oxidation during ripening, the 2-thiobarbituric acid was determined by the method described by Salih *et al.* (1987). Free amino acids and organic acidswere analyzed by HPLC as described by Bruna *et al.* (1999b).

Sensory analysis was carried out at the end of ripening by a panel of 18 tasters. Colour, texture, odour and taste were assessed using a non-structured hedonic scale in which samples were given scores of 1 (very poor) to 10 (excellent). The global quality was calculated from the expression: general acceptability = (Color x 0.1) + (Texture x 0.25) + (Odour x 0.15) + (Flavour x 0.5).

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.

Results and discussion

In relation to the lipolytic activiy of the mould (Figure 1), a significant increase in diglyceride content and, especially free fatty acids, was observed in batches inoculated with *P. aurantiogriseum* on the surface.

From TBARS (Table 1) it can be observed that, when inoculated on the surface, *P. aurantiogriseum* showed a significant antioxidative activity. Similar results have been obtained by other authors with *P. aurantiogriseum* strains isolated from dry cured ham (Núñez *et al.*, 1996, 1998; Rodríguez *et al.*, 1998). 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). The strain used in this work showed a noticeable catalase activity.

Table 1 shows that the superficial inoculation with the mould gave rise to a decrease in the lactic acid content and an increase in acetic, propionic and n-butyric acid contents. The decrease of lactic acid has also been observed by other authors in dry fermnented sausages (Grazia *et al.*, 1986, Bruna *et al.*,2000b). The addition of *P. aurantiogriseum* ICFE to sausages determined an increase in the final content of all organic acids. 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. Similar results have been reported in previous work (Bruna *et al.*, 2000a).

The mould inoculation on the surface lead to an increase in the free amino acid content in batches S and E+S compared to their respective controls (batches C and E) (Table 1). These results reflect an intense proteolytic activity of the mould as expected from previous experiences *in vitro* (Trigueros *et al.*, 1995) and in dry fermented sausages (P-3 in Toledo *et al.*, 1997). On the other hand, addition of ICFE of *P. aurantiogriseum* (batches E and E+S), caused a reduction in the free amino acid content in comparison with their respective controls (batches C and S). A drop in the content of most of the free amino acids was noticed due to the ICFE (comparing batches E *vs* C), specially Ala+Arg, Asp, Cis, His, Ile, Lys, Tau+GABA and Tyr.

Table 1 also shows the changes in ammonia content of the experimental sausages during ripening. 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 agreement with the proteolytic activity registered in the mould inoculated sausages, since ammonia is one of the main products of protein breakdown (Demeyer *et al.*, 1984, Montel *et al.*, 1998). 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 (Bruna *et al.*, 1999) suggests that deamination is one of the main pathways of amino acid breakdown (Wellner and Lichtemberg, 1971).

pH at the end of the ripening was comprised between 4.7 and 5.9 (Table 1). The higher values were reported in batches S and E+S. These observations, previously decribed by other authors in mould covered meat products (Grazia *et al.* (1986), Roncalés *et al.* (1991), Rödel *et al.* (1993) Cook (1995) *et al.* (1995)) can be attributed to the utilisation of lactate, acetate and/or the production of ammonia from protein hydrolysis due to the mould activity.

Sensory analysis (Table 3) showed that both the superficial inoculation with *P. aurantiogriseum* and the addition of the ICFE gave better scores for odour and taste and consequently for global quality. The combination of both treatments produced a higher improvement in the quality of sausages.

Conclusion

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 the 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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Table 1. Changes in organic acids (mg/100 g D.M.), pH and TBARS (mg MDA/kg D.M.) after 22 days of ripening.

Organic acid	C*	E*	S*	E+S*
Citric	38.74^a	46.27^b	45.21^b	49.22^c
Pyruvic	4.00^a	7.21^b	5.58^c	6.21^b
Succinic	0.04^a	0.11^a	0.09^a	0.12^a
Lactic	2574.14^a	2896.33^b	2165.71^c	2210.01^c
Formic+ Uric	59.37^a	78.52^b	68.41^c	79.32^b
Acetic	76.21^a	110.71^b	112.96^b	141.25^c
Propionic	1221.7^a	1632.23^b	1521.36^c	1874.36^d
N-butyric	29.21^a	49.98^b	41.00^b	42.85^b
Total	4003.41^a	4821.36^b	3960.32^a	4403.34^c
pH	4.94^a	4.62^b	5.21^c	5.84^d
TBARS	0.756^a	0.756^a	0.496^b	0.504^b

Table 2. Free amino acids and ammonia (mg/100g D.M.) after 22 days of ripening.

Amino acid	C*	E*	S*	E+S*
Asp	19.3^a	11.5^b	20.0^a	35.1^c
Glu	72.2^a	95.4^b	150.4^c	116.2^d
Hxp	n.d.	0.7^a	1.7^b	0.2^c
Ser	7.1^a	8.4^b	12.7^c	12.4^c
Asn	4.2^a	4.7^a	6.6^b	4.6^a
Gly	27.1^a	26.2^a	40.2^b	43.4^b
Gln	49.7^a	88.6^b	128.5^c	64.1^d
His	25.1^a	13.2^b	21.3^c	37.1^d
Tau+GABA	81.1^a	50.0^b	74.7^c	96.8^d
Thr	11.7^a	17.7^b	28.0^c	1.1^d
Ala+Arg	226.5^a	164.1^b	248.4^c	273.9^d
Pro	115.6^a	160.2^b	161.0^b	221.1^c
Tyr	16.2^a	10.7^b	14.7^c	19.2^d
Val	34.9^a	30.5^b	47.5^c	50.0^c
Met	24.1^a	27.3^a	13.4^b	33.2^c
Cys	5.4^a	1.4^b	2.4^c	8.0^d
Ile	41.3^a	30.4^b	46.0^c	52.9^d
Leu	38.8^a	41.5^a	66.2^b	57.0^c
Phe	24.4^a	23.4^a	35.8^b	37.4^b
Trp	34.3^a	25.1^b	38.5^a	50.2^c
Lys	50.1^a	38.5^b	61.7^c	64.3^c
Cis	1.2^a	3.3^b	3.5^b	3.0^b
Total	910.1^a	872.8^b	1353.2^c	1281.4^d
NH_3	55.4^a	65.1^a	80.2^c	89.9^c

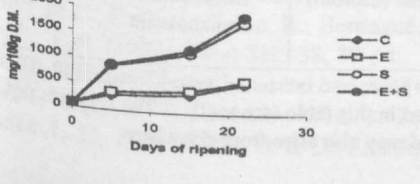
a, b, c, d: values in a row at the same ripening day with different letters are significantly different ($p < 0.05$); n.d.: not detected

*(C) Control batch, (E) Control batch added with a intracellular cell free extract of *Penicillium aurantiogriseum* (100.87 mg protein/kg), (S) Control batch superficially inoculated with a spore suspension of *Penicillium aurantiogriseum* and (E+S) Batch E superficially inoculated with the spore suspension extract of *Penicillium aurantiogriseum*.

Table 3. Sensory analysis after 22 days of ripening.

	C*	E*	S*	E+S*
Odour	4,9± 1.2^a	5.8± 0.7^b	7.9± 2.0^c	9.3± 1.1^c
Colour	7.8± 1.5^a	7.7± 1.3^a	8.0± 1.6^a	8.0± 0.8^a
Texture	6.9± 1.6^a	7.3± 1.6^a	7.7± 2.0^a	7.8± 0.8^a
Flavour	5.5± 1.7^a	6.9± 1.2^b	7.2± 1.2^b	9,1± 0,9^c
Overall quality	6.1± 1.2^a	6.9± 0.9^b	7.5± 1.1^b	8.7± 0.9^c

Figure 1. Changes in free fatty acids (mg/100g D.M.) during the ripening of the dry fermented susages



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