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Dry fermented sausages made with a protease from *Aspergillus oryzae* and/or a starter culture

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Abstract

Sausages with a starter culture (*Lactobacillus plantarum* + *Staphylococcus carnosus*), a protease (Fungal Protease from Solvay Enzymes) and both enzyme/starter were produced from the same raw matter under the same conditions for 15 days of ripening. The lowest pH values were found in Fungal/starter sausages. Significantly higher amounts of free amino acids (FAA) were seen in sausages containing enzyme from the 3rd day of ripening, whereas amino acids from peptides (PAA) were significantly higher only after 15 days of ripening. Enzyme addition gave rise to changes in 10 of the 15 FAA analyzed. Histidine was the main amino acid from the peptide fraction that increased in both sausages containing added enzyme. Although trained panelists detected some sensorial benefits in the sausages with added enzyme, the effects were not as marked as might have been expected.

Keywords: Dry fermented sausages; Fungal protease; Free amino acids; Peptide amino acids

1. Introduction

The use of starter cultures in the manufacture of dry fermented sausages achieves a controlled fermentation that ensures and enhances the production of desired end products (Bacus, 1984; Leistner, 1992). The main microorganisms used as starters are *Lactobacilli, Pediococci, Micrococci* and *Staphylococci*. Lactic Acid Bacteria (LAB) reduce the pH of the medium as a result of lactic acid production. This contributes to the preservation, aroma, texture and drying of the raw sausages. Furthermore, they produce protective agents such as acids and antibiotic-like substances (bacteriocins) (García, Bartín, Sanz, & Hernández, 1995; Rodriguez et al., 1995; Schillinger & Lücke, 1990). The reduction of nitrate and catalase formation has been attributed to *Micrococcaceae* action (Leistner, 1992).

Proteolytic and lipolytic processes, that contribute to the flavor and texture of these types of products, are catalyzed by lipases and proteases of endogenous and/or microbial origin (Arbolés & Julia, 1992; Berdagué, Monteil, Montel, & Talon, 1993; Berdagué, Montel, Talon, & Monteil, 1992; Montel, Talón, Berdagué, & Cantonnet, 1993). Regarding proteolytic processes, microbial proteinases have little influence on the formation of polypeptides during dry sausage production. However, the formation of smaller degradation products such as peptides and free amino acids is from bacterial as well as endogenous origin (Toldrá, 1992).

Addition of lipases or proteases to sausages can in some cases be used to accelerate aroma development. Several studies on the use of lipases (Fernández, Díaz, Cambero, De la Hoz, & Ordoñez, 1991; Fernández, De la Hoz, Díaz, Cambero, & Ordoñez, 1995a,b; Zalacain, Zapelena, Astiasarán, & Bello, 1995, 1996; Zalacain, Zapelena, De Peña, Astiasarán, & Bello, 1997a,b,c) and proteases (Díaz, Fernández, García de Fernando, De la Hoz, & Ordoñez, 1992, 1993, 1996; Hagen, Berdagué, Holck, Naes, & Blom, 1996; Melendo, Beltrán, Jaime, Sancho, & Roncalés, 1996; Naes, Holck, Axelsson, Andersen, & Blom, 1995) in dry fermented sausages manufacture have been published. In most of these studies, the effect of the enzyme was independently assessed of the starter culture. Gossling (1990) comparing sausages made with starter culture (*Pediococcus* and *Micrococcus*) to sausages made with an enzymatic extract from *Lactobacillus plantarum* plus 0.3% glucose and 1% lactose-maltodextrin concluded that the starter culture could be replaced by enzymes without loss of

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quality and this would reduce the time of ripening. The replacement of a starter culture by some lipases has been studied previously (Zalacain, Zapelena, Astiasarán et al., 1995; Zalacain, Zapelena, De Peña et al. 1997c). The objective of this work was to compare the effect of the addition of a selected dose of a commercial protease from *Aspergillus oryzae* (Fungal Protease), with exo- and endopeptidase activity, to a starter culture. Sausages containing both enzyme and starter were also studied.

2. Material and methods

Three types of dry fermented sausages (with starter culture, with enzyme and with both starter and enzyme) were manufactured with a standard formulation of 75% lean pork meat, 25% pork back fat, 30 g/kg NaCl, 15 g/kg dextrin, 20 g/kg lactose, 3 g/kg dextrose, 2 g/kg polyphosphates, 0.5 g/kg sodium ascorbate (E301), 0.2 g/kg $NaNO_2$ (E250), 0.15 g/kg Ponceau 4R (E124) and 1 g/kg oregano. The starter culture was a mixture of *L. plantarum* L115 (10%) plus *Staphylococcus carnosus* M72 (90%) from Lacto-Labo (TEXEL). Added enzyme was a commercial protease (Fungal Protease P from Solvay enzymes GMBH & CO.KG) with exo- and endopeptidase activity.

Fungal Protease is a mixture of acid, neutral and alkaline proteases from *A. oryzae*. It has optimal activity at 45–55°C and pH = 4.5–9.0; 0.90 HU of Fungal Protease P per g of moisture were added as described previously (Zapelena, Ansorena, Zalacain, Astiasarán, & Bello, 1998), 1 HU (Hemoglobin Unit) is the amount of enzyme that releases 0.447 mg of non protein nitrogen.

Lean pork meat and back fat were minced and all ingredients were mixed in a vacuum kneading machine. The mixture was stuffed into artificial casings of stiff collagenous material derived from beef split (60 mm diameter). Starter and/or enzyme was added to the mixture at the same time as the other ingredients. The sausages were fermented in a laboratory ripening cabinet (Kowel Model CC-I) at a constant temperature of 22°C and 85% RH for 72 h. The sausages were then transferred to a drying chamber at 15°C and 77% RH till the end of the ripening (15 days).

Four batches of each type of sausages were made. Samples were taken on the 3rd, 9th and 15th day of ripening and two sausages from each batch were analyzed at each time.

2.1. Analytical methods

2.1.1. General parameters

pH was determined using the potentiometer Orion Research Microprocessor Ionalyzer-901 using needle electrodes for solid samples (ISO, 1974). Moisture was determined by the ISO (1973) method. Water activity was determined using a Novasina Model 5803 meter.

2.1.2. HPLC analysis

Free amino acids (FAA) and amino acids from peptides were identified and quantified by reverse-phase high-performance liquid chromatography, following derivatization with phenyl isothiocyanate. Extraction of free amino acids was carried out with citrate buffer, and TCA was added to precipitate the proteins (Massi, 1963).

Non-Protein N (NPN) extraction was as described by Astiasarán, Villanueva, and Bello (1990) and amino acids from peptides were obtained from the NPN fraction by hydrolysis with 6 M HCl (110°C/22 h) (Zapelena, Zalacain, De Peña, Astiasarán, & Bello, 1997a). After chromatographic analysis, the amount of each peptidic amino acid was determined by subtracting the relative amount of free amino acid.

Phenylthiocarbamil derivatives of the different amino acid types, obtained according to Yang and Sepulveda (1985), were injected into a Perkin–Elmer (PE Nelson) high-performance liquid chromatograph equipped with a Rheodyne manual injector with a 20-μl loop, a series 200 LC pump quaternary version, and a diode array Detector operating at 255 m. A column Nova Pack Cl 8 (3.9×300 mm) from Waters was employed. The pump and detector were connected to a series 600 link, and conditions were controlled by a Turbochrom Navigator program (PE Nelson). Resolution of the peaks was accomplished using gradient elution (Zapelena, et al., 1997a) Amino acids were identified by comparison of retention times with standards (Sigma) and were quantified using the internal standard L-norleucine (Sigma). Arginine could not be quantified in the free amino acids as its peak sometimes overlapped with that of carnosine and was thus excluded from both the FAA and peptide fractions.

2.1.3. Microbial analysis

Ten grams of sausage was homogenized with 90 ml of peptone water (under sterile conditions for 2 min with a Stomacher). From this suspension decimal dilutions, in peptone water, were prepared and spread on the corresponding plates.

De Man Rogosa and Sharpe Agar No. 1 (MRS, Oxoid) were used to enumerate lactic acid bacteria (30°C/72 h) in an anaerobic jar with a CO_2-enriched atmosphere (Gaspack, BBL). *Staphylococcus* medium No. 110 (Oxoid) was used for enumeration of *Micrococcaceae* (*Staphylococcus* and *Micrococcus*; 30°C/48 h) (Pascual, 1992).

2.1.4. Sensory analysis

Quantitative descriptive analysis (QDA) was carried out as described by Zapelena, et al. (1997a) using a

trained panel. Sausages with Fungal Protease were compared with controls, which were taken as the reference samples (five for all parameters on a 1–9 scale). The selected parameters were: odor intensity (degree of odor), pleasant odor (hedonic parameter that evaluated the degree of pleasure), saltiness (amount of salt perceived during chewing), spiciness (degree of spicy taste perceived during chewing), astringency (degree of astringency perceived after swallowing) and overall acceptability.

2.1.5. Data analysis

Data analysis was carried out with Statgraphics STSC Inc. program (version 4.0). This is a registered trademark of Statistical Graphics Corp. Analysis of variance (ANOVA) was used to study significant differences ($p < 0.05$) for each parameter at the same time for the three types of sausage and in the same type of sausage as a function of ripening time.

Student's *t*-test was used for each sensory parameter to determine whether there were significant differences between sausages.

3. Results and discussion

The pH, water activity (Aw) and moisture of the sausages are shown in Table 1. No differences were observed in water activity and moisture between the three types of sausages (Table 1). Naes et al. (1995) observed a greater water loss in sausages containing NCDO 151 proteinase than in controls.

In the finished products, sausages containing starter had a lower pH than sausages without starter culture. Obviously, this is due to the lower lactic acid bacteria counts ($< 10^8$ u.f.c./g) in sausages without starter (Fig. 1). Although no increase in lactic acid bacteria counts were observed in sausages with both starter and enzyme, the lowest pH values were found in these products. It could be that the simultaneous addition of starter and enzyme increased bacterial metabolic activity. Hagen et al. (1996) pointed out that NCDO 151 proteinase had a stimulating effect on the metabolism of the starter (*L. sake* L45) as a consequence of the products of protease activity increasing in number. In a previous paper (Zapelena, Zalacain De Peña, Astiasarán, & Bello, 1997b) no significant effect on the pH was observed when a metalloproteinase from *Bacillus subtilis* (Neutrase) plus a starter culture (*L. plantarum* + *S. carnosus*) were added to dry fermented sausages. Other authors have observed that proteinases can affect the pH of these types of products (Díaz et al., 1993; Naes et al., 1995).

Larger differences were found in *Micrococcaceae* counts than in lactic acid bacteria. Sausages without starter had smaller *Micrococci* counts during ripening (Fig. 1). Similar results were found when a lipase from *Aspergillus* was added to sausages without starter (Zalacain, et al., 1997c). *Micrococacceae* sensitivity to lack of oxygen and acid (Leistner, 1992) could be the reason for the lower growth of these bacteria. Gossling (1990) found smaller counts of both lactic acid bacteria and *Micrococci* in sausages containing an enzymatic extract from *L. plantarum* than in those made with starter culture.

Profiles of free amino acids (FAA) and amino acids from peptides (PAA) are shown in Tables 2 and 3, respectively. Higher amounts of total FAA and PAA were found in sausages with added enzyme. Similar results were found when Fungal Protease was used in 3 mm particle size sausages (Ansorena, Zapelena, Astiasarán, & Bello, 1998). The addition of Pronase E, with exo and endopeptidase activity, also gave rise to increases in these nitrogen fractions (Díaz et al., 1993). Neutrase, a metalloproteinase with only endopeptidase activity, caused significant increases in amino acids and α-NH_2–N from peptides but not in FAA and free α-NH_2–N (Zapelena, Zalacain et al., 1997a,b). Naes et al. (1995) found similar results with a serinproteinase from *L. paracasei* NCDO151. Other authors have observed increases in free AA and free α-NH_2–N when other endoproteases have been added to dry fermented sausages (Díaz et al., 1996; Melendo, Beltrán, Jaime, Sancho, & Roncalés, 1996). They suggested that the higher concentration of large peptides may give a greater availability of substrate for degradation by the exopeptidases present. The addition of a proteolytic extract with exopeptidase activity could ensure the increase of FAA content.

Table 1
pH, water activity (Aw) and moisture in control (C + St), Fungal (FP) and Fungal/starter (FP + St) sausages

	pH			Aw			Moisture (%)		
	C + St	FP + St	FP	C + St	FP + St	FP	C + St	FP + St	FP
3 days	5.45bA[a]	5.47bA	5.68bB	0.944cA	0.940cA	0.933cA	46.96cB	44.64cA	45.22cA
9 days	5.42bB	5.06aA	5.64abC	0.906bA	0.895bA	0.906bA	38.20bB	35.02bA	35.58bA
15 days	5.29aB	5.02aA	5.60aC	0.865aA	0.858aA	0.870aA	32.03aA	33.19aA	33.73aA

[a] Within a column, different lowercase letters denote significant differences ($p < 0.05$). Within a row different uppercase letters denote significant differences ($p < 0.05$) between products.

Table 2
Free amino acids (mg/100 g of dry matter) in control (C + St), Fungal (FP) and Fungal/starter (FP + St) sausages

	3 days			9 days			15 days		
	C + St	FP + St	FP	C + St	FP + St	FP	C + St	FP + St	FP
Asp	trace aA[a]	5.05bC	3.79aB	trace aA	7.77cC	3.72aB	0.07bA	3.01aB	2.84aB
Glu	13.63aA	12.26aA	34.48bB	30.74bB	24.84bA	31.91abB	50.16cB	25.07bA	30.50aA
His	27.17aA	23.52aA	47.80aB	40.88bB	31.77bA	47.80aB	46.06bA	46.75cA	53.55bA
Lys	46.57aA	54.57aB	78.47aC	71.12bA	87.47bA	85.63aA	90.10cA	99.06cAB	106.80bB
Thr	59.81bB	81.68ac	43.91aA	29.06aA	108.97bB	42.36aA	32.50aA	100.99bB	41.07aA
Ser	8.36aA	11.29aAB	16.17bB	12.99aB	28.13bc	11.06aA	28.78bA	26.07bA	27.38cA
Gly	38.41bB	13.24aA	56.95cC	48.77cB	24.12bA	50.84bB	30.08aA	28.07bA	45.21aB
Ala	42.22aB	19.30aA	94.34aC	83.47bB	27.52abA	93.86aB	111.34cB	35.71bA	102.64aB
Met	24.95aA	35.29aB	32.15aAB	32.92bA	46.57abA	38.18aA	37.58bA	51.97bC	44.80bB
Val	32.69aA	56.98aB	49.52aB	51.24bA	82.19bB	63.50abA	63.06cA	79.37bB	82.57bB
Ile	32.58aA	57.71aB	45.74aAB	44.70bA	78.00bB	58.95aA	50.34bA	80.48bB	73.96bB
Leu	41.81aA	68.66aB	58.09aB	56.92bA	83.83bB	70.43abAB	65.32cA	89.08bB	87.02bB
Tyr	32.87aA	48.30aB	39.55aA	39.18bA	59.64bB	44.71aA	45.00cA	56.52bB	50.17aAB
Phe	49.35aA	65.31aB	47.64aA	66.56bA	88.67bA	61.92abA	75.96bA	83.34bA	75.42bA
Pro	52.83aA	87.59aB	59.94aA	64.18abA	107.52bB	58.77aA	72.12bA	101.84bB	68.55aA
Total	503.25aA	640.75aB	688.48aB	672.73bA	887.01bB	748.84abAB	798.47cA	907.33cB	892.48bB

[a] Within a row, different lowercase letters denote significant differences ($p < 0.05$) with regard to time and different uppercase letters between products at each time.

Table 3
Amino acids from peptides (mg/100 g of dry matter) in control (C + St), Fungal (FP) and Fungal/starter (FP + St) sausages

	3 days			9 days			15 days		
	C + St	FP + St	FP	C + St	FP + St	FP	C + St	FP + St	FP
Asp	170.02aC[a]	102.69aA	146.89aB	198.29aB	131.36bA	171.62bB	181.62aA	170.75cA	171.23bA
Glu	365.77aC	282.72aA	322.86aB	453.09bA	485.94cA	400.84bA	430.79bA	430.97bA	418.94bA
His	530.83bA	810.42aB	755.54aB	536.76bA	787.68aB	804.22aB	429.58aA	704.12aB	805.21aB
Lys	115.36aB	51.95aA	88.65aB	128.88aA	111.97cA	127.72bA	130.24aC	78.91bA	117.46bB
Thr	–A	–aA	15.65aB	3.75aA	–aA	31.95cB	13.82bB	–aA	25.96bC
Ser	45.99bA	58.66bA	49.20aA	17.49aA	19.90aA	54.32aB	25.96aA	60.51bB	44.16aB
Gly	148.99aC	53.04aA	62.75aB	163.95bC	116.36bB	83.87bA	167.69bC	117.12bB	90.89bA
Ala	139.07bB	118.60aB	41.83aA	139.53bB	159.31cC	80.32bA	112.87aB	143.82bC	80.33bA
Met	18.84bB	–aA	0.49aA	13.82aB	–aA	26.71bC	12.50aB	–aA	–aA
Val	40.47abA	37.37aA	37.25abA	56.48cB	38.94aA	47.27bAB	44.92bcA	31.68aA	33.92aA
Ile	20.97aB	–aA	21.57bB	30.27aC	13.34bA	20.66bB	21.12aC	1.57aA	10.53aB
Leu	27.92abC	–aA	14.13bB	37.78bB	9.26bA	15.21bA	22.57aC	0.46aA	4.64aB
Tyr	11.63bB	1.09bA	–aA	4.82aB	–aA	10.56bC	–A	1.31bB	–aA
Phe	17.18bB	–aA	11.53bB	22.07bB	–aA	22.78cB	8.38aB	–aA	–aA
Pro	68.14aA	54.39aA	55.09aA	78.37bB	58.14aA	83.32bB	85.02bB	51.10aA	104.03cC
Total	1721.18aB	1570.93aA	1623.4aAB	1885.35bA	1932.21bA	1981.38bA	1687.08aA	1792.30abB	1907.31bC

[a] Within a row, different lowercase letters denote significant differences ($p < 0.05$) between sausages with regard to time and different uppercase letters between products at each time.

The exopeptidase activity of Fungal Protease was observed from the 3rd day of ripening as higher amounts of FAA were observed in the sausages made with enzyme. Amounts of PAA in sausages with added enzyme were significantly higher than the controls at the end even though the contents were significantly lower at the 3rd day than in the controls. In the last week the total amount of PAA decreased in all products although being this decrease was lower in the sausages with added enzyme. This suggests that the Fungal Protease shows exopeptidase activity throughout ripening, whereas the endopeptidase activity is only observed on the 9th day of ripening.

Differences in the amounts and profiles of FAA and PAA have direct effects on taste since many of these compounds are sweet, bitter, sour, salty or yield a umami taste (Kato, Rhue, & Nishimura, 1989; McLeod, 1994). They may also lead to different metabolic compounds

(Davidek, Velisek, & Podarny, 1990; Mateo & Zumalacárregui, 1996) which accelerate or change the aroma of these products.

Although total FAA was similar between enzyme and enzyme plus starter containing sausages, different amino acids profiles were found in these products. Fungal/starter sausages showed differences in 10 of the 15 FAA found in control sausages. The amounts of Asp, Thr, Met, Val, Ile, Leu, Tyr and Pro were higher whereas those of Glu and Ala were lower. Met, Val, lle, Leu, Tyr and Pro, which are some of the eight amino acids that increased, have a bitter taste (Kato et al., 1989). Starter culture addition when Fungal Protease was used gave rise to changes only in five amino acids compared to sausages containing only enzyme. Sausages with Fungal/starter had higher amounts of Thr, Met and Pro and lower amounts of Gly and Ala than sausages containing only enzyme.

In the PAA fractions the use of enzyme gave rise to changes in 12 of the 15 analyzed AA. This was the major amino acid that increased in both sausages containing enzyme. The peptides of Fungal/starter sausages had higher amounts of Gly and Ala, and lower amounts of Lys, Thr, lle, Leu and Pro than the Fungal Protease containing ones.

When an exogenous protease is used to accelerate proteolysis an increase in flavor is expected. Díaz et al. 1997; Fernández, García de Fernando, De la Hoz & Ordoñez (1997) did not observe significant differences in flavor between control and sausages containing Pronase E, papain and Aspartyl proteinase. Zapelena, Zalacain et al. (1997a) found a slightly better score for pleasant odor, taste and overall acceptability in sausages containing Neutrase. Bromelain was found to give a more intense flavor in sausages, but texture defects were observed (Melendo et al., 1996). NCDO 151 proteinase gave significantly better scores for flavor intensity,

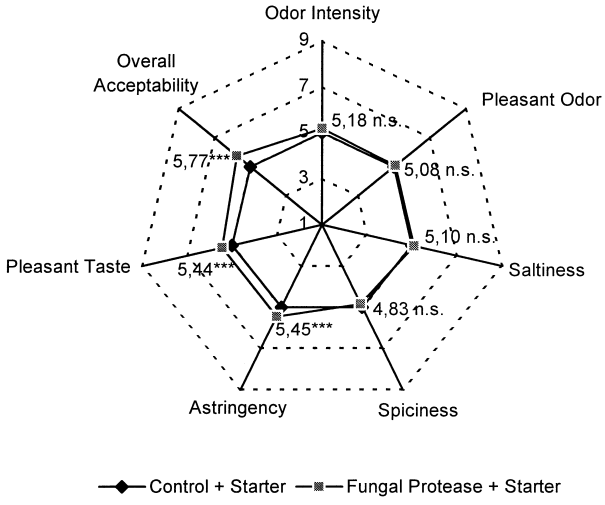

Fig. 2. Sensory analysis of sausages made with Fungal protease and starter culture.

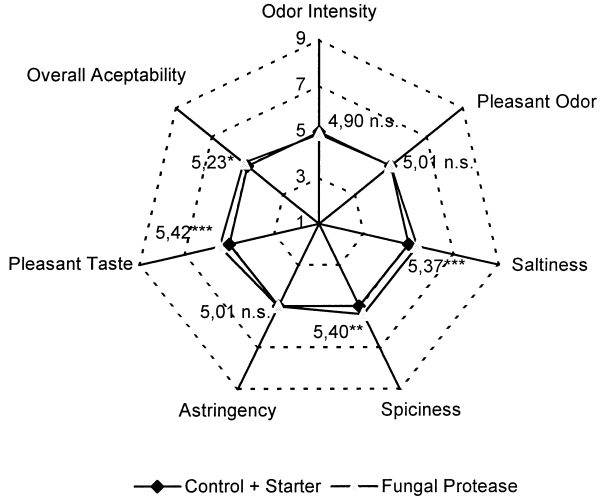

Fig. 3. Sensory analysis of sausages made only with Fungal protease.

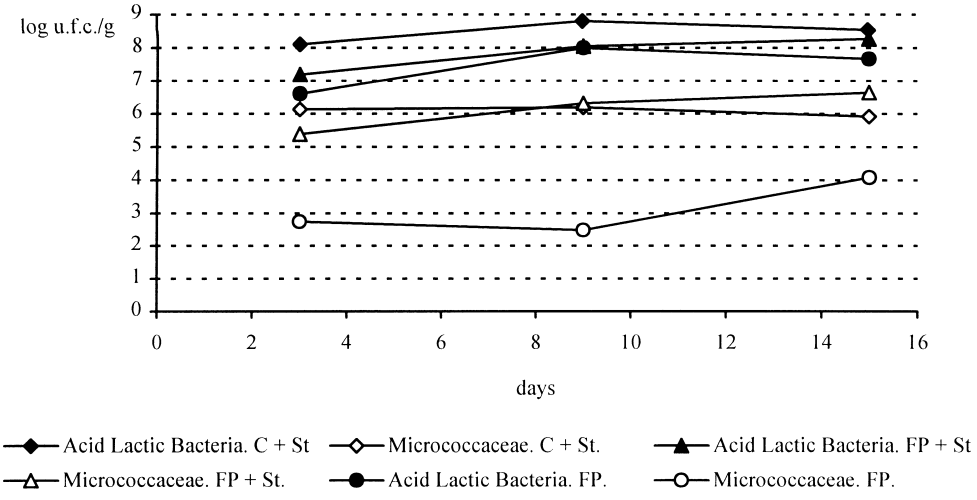

Fig. 1. Lactic acid bacteria and *Micrococcaceae* evolution in control (C + St), Fungal (FP) and Fungal/starter (FP + St) sausages.

acidic and bitter taste in dry fermented sausages after 14 days of ripening (Naes et al., 1995). In our study, Fungal/starter sausages were scored as more astringent (Fig. 2) and sausages containing Fungal as significantly more salty and spicy (Fig. 3) than control sausages. Both products scored better for taste and acceptability. The overall acceptability for Fungal/Starter sausages was higher than obtained with Neutrase under similar conditions (Zapelena, et al., 1997a). Although these sensorial benefits were statistically significant they would probably not have been detected by most consumers.

In summary, the added enzyme gave rise to significant differences in pH, free amino acids and amino acids from peptides. However, the effect of these changes on the sensory quality of dry fermented sausages although statistically significant, may not be meaningful.

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