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Use of Lipase from *Rhizomucor miehei* in Dry Fermented Sausages Elaboration: Microbial, Chemical and Sensory Analysis

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ABSTRACT

Three different amounts of lipase (0.075, 0.100 and 0.150 LU/g) from Rhizomucor miehei *(Palatase M 200L Novo Nordisk) were used to determine the correct amount to use in dry fermented sausages. Determination of acidity values through fourteen days of ripening showed that 0.100 LU/g was the most appropriate.*

Two types of fermented sausages were manufactured, addition of the enzyme being the only difference between them. Addition of Palatase did not affect product stability (pH and A_w), and the growth of micro-organisms. In spite of the increase in acidity value, no rancidity developed as determined by both chemical and sensory analysis. Increases in the liberation of palmitic, palmitoleic, stearic, oleic and linoleic acids were found when lipase was used. Juiciness and taste were slightly better in the sausages with Palatase than in those without, but these differences were not reflected in the overall acceptability. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

There are few papers in the literature about the use of enzymes in the manufacture of dry fermented sausages. In contrast, other dry fermented products such as cheeses have been the subject of extensive studies as they give rise to the possibility of accelerating ripening and obtaining high quality products.

Bacus (1986) pointed out that, although similar in principle to fermented dairy products, fermented meat technology has progressed at a much slower pace. This is to be expected since meat is more difficult to standardise and control due to its nonuniformity and non-fluid nature (Nurmi & Niinivaara, 1964).

Gossling (1990) used a lactic acid bacterial enzyme extract in the manufacture of dry fermented sausage and found that high quality products could be obtained in less time than with the traditional process. Fernández *et al.* (1991, 1995*a*,*b*) studied the effect of the

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addition of pancreatic lipase on the ripening of dry-fermented sausages and found addition of 60 and 90 units of pancreatic lipase was useful in enhancing flavour and reducing the ripening time. They also suggested that lipases from other sources, different ripening conditions and/or the modification of other variables in the ripening process may improve the results.

In a previous study (Zalacain *et al.*, 1996) we employed a lipase from *Candida cylindracea* to produce dry fermented sausages and showed there was no effect on the oxidative processes due to higher lipolytic activity.

In this paper we have studied the use of different amounts of a *Rhizomucor miehei* lipase in the production of dry fermented sausages. Furthermore, we describe differences in the microbial characterisitics, lipidic fraction and sensory evaluation of sausages when a specific amount of this lipase is added to a traditional formulation.

MATERIAL AND METHODS

For the selection of the most convenient lipase amount, three types of sausages with different amounts of lipase (0.075 LU/kg, 0.100 LU/kg and 0.150 LU/kg mixture) were produced. For each sausage containing enzyme, one without lipase was made at the same time.

The lipase utilised was Palatase M 200L, a microbial enzyme from *Rhizomucor miehei* (Novo Nordisk). The activity of the enzyme was 200 LU/g. [One Lipase Unit (LU) is the amount of enzyme which liberates one micromol of butyric acid per minute from an emulsified tributyrin substrate at pH 7.0 and 30°C.] The enzyme was added at the same time as the other ingredients.

The different types of sausages were made with a standard formulation of lean pork meat 75%, and pork back fat 25%. Other ingredients were added as follows: NaCl 30 g/kg mixture, Dextrin 15 g/kg, lactose 20 g/kg, dextrose 3 g/kg, polyphosphates 2 g/kg, sodium ascorbate 0.5 g/kg, $NaNO_2$ 0.2 g/kg, red pepper 20 g/kg, cayenne pepper 0.5 g/kg, garlic 6 g/kg, Ponceau 4R 0.15 g/kg and oregano 1 g/kg. Lean pork meat and pork back fat were minced in a cutter and subsequently mixed with the other ingredients in a vacuum kneading machine and stuffed into artificial casings (60 mm diameter). The sausages were fermented in a laboratory ripening cabinet (Kowel Model CC-I) at 22°C and RH 85% for 72 h, after which the sausages were transferred to a drying chamber at 15°C and RH 77% until the end of ripening (15 days).

Samples were taken on the 3rd, 7th, 10th and 14th day, and the acidity values measured.

For the study of the addition of Palatase in a traditional formulation, two types of sausages were manufactured. One with starter culture and the other with starter and the commercial lipase. The starter culture was a mixture of *Lactobacillus plantarum* L115 (10%) + *Staphylococcus carnosus* M72 (90%) from Lacto-Labo (TEXEL). The amount added was 10^6–10^7 cfu/g of mixture

These sausages were made and ripened as before and samples were taken on day 0 and on the 3rd, 9th and 15th days.

Analytical methods

Microbial analysis

Ten grams of sausage were homogenised with 90 ml 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 appropiate plates.

Plate count Agar No 1 (PCA, Oxoid) for total counts of aerobic mesophiles (30°C/48 h), De Man Rogoso and Sharpe Agar No 1 (MRS, Oxoid) for lactic acid bacteria (30°C/72 h) in an anaerobic jar with a CO_2-enriched atmosphere (Gaspack, BBL), Violet Red Bile Glucose (VRBG, Oxoid) for Enterobacteriaceae (30°C/24), and *Staphylococcus* medium No 110 (Oxoid) for Micrococcaceae, *Staphylococcus* and *Micrococcus* (30°C/48 h).

Chemical analysis

pH was determined with a pH meter micropH 2000 with Needle electrode (CRISON Instrument S.A., Barcelona). Water activity (A_w) was determined using a Novasina Model 5803meter. Humidity was determined using Method ISO R-1442 (ISO, 1973).

Thiobarbituric acid value (TBA) was determined according to Tarladgis *et al.* (1960, 1964). Qualitative extraction of fat was made according to Bligh & Dyer (1959). Peroxide value was determined using Method ISO 3960 (1977). Acidity value (g oleic acid/100 g fat) was determined using Method ISO 1740 (1980). Carbonyl compounds were determined according to Henick *et al.* (1954).

To determine free fatty acids (FFA), lipid was extracted from the sausages and FFA were recovered by shaking the extract with anion exchange resin Amberlyst A-26 (Needs *et al.*, 1983). The resin-bound FFAs were methylated directly and the individual acids quantified using as internal standard of heptadecanoic acid (Sigma), by gas chromatography.

Determination was carried out using a Perkin Elmer Model Sigma-300/ Dual FID (oven temp. 185°C, detector temp. 240°C, injector temp. 250°C). The fatty acids were identified and quantified with appropiate standards (Sigma).

Total fatty acids were extracted and methylated using Method UNE 55-118 (1979). An aliquot (0.25 g) of the lipid extract was dissolved in 4 ml of hexane (Merck) and the internal standard [methyl heptadecanoate (Sigma)] added. The methyl esters were formed by addition of 4 ml of 2M KOH in methanol. The mixture was shaken and allowed to stand for 10 min. Determination was carried out using the same conditions as for the free fatty acids.

Sensory analysis

Quantitative Descriptive Analysis (QDA) was carried out on the two types of sausages as described in Zook & Pearce (1988).

The panel was composed by 10 judges previously selected by triangle tests and trained.

Fat cleanness, juiciness, odour intensity, acidity, rancidity, spiciness, and pleasant taste and overall acceptability were evaluated on a 1–9 hedonic scale.

Four panel replications were carried out on each sample.

Statistical analysis

The values shown in the tables are the means of eight determinations and their standard errors.

Analysis of variance was used to determine significant differences ($p < 0.05$) for every parameter at the different times, in the same type of sausage.

Student's *t*-test was used to determine whether there were differences in the sensory scores.

RESULTS AND DISCUSSION

Selection of the lipase amount

Sausages with lipase showed higher acidity values than their controls at all sampling times (Table 1). It can be seen from Table 1 that the greater the lipase content, the higher the

acidity value. Demeyer *et al.* (1974) stated that FFA content may reach between 1 and 7% in the finished product. 0.150 LU/g produced high acidity (6.57 g oleic acid/100 g fat), so 0.100 LU/g (acidity 5.36 g oleic acid/100 g fat) was considered more appropriate for further study.

Effects of lipase addition

The addition of Palatase had no effect on the growth of micro-organisms (Table 2). This is important because of the relevance of lactic acid bacteria and micrococci to product quality. A similar result was found between control and batches to which pancreatic lipase had been added (Fernández *et al.*, 1995*a*). The similarity in growth of Lactobacilli gave rise to a similar degrees of acidification reaching pH values of 4.95 and 4.76 in the products with and without lipase at the end of the process. Thus, no problems should be seen in relation to product stability and protein coagulation, which are related to acidification. The use of lipase from *Candida cylindracea* also gave a normal pH value for this type of sausage (Garriga *et al.*, 1988; Astiasarán *et al.*, 1990; Marquina *et al.*, 1993).

There were no differences in moisture contents, decreasing from 54.8% to 39.9% in the product without lipase and from 55.0% to 38.0% in the product with lipase. No significant differences were found in the water activity (0.90 and 0.92 at the end of the process for products with and without lipases). These results agree with those of Fernández *et al.* (1995*a*) who found no effect of pancreatic lipase on water loss and activity in fermented sausage.

The important role of lipid oxidation on sensory characteristics has been studied by several authors. Berdagué *et al.* (1993) showed that the oxidation of lipids accounted for about 60% of the total compounds responsible for dry sausage flavour. However, if lipid oxidation is excessive, it can limit shelf life. TBA and peroxide values and the quantity of

TABLE 1
Acidity Value (g oleic acid/100 g fat) in Sausages Prepared Using Different Amounts of Lipase

Time (days)	*Type of sausage*					
	Control	*0.075 LU*	*Control*	*0.100 LU*	*Control*	*0.150 LU*
3	1.70 ± 0.01	2.30 ± 0.05	1.75 ± 0.01	2.64 ± 0.08	1.99 ± 0.01	2.65 ± 0.01
7	2.42 ± 0.01	3.79 ± 0.01	2.27 ± 0.04	4.02 ± 0.11	3.00 ± 0.15	3.79 ± 0.03
10	2.94 ± 0.04	4.27 ± 0.06	2.93 ± 0.06	4.94 ± 0.07	3.68 ± 0.15	5.20 ± 0.09
14	3.56 ± 0.05	5.34 ± 0.01	3.15 ± 0.02	5.36 ± 0.02	4.18 ± 0.00	6.57 ± 0.07

Values are mean of eight determinations ± standard errors.

TABLE 2
Evolution of Microorganisms During the Ripening in Both Types of Sausages [log (cfu/g)]

	Aerobic mesophiles		*Lactobacilli*		*Micrococcaceae*		*Enterobacteriaceae*	
	Starter	*Starter + Palatase*	*Starter*	*Starter + Palatase*	*Starter*	*Starter + Palatase*	*Starter*	*Starter + Palatase*
0 days	7.0	6.6	4.4	4.2	6.6	6.5	1.7	1.7
3 days	8.1	8.7	8.0	8.5	6.7	6.0	0	0.4
9 days	8.6	8.5	8.3	8.4	6.6	5.6	0	0
15 days	8.3	8.6	8.4	8.4	6.2	5.3	0	0

carbonyl compounds are traditional parameters used to quantify lipid oxidation. No increase in these parameters was observed as a consequence of the use of lipase. At the end of the process values for products with and without lipase were respectively: TBA number 0.45 and 0.50 mg MA/g d.m., peroxides 2.17 and 4.19 meq O_2/g fat, carbonyl compounds 18.08 and 19.92 μmol/g fat. In fact, slightly higher values were found for the three parameters at the end of the process in the product without lipase. The scores obtained in the sensory analysis for rancidity agree with these results (Table 5). In agreement with these results no rancidity problems were found with the use of lipase from *Candida cylindracea* as measured by chemical parameters (Zalacain *et al.*, 1995), or by the use of pancreatic lipase as measured by sensory evaluation (Fernández *et al.*, 1995*b*). This is important because the increase in lipolytic activity due to lipases could give rise to increased lipid oxidation.

Increased lipolysis in the sausage with Palatase caused a greater increase in the acidity values during ripening, although in the sausage without lipase the acidity value in the initial phase was higher (1.16 and 1.42%, respectively). At all other times it was lower, the differences between both sausages being of 0.69, 0.79 and 1.58 at 3, 9 and 15 days, respectively. However, the final value at the end of the drying process in the product with lipase (5.81%) is normal (Johansson *et al.*, 1994).

Evolution of free fatty acids during ripening is shown in Table 3. Increases in free palmitic, palmitoleic, stearic, oleic and linoleic acids were found when lipase was used. Lipolysis caused a more pronounced release of FFA in the sausage with lipase, with the amounts of every FFA being higher on the third day of fermentation. However, from the ninth day myristic, linoleic and linolenic acids did not show significant differences between the products.

In both types of sausage, free fatty acid contents increased as oleic > palmitic > stearic > linoleic during ripening. A lower release of linoleic acid was observed in the sausage with lipase. This was also seen for pancreatic lipase (Fernández *et al.*, 1995*a*,*b*) who

TABLE 3
Evolution of Free Fatty Acids During Ripening (mg/100 g dry matter)

		0 days	*3 days*	*9 days*	*15 days*	Δ
Myristic	St	5.8 ± 0.5^a	6.0 ± 0.3^a	12.2 ± 1.3^b	22.6 ± 1.1^c	16.8
	St + Palatase	3.8 ± 0.4^a	8.9 ± 0.4^b	15.3 ± 1.0^c	19.9 ± 0.6^d	16.1
Palmitic	St	62.7 ± 1.2^a	73.7 ± 1.6^a	169.4 ± 2.1^b	215.9 ± 6.8^c	153.2
	St + Palatase	39.3 ± 0.6^a	123.1 ± 1.2^b	221.1 ± 5.5^c	295.4 ± 2.6^d	256.1
Palmitoleic	St	7.2 ± 0.7^a	12.6 ± 0.3^b	33.3 ± 0.6^c	43.3 ± 0.6^d	36.1
	St + Palatase	7.4 ± 0.3^a	28.0 ± 0.3^b	54.4 ± 1.0^c	69.9 ± 1.1^d	62.5
Stearic	St	16.9 ± 1.5^a	38.0 ± 2.1^b	94.5 ± 0.9^c	110.9 ± 1.6^d	94.0
	St + Palatase	10.3 ± 0.4^a	69.2 ± 1.8^b	115.5 ± 0.2^c	119.7 ± 2.4^c	109.4
Oleic	St	34.4 ± 1.1^a	67.6 ± 2.2^a	424.2 ± 5.8^b	601.3 ± 16.6^c	566.9
	St + Palatase	43.7 ± 0.9^a	228.5 ± 2.3^b	607.4 ± 12.2^c	722.8 ± 10.2^d	679.1
Linoleic	St	41.7 ± 0.7^a	52.3 ± 0.9^b	114.7 ± 1.3^c	126.5 ± 3.3^d	84.8
	St + Palatase	36.6 ± 0.7^a	67.4 ± 0.9^b	113.6 ± 1.6^c	118.5 ± 2.3^c	81.9
Linolenic	St	11.0 ± 1.0^a	11.1 ± 1.1^a	22.6 ± 0.8^b	25.5 ± 1.6^b	14.5
	St + Palatase	5.2 ± 0.1^a	16.7 ± 0.8^b	25.4 ± 1.3^c	28.8 ± 0.7^c	23.6

Values are mean of eight determinations ± standard errors.
In the same row, different superscripts denote significant differences ($p < 0.05$) between the ripening times.
Δ: increment observed between 0 days and 15 days.
St: control sausage. St + Palatase: sausage with Palatase.

pointed out that as this fatty acid is the main substrate for lipid oxidation (Forss, 1972), its accumulation may not be detected. Thus, the differences in rates of liberation of different FA between the two sausages may be related to the specificity of the enzymes, the degradation and further oxidation of these acids or different amounts of total fatty acids. In relation to the last point, the amounts of free fatty acids as a proportion of the total fatty acids are shown in Table 4. It can be seen that the percentage FFA to Total FA content for every fatty acid were greater in the sausage with lipase, in spite of its lipolytic specificity (1,3-specific). Dugan (1976) reported that saturated fatty acids normally occupy position 1, short unsaturated acids and palmitic acid position 2 and long unsaturated fatty acids position 3 in the triglycerides of pork fat. Stahnke (1995) showed that unsaturated fatty acids were more prone to hydrolysis than saturated fatty acids during sausage ripening. This was because these two unsaturated fatty acids were at the outer positions (1 + 3) (Coleman, 1961), and were more prone to attack by lipases.

Table 5 shows the results of the sensory evaluation. The addition of Palatase did not produce significant differences in fat cleanness, odour intensity, acidity, rancidity, spiciness and overall acceptability. The only differences between the sausages were in juiciness and pleasant taste, both being significantly higher in the sausage with added lipase.

TABLE 4
Total Fatty Acids (TFA) (mg/100 g dry matter) and Percentage Free Fatty Acid for Each Fatty Acid (free fatty acid×100/total fatty acid) at 15th day

Fatty acids	*Type of sausage*			
	Starter		*Starter + Palatase*	
	TFA	*FFA×100/TFA*	*TFA*	*FFA×100/TFA*
Myristic	46.0 ± 0.4	49.2	29.2 ± 0.7	68.3
Palmitic	6568.3 ± 64.4	3.3	3660.2 ± 35.5	8.1
Palmitoleic	1220.3 ± 12.2	3.5	872.8 ± 7.0	8.0
Stearic	3216.2 ± 87.7	3.4	1667.0 ± 13.6	7.2
Oleic	14024.6 ± 44.9	4.3	7731.3 ± 22.7	9.3
Linoleic	4509.9 ± 19.9	2.8	2908.2 ± 45.7	4.1
Linolenic	375.3 ± 14.4	6.6	282.6 ± 4.6	10.2

All values are means ± standard error of eight determinations.

TABLE 5
Sensory Evaluation of the Two Types of Sausages at 15th Day of Ripening

	Type of sausage		*L.S.*
	Starter	*Starter + Palatase*	
Fat cleanness	6.4 ± 0.3	6.8 ± 0.2	ns
Juiciness	5.0 ± 0.3	6.0 ± 0.2	**
Odour intensity	5.5 ± 0.2	5.1 ± 0.2	ns
Acid taste	4.4 ± 0.4	4.0 ± 0.4	ns
Rancid taste	2.2 ± 0.3	1.7 ± 0.2	ns
Spicy taste	3.8 ± 0.4	3.5 ± 0.4	ns
Pleasant-taste	5.1 ± 0.2	5.7 ± 0.2	*
Overall acceptability	5.3 ± 0.2	5.8 ± 0.2	ns

Mean values (± standard error) from the scores of the 10 judges.
L.S.: level of significance; ns: not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$.

These results support the suitability of using enzymes in dry fermented sausages. Obviously, more studies need to be carried out, especially to establish if this new technology can reduce the ripening time, and thus bring economic benefits with no detriment to quality.

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