LIPOLYTIC ACTIVITY OF MICROORGANISMS ISOLATED FROM DRY-CURED HAM.

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INTRODUCTION.

Dry-cured ham is a typical Spanish meat product, produced and consumed extensively. The Spanish Meat Industries Association estimates 1988 production at some 25 million units.

During the curing process significant physical and enzymatic chemical changes occur, mainly proteolytic and lipolytic, directly affecting the development of aroma and taste. It has not been specifically established whether the origin of these changes is basically microbial or tissular (Cantoni et al., 1970a; 1971; Flores et al., 1984; 1985; 1988).

During the last few years studies of the evolution of microbial flora along curing process have been carried out, on both Parma ham (Giolitti et al., 1971; Baldini et al., 1977; Dellaglio et al., 1984) and Spanish ham (Francisco et al., 1983; Jociles et al., 1983; Hugas and Monfort, 1986; Carrascosa et al., 1988). However there is insufficient information concerning the functional properties of these microorganisms that are directly related to the development of the typical sensory characteristics of ham. Mourey (1978) has isolated lipolytic flora from different meat products, including ham, finding values of 10^3 and 10^4 cfu/g. Cantoni study the lipolysis from the subcutaneous adipose tissue of raw and cured ham (1970a,b) and indicates that it is due to microbial lipases and, to a lesser extent, tissular lipases. Comi et al. (1983) have studied the lipolytic activity of yeasts isolated from Parma ham on various substrates.

In a previous study, during two industrial curing processes (slow and fast), the following microorganisms were isolated: micrococcacea (Staphylococcus xylosus), lactic acid bacteria (Pediococcus pentosaceus and Lactobacillus curvatus) and yeasts (Cryptococcus albidus) (Silla et al., 1989; Molina et al., 1989a, b, c).

Taking into account the numerous studies of lipolytic activity of microorganisms isolated from diverses foods (Mourey et al., 1977; Alifax, 1979; Delarras, 1982; Banerjee et al., 1985; Papon and Talon, 1988; Papon et al., 1988), the aim of this work is to examine the lipolytic activity of the microorganisms previously isolated in order to contribute to the information about their possible participation in the development of aroma in ham.

MATERIAL AND METHODS.

The microorganisms used in this study were isolated and identified along ham dry-curing process, and comprise micrococcacea (Staphylococcus xylosus), lactic acid bacteria (Pediococcus pentosaceus and Lactobacillus curvatus) and yeasts (Cryptococcus albidus) (Silla et al., 1989; Molina et al., 1989a, b, c).

1- Preparation of the model substrate.

The model substrate consists of fat from the subcutaneous ham tissue (4 gr.) plus 20 ml. of a supplement, depending on the microorganism being used:

a- Micrococcacea: peptone (10 gr.), yeast extract (5 gr.), meat extract (5 gr.), 4% NaCl, and 1000 ml. of water (Soncini et al., 1982).

b- Lactic acid bacteria: 5.2% MRS-broth from which the sodium acetate, Tween 80 and ammonium citrate have been removed.

c- Yeasts: peptone (5 gr.), yeast extract (5 gr.) and 1000 ml. of water. The medium used by Comi et al. (1983), but without Tween 80 and agar.

All the substrates are sterilized at 121ºC for 20 minutes.

2- Microbiological analysis.

Preparation of the inocculation

The concentration of micro-

organisms inoculated is adjusted to correspond to point 3 on the McFarlan scale. Subsequently the inoculated substrates and non- inoculated controls are incubated at 37ºC for 12 days. This incubation temperature is choosen in order to keep the fat fluid.

Microbial counts

Viables of the different strains are counted on days 0, 4, 8 and 12 in order to check microbial development, using the following culture media: Chapman agar (micrococcacea), MRS agar (lactic acid bacteria) y Malt agar (yeasts).

3- Chemical analysis.

Lipolytic activiy is evaluated by measuring the volatile and non-volatile acidity in the controls and inoculated samples on days 0 and 8.

Measurement of volatile acidity

The steam distillation method is used (Halvarson, 1972). The distillate is collected in excess alkali and evaluated with hydrochloric acid 0.05M, using phenolphthalein as indicator. The results are expressed in mgr. of acetic acid/100 gr. of fat.

Measurement of non-volatile acidity

The lipids are extracted using chloroform/methanol, following Bligh and Dyer's method (1959). The acidity of the lipids extracted is determined with KOH (AOAC, 1980). The results are expressed in mgr. of oleic acid/gr. of lipid extracted.

4- Statistical analysis of the results.

With the microbial counts and the results for volatile and non-volatile acidity, a mean comparison test (Student's t test) is performed to see whether there are significant differences between the controls and the samples inoculated.

RESULTS AND DISCUSSION.

Microbial growth in model substrates.

The lipolytic activity of the microorganisms isolated from the ham is studied on model substrates suitable for their specific growth, with the addition of fat from subcutaneous adipose ham tissue. The suitability of the model substrates used and the optimum conditions for development of microorganisms are determined from the growth curves, which comprise mean values from six experiments (figure 1). The results obtained show that all the microorganisms growth on the model substrates. The lactic acid bacteria (Lactobacillus curvatus and Pediococcus pentosaceus) and the yeast (Cryptococcus albidus) show similar behaviour during the incubation period, on the fourth day reaching a maximum which is maintained until the eighth. On the other hand, the growth of the micrococcacea (Staphylococcus xylosus) during the incubation period is progressive. Several authors (Vanderzant et al., 1986, and Talon and Montel, 1986) have studied the development on different substrates of microorganisms isolated from meat products and also observe that micrococcacea and lactic acid bacteria (L. plantarum and L. curvatus) growth on pork fat.

In order to standardise the experiments, it was necessary to carry out a study of lipolytic activity on the model substrates indicated for an incubation period of 8 days at 37ºC. In each experiment microbe counts are taken in order to check growth of the microorganisms.

Evaluation of lipolytic activity.

Lipolytic activities were evaluated from changes in the level of volatile and non-volatile free fatty acids in both the control and inoculated substrates at the start and at the end of the incubation period.

1. Micrococcacea.-

Table I shows the chemical and microbiological results, at the start and after 8 days of incubation at 37ºC, for the control model substrate and the substrate inoculated with Staphylococcus xylosus.

At the end of the incubation period the inoculated samples show a volatile acidity (P.0.01) and non-volatile acidity (P.0.05) which is noticeably different from the control. Specifically, the increase in volatile

acidity is about 3 times greater in the inoculated samples, while the increase in non-volatile acidity is only 11%. The differences observed in the acidity of the control samples before and after inoculation are not significant. The count of viables confirms that Staphylococcus xylosus develops well on subcutaneous adipose ham tissue, with a count of 10^9 cfu/g on day 0 and 10^{11} cfu/g on day 8.

Diverses authors have studied the lipolytic activity of Micrococcaceae isolated from meat products (Mourey, 1978; Cantoni et al., 1966; Debevere et al., 1976). Recently, Soncini et al. (1982), have experimented with micrococci isolated from cured sausages and observe very variable lipolytic behaviour amongst the various strains, from very lipolytic strains which increase acidity 18 times compared with the control to strains which only double the acidity with the same incubation conditions, 7 days at 35ºC. Delarras (1982) has determined the lipolytic activity of micrococcacea isolated from meat products, establishing that staphylococci are more lipolytic than micrococci, with Staphylococcus xylosus showing a high level of lipolytic activity. Dineva et al. (1985) have performed a study of changes in lipids during curing of salami-type sausages using starters, noting that Micrococcaceae are the microorganisms responsible for lipolysis during curing of the sausages and that lipolytic activity depends on the substrate used.

However there are very few studies of lipolytic activity of Micrococcaceae isolated from dry-cured ham. Cantoni et al. (1970b) have carried out a study of the content of volatile and non-volatile fatty acids and carbonylic compounds in fresh and cured subcutaneous adipose ham tissue and report that production of free fatty acids from the ham fat covering is due to the action of microbial and tissular lipases, thus demonstrating the existence of a tissular lipase.

2. Lactic acid bacteria.-

Tables II and III show the results for the experiments with lactic acid bacteria, Pediococcus pentosaceus and Lactobacillus curvatus respectively.

Statistical analysis indicates significant differences (P.0.01) in the content of volatile and non-volatile acidity between the control and inoculated samples at the end of the incubation period. Pediococcus pentosaceus (Table II) increases the volatile and non-volatile acidity of the samples four and two times respectively, compared with the control. Lactobacillus curvatus (Table III) shows a similar pattern of lipolytic activity, with increases in the content of volatile and non-volatile acidity of 2.5 and 1.8 times respectively. The microbial behaviour is also similar, progressing from an initial concentration of 10^7 and 10^8 cfu/g for L. curvatus and P. pentosaceus respectively to viable scores after 8 days of 10^8 and 10^9 cfu/g.

Some authors indicate significant lipolytic activity in lactic acid bacteria, comparable to that of micrococci. However there is little documentation for this. Papon et al. (1988) and Papon and Talon (1988) observe the presence of an active lipase in Lactobacillus curvatus on natural fat (pork fat); Nordal and Slide (1980) do not detect appreciable lipolytic activity in lactic acid bacteria when used as a starter for curing salami-type sausage; however, Bersani and Cantoni (1983) observe that Pediococci affect the aroma of salami-type sausage, due to their ability to hydrolyze fat.

3. Yeasts.-

In a study of lipolytic activity of yeasts isolated from various foods, Alifax (1979) concludes that the most lipolytic species are those belonging to the genera Candida, Cryptococcus and Trichosporum. As a result of this study, Cryptococcus albidus was selected from the yeasts isolated from ham to determine its lipolytic activity.

The results of the analyses for the experiments with the yeast

Cryptococcus albidus are shows on Table IV.

The checks carried out after incubation for eight days at 37ºC confirm significant differences (P.0.01) between the volatile and non-volatile acidity contents of the inoculated samples compared with the controls. These differences amount to increases of 13.7% and 28.1% in the volatile and non-volatile acidity, respectively, in the inoculated samples. It must be emphasised that in this case, as with the lactic acid bacteria, there are significant differences in acidity in the controls before and after incubation, probably due to the characteristics of the culture media used for the model substrates. The yeasts count shows that C. albidus develops well on subcutaneous pork fat, progressing from 10^7 cfu/g to 10^8 cfu/g after incubation for 8 days.

Several authors have studied lipolytic activity in yeasts. Mourey et al. (1977) use model systems of tributyrin and Tweens, and note that a number of strains isolated from meat products exhibit activity on tributyrin but very few on Tweens. Tan and Gill (1985) have studied the development of Saccharomycopsis lipolytica on animal fats and note that most of the saturated fatty acids were consumed during the growth period of the yeasts. Nestorov et al. (1984) and Miteva et al. (1986) indicate that sausages inoculated with yeasts present a greater degree of lipolysis, manifested in a more intense development of aroma. Comi et al. (1983) have studied lipolytic activity in yeasts isolated from Parma ham on various substrates, including ham fat. These authors conclude that the level of lipolytic activity in yeasts isolated from ham is low, and much less than in the case of micrococci and lactic acids.

The results of the present study agree with the observations of Comi et al. (1983).

Figure 2 shows a comparison of the lipolytic activity of microorganisms isolated from ham. As can be seen, the yeast Cryptococcus albidus shows the lowest level of lipolytic activity, the lactic acid bacteria (L. curvatus and P. pentosaceus) present significant activity of similar levels, and Staphylococcus xylosus produces the highest content of volatile acidity.

These results indicate that the microorganisms isolated from Spanish dry-cured ham have a remarkable lipolytic effect and perhaps can play an important role in the sensorial characteristics development. St. xylosus can have a significant effect on the development of aroma, given its high counts and marked lipolytic activity with influence on the development of volatile components. However, yeasts and lactic acid bacteria are only present in small numbers in ham (Silla et al., 1989) and probably may have a little influence on the curing process.

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TABLE III- Initial and final values of volatile acidity, non-volatile acidity and microbial counts of Lactobacillus curvatus in fat from subcutaneous ham tissue, incubated 8 days at 37ºC.

EXPERIENCE NUMBER	INITIAL VALUES			FINAL VALUES				
	CONTROL		INOCULATED SAMPLES	CONTROL		INOCULATED SAMPLES		
	Non-volatile acidity(1)	Volatile acidity(2)	Microbial counts(3)	Non-volatile acidity(1)	Volatile acidity(2)	Non-volatile acidity(1)	Volatile acidity(2)	Microbial counts(3)
1	9,50	116,43	$8,4 \times 10^7$	9,47	211,68	17,18	562,68	$1,5 \times 10^8$
2	8,53	128,56	$7,9 \times 10^7$	10,17	237,32	18,05	535,00	$1,9 \times 10^8$
3	8,61	113,63	$8,6 \times 10^7$	9,44	209,68	16,03	527,79	$2,0 \times 10^8$
4	8,11	116,45	$6,5 \times 10^7$	8,28	203,68	16,71	496,31	$3,8 \times 10^8$
5	8,65	105,75	$9,3 \times 10^7$	9,00	209,77	16,60	528,75	$2,5 \times 10^8$
6	8,85	116,44	$8,5 \times 10^7$	9,65	203,17	17,17	524,51	$1,9 \times 10^8$
$\bar{x}$ (4)	8,70	116,21	$8,2 \times 10^7$	9,33	212,55	16,95	529,17	$2,3 \times 10^8$
s (4)	0,46	7,33	$0,95 \times 10^7$	0,64	12,63	0,68	21,26	$0,82 \times 10^8$
C.V.(%)(4)	5,2	6,3	11,58	6,8	5,9	4,0	4,0	35,65

(1) mg. oleic acid/g. lipid, (2) mg. acetic acid/100g. adipic tissue, (3) cfu/g (colonie forming units per gramme)
(4) $\bar{x}$ (mean values), s (standard desviation), c.v. (coefficient of variation $s/\bar{x}.100$).

TABLE I- Initial and final values of volatile acidity, non-volatile acidity and microbial counts of Staphylococcus xylosus in fat from subcutaneous ham tissue, incubated 8 days at 37ºC.

EXPERIENCE NUMBER	INITIAL VALUES			FINAL VALUES				
	CONTROL		INOCULATED SAMPLES	CONTROL		INOCULATED SAMPLES		
	Non-volatile acidity(1)	Volatile acidity(2)	Microbial counts(3)	Non-volatile acidity(1)	Volatile acidity(2)	Non-volatile acidity(1)	Volatile acidity(2)	Microbial counts(3)
1	5,17	124,93	$12,0 \times 10^9$	5,90	103,98	6,28	387,91	$6,3 \times 10^{11}$
2	6,76	120,52	$8,0 \times 10^9$	5,24	99,88	6,62	333,23	$7,0 \times 10^{11}$
3	5,13	112,70	$6,9 \times 10^9$	5,38	102,22	6,33	343,23	$5,7 \times 10^{11}$
4	5,71	101,73	$8,6 \times 10^9$	5,97	125,05	6,27	372,64	$6,0 \times 10^{11}$
5	5,48	117,28	$5,3 \times 10^9$	6,01	103,12	6,35	346,60	$5,1 \times 10^{11}$
6	5,79	103,09	$15,0 \times 10^9$	6,45	105,51	7,01	388,36	$6,7 \times 10^{11}$
$\bar{x}$ (4)	5,67	113,37	$9,3 \times 10^9$	5,82	107,31	6,47	362,01	$6,1 \times 10^{11}$
s (4)	0,59	9,39	$3,6 \times 10^9$	0,44	9,25	0,29	23,98	$0,69 \times 10^{11}$
C.V.(%)(4)	10,5	8,3	38,38	7,6	8,6	4,5	6,6	11,31

(1) mg. oleic acid/g. lipid, (2) mg. acetic acid/100g. adipic tissue, (3) cfu/g. (colonie forming units per gramme)
(4) $\bar{x}$ (mean values), s (standard deviation), c.v. (coefficient of variation $s/\bar{x}.100$).

TABLE IV- Initial and final values of volatile acidity, non-volatile acidity and microbial counts of Cryptococcus albidus in fat from subcutaneous ham tissue, incubated 8 days at 37ºC.

EXPERIENCE NUMBER	INITIAL VALUES			FINAL VALUES				
	CONTROL		INOCULATED SAMPLES	CONTROL		INOCULATED SAMPLES		
	Non-volatile acidity(1)	Volatile acidity(2)	Microbial counts(3)	Non-volatile acidity(1)	Volatile acidity(2)	Non-volatile acidity(1)	Volatile acidity(2)	Microbial counts(3)
1	6,23	95,09	$1,8 \times 10^7$	9,31	153,29	13,32	175,99	$3,5 \times 10^8$
2	5,55	98,33	$1,2 \times 10^7$	11,03	146,42	13,86	168,98	$2,3 \times 10^8$
3	5,54	92,62	$1,0 \times 10^7$	9,46	142,90	11,76	172,05	$2,9 \times 10^8$
4	7,50	91,07	$1,5 \times 10^7$	11,16	157,10	12,93	166,66	$2,5 \times 10^8$
5	5,62	97,47	$1,9 \times 10^7$	9,17	161,32	11,20	179,11	$2,3 \times 10^8$
6	6,42	91,63	$1,3 \times 10^7$	9,16	148,69	12,89	171,88	$3,4 \times 10^8$
$\bar{x}$ (4)	6,14	94,36	$1,4 \times 10^7$	9,88	151,62	12,66	172,44	$2,8 \times 10^8$
s (4)	0,76	3,07	$0,35 \times 10^7$	0,95	6,91	0,99	4,54	$0,54 \times 10^8$
C.V.(%)(4)	12,4	3,2	25,00	9,6	4,6	7,8	2,6	19,28

(1) mg. oleic acid/g. lipid, (2) mg. acetic acid/100g. adipic tissue, (3) cfu/g. (colonie forming units per gramme)
(4) $\bar{x}$ (mean values), s (standard deviation), c.v. (coefficient of variation s/x.100).

TABLE II- Initial and final values of volatile acidity, non-volatile acidity and microbial counts of Pediococcus pentosaceus in fat from subcutaneous ham tissue, incubated 8 days at 37ºC.

EXPERIENCE NUMBER	INITIAL VALUES			FINAL VALUES				
	CONTROL		INOCULATED SAMPLES	CONTROL		INOCULATED SAMPLES		
	Non-volatile acidity(1)	Volatil acidity(2)	Microbial counts(3)	Non-volatile acidity(1)	Volatil acidity(2)	Non-volatile acidity(1)	Volatil acidity(2)	Microbial counts(3)
1	10,25	105,54	$9,8 \times 10^8$	13,16	129,63	25,83	435,57	$4,8 \times 10^9$
2	11,31	116,28	$8,9 \times 10^8$	13,69	133,47	24,54	402,55	$4,2 \times 10^9$
3	11,51	113,66	$6,9 \times 10^8$	13,38	146,27	24,75	421,16	$8,2 \times 10^9$
4	10,30	105,25	$8,3 \times 10^8$	12,71	160,54	25,70	405,39	$7,2 \times 10^9$
5	11,85	108,32	$9,5 \times 10^8$	11,41	150,25	26,20	430,54	$9,5 \times 10^9$
6	11,85	109,81	$7,0 \times 10^8$	12,87	144,03	25,40	419,04	$5,4 \times 10^9$
$\bar{x}$ (4)	10,84	109,81	$8,4 \times 10^8$	12,87	144,03	25,23	419,04	$6,5 \times 10^9$
s (4)	0,51	4,43	$1,2 \times 10^8$	0,79	11,27	0,51	13,17	$2,08 \times 10^9$
C.V.(%)(4)	4,70	4,00	14,64	6,2	7,8	2,0	3,1	32,00

(1) mg. oleic acid/g. lipid, (2) mg. acetic acid/100g. adipic tissue, (3) cfu/g. (colonie forming units per gramme)
(4) $\bar{x}$ (mean values), s (standard deviation), c.v. (coefficient of variation s/x.100).

Log cfu/g

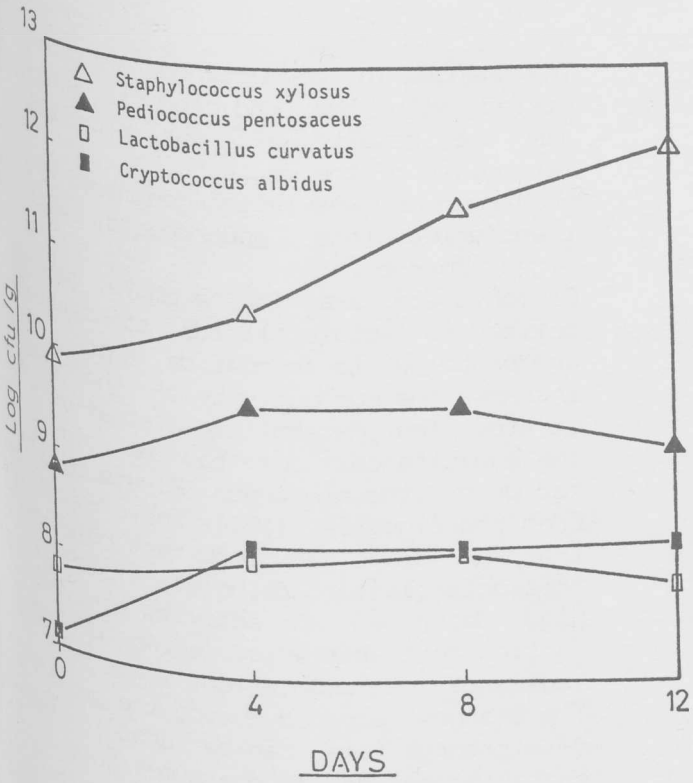

Figure 1.- Microbial growth curves at 37ºC.

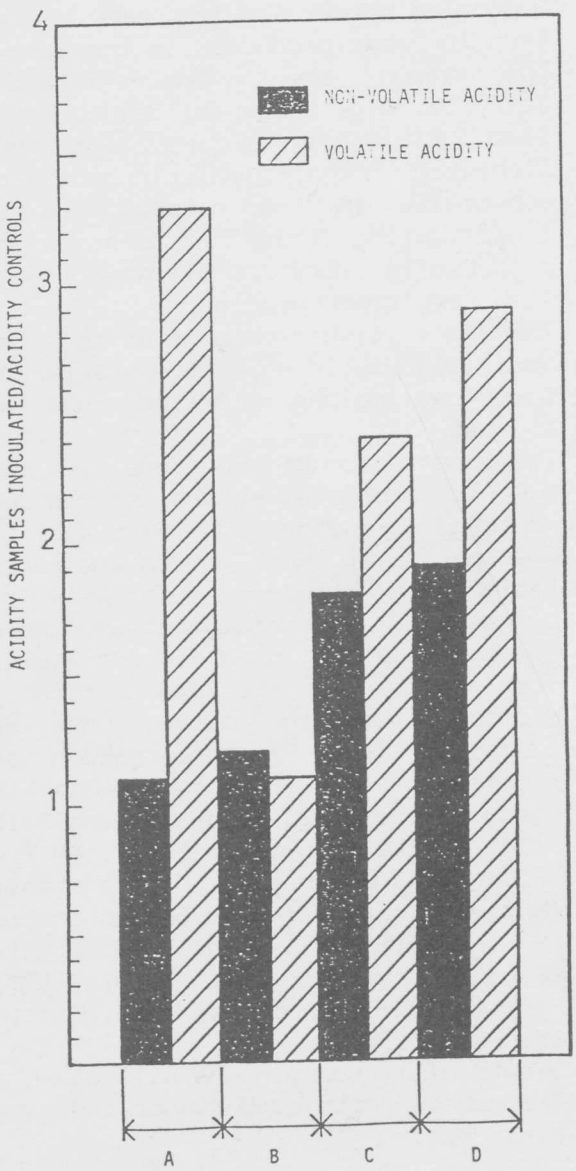

Figure 2.- Lipolytic acidity (A:St. xylosus, B:C. albidus C:L. curvatus, D:P. pentosaceus).