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ORIGINAL ARTICLE

Characterization of *Enterobacteriaceae* strains isolated from spoiled dry-cured hams

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Microbiological and physicochemical aspects of spoiled specimens of dry-cured hams affected by deep putrefaction were studied. Total aerobe (10^3–10^4 cfu g^{-1}), Micrococcaceae(10^4 cfu g^{-1}) and lactic acid bacteria ($<10^2$ cfu g^{-1}) were present at the same levels as in unspoiled control hams. The overall hygienic quality of the hams was good, even when spoiled. Only Enterobacteriaceae counts (10^2–10^3 cfu g^{-1}) were higher in spoiled dry-cured hams. pH (5·85–6·09) and A_w (0·888–0·909) were similar in spoiled and unspoiled hams. Thirty strains of the family Enterobacteriaceae were isolated and characterized. The strains were identified as Serratia liquefaciens *and* Proteus vulgaris*. The strains of* S. liquefaciens *were slightly lipolytic, proteolytic, and psychrotrophic. Only two strains of this species were able to grow at an A_w level of 0·949. The strains of* P. vulgaris *were not lipolytic, were strongly proteolytic and only slightly psychrotrophic, and were able to grow at an A_w level of 0·949. None of them were able to grow at an A_w level of 0·929. The results indicate that the isolated strains could have caused deep putrefaction of dry-cured hams, growing during the first non-refrigerated steps of the curing process before the decrease of A_w. Enterobacteriaceae ought therefore to be considered a microbial quality-related hazard in the development of HACCP systems for dry-cured ham.*

Introduction

The production of Spanish dry-cured ham — a traditional intermediate-moisture meat product which is called Serrano ham when made from various breeds of white hogs, and Iberian ham when made from Iberian (black) hogs — totalled 183 955 tonnes in 1995 (Anonymous 1996), that is, more than 30 million hams per year. In order to assure microbial quality of the ham, it is recommended to apply the HACCP system to control the development of microorganisms that could cause epidemiological and quality related hazards (ICMSF 1988).

Microbiological spoilage of Spanish dry-cured ham has a very unusual occurrence and has therefore been little studied. However, several kinds of defect have been described, among them deep putrefaction. Ham subject to this spoilage is swollen and smells bad when cut open. It presents a large pocket of gas, a soft texture and off-colour. Such hams are easily recognizable and are always weeded out by quality control before they are put up for sale. Cantoni et al. (1987) stated that deep putrefaction was the most common spoilage form in Italian dry-cured ham.

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It has been suggested that enterobacteria could cause deep putrefaction in meat products similar to Spanish dry-cured ham, but the characteristics of the micro-organisms present in these spoiled products have not been comprehensively studied (Simonetti et al. 1983, Cantoni et al. 1994, Papa 1994). On the other hand, there has been comprehensive study of the physicochemical characteristics of similar ham defects (Blanco et al. 1997). In this case the pH values of the spoiled hams were higher.

Micrococcaceae are the prevalent microbiological group in the processing of Spanish Serrano and Iberian dry-cured hams. *Staphylococcus xylosus* is the predominant species, and the presence of pathogenic micro-organisms has not been reported (Silla et al. 1989, Molina et al. 1989, Rodríguez et al. 1994, Sanabria et al. 1997, Marín et al. 1994). In Serrano ham, there is a description of the evolution, at species level, of Fam. Enterobacteriaceae, which are considered to be hygiene indicators (Marin et al. 1996). However, there are no deep studies on characterization of enterobacteria in spoiled Spanish dry-cured ham.

The aim of this work was to study several microbiological and physicochemical aspects of Serrano and Iberian hams affected by deep putrefaction, with particular focus on isolating and characterizing the species names and the growth capabilities of the Enterobacteriaceae strains present. This is important in order to ascertain whether and to what extent they are the cause of such spoilage, and the consequences for a possible HACCP system design.

Materials and Methods

Hams

Deep putrefied hams were analysed following the production processes described previously for Serrano (Marín et al. 1996) and Iberian (Rodríguez et al. 1994) ham. The spoilage was detected during the stages in which the hams were stored at more than 4°C. The signs were strong off odours and an interior gas pocket, with partially liquefied musculature around the gas pocket, visible when the ham was cut transversally near the hip bone, mainly at the *biceps femoris* muscle. The location of the spoilage was always near the femur and femoral artery.

Samples

Samples were taken from spoiled and unspoiled (control) hams, from the affected areas, using a sterile trier under a laminar air-flow hood. After sampling, 10 g per sample was weighed out and diluted by homogenizing under aseptic conditions in a Masticator blender (IUL) in 90 ml of 0·1% sterile peptone water (Pronadisa, Spain). Successive dilutions were performed using the same diluent.

One sample per ham was taken. A total of 12 samples were analysed. These were taken from six Serrano hams and six Iberian hams, three spoiled and three unspoiled in either case.

Microbiological Methods

Microbiological counts. Appropriate dilutions were used to obtain microbial counts of total aerobes, Micrococcaceae, Enterobacteriaceae, Lactic acid bacteria, total and faecal coliforms, sulphide-reducing clostridia, *Clostridium perfringens* and enterotoxigenic *Staphylococcus aureus* and *Salmonella* detection, following the methodology described previously (Marin et al. 1992, Sanabria et al. 1997).

Characterization of Enterobacteriaceae strains. Strains were isolated from the VRBG count plates following the methodology previously described (Marín et al. 1992; Sanabria et al. 1997), and subjected to preliminary taxonomic assays to verify their membership to Fam. Enterobacteriaceae, following the methodology described by Cowan and Steel (1993).

Next, overnight cultures (37°C, 200 rpm) were prepared in brain heart infusion (BHI) (Pronadisa) with the strains belonging to Fam. Enterobacteriaceae. These were used to study the following characteristics:

Proteolytic activity: in gelatin and caseinate agar (Pronadisa), seeding by puncture in the centre of plates, incubation for 48 h at 37°C. The resulting proteolysis haloes were measured in mm, employing a Haloes caliper (IUL, Germany).

Lipolytic activity: in tributyrin agar (Mourey and Klibertus 1986), and Tween 40 and 80

(Merck, Germany) agar (Sierra 1957), seeding in furrows, incubation for 48 h at 37°C. The resulting lypolisis haloes were measured in mm using a Haloes caliper (IUL).

Estimation of minimum water activity (A_w): By the method of McMeekin et al. (1987), seeding with 100 µl of culture in BHI broth with $A_w = 0{\cdot}949$, 0·928 and 0·909, and static incubation for 48 h at 37°C. Growth was considered positive when the turbidity was visible to the naked eye.

Psychrotrophy: in plate count agar, seeding by furrow, incubation for 15 days at 4°C, noting the day that there was growth in the entire seeded furrow.

Finally, Enterobacteriaceae strains were identified at species level following the methodology described previously (Marín et al. 1996).

Physicochemical methods

Physicochemical parameters were analysed following the methodology described below:

- pH: using the Bacus method (Bacus 1984).
- A_w: using a Rotronic-Hygroskop DT apparatus with temperature probe WA-40 maintained at 25°C.

Statistical methods

Analysis of variance were made in a personal computer with the Statgraphics® program. Microbiological data were previously transformed to logarithm.

Results

The results of the microbiological counts are summarized in Table 1. The total aerobic count was very similar ($P > 0{\cdot}05$) in all the samples analysed, in the range 10^3–10^4 cfu g^{-1}. The micrococcaceae count was of roughly the same magnitude; the lactic bacteria count was $< 10^2$ cfu g^{-1}. The enterobacteria count (Table 1) was 10^2–10^3 cfu g^{-1} in the spoiled hams and < 10 cfu g^{-1} in the controls.

Total and faecal coliforms, sulphide-reducing clostridia, *Clostridium perfringens* and Enterotoxigenic *Staphylococcus aureus* counts were < 10 cfu g^{-1} (data not shown). The presence of *Salmonella* was not detected (absence in 25 g) in any samples.

Only 30 of the 40 strains isolated from VRBG plates of spoiled ham counts belonged to Fam. Enterobacteriaceae, 15 from Serrano and 15 from Iberian spoiled hams. The characterization of the strains isolated from spoiled Serrano hams is shown in Table 2. The study of lipolytic capacity showed that these strains possessed little capacity, although all except strain IFI61 presented activity vs at least one of the substrates assayed. None presented activity versus all three substrates. The haloes never exceeded 3 mm, and this size was attained most frequently on the Tween 40 plates. The definition of the lipolytic halo was much greater on the tributyrin agar plates than on the Tween plates.

Only strains IFI61, IFI65, IFI66, IFI67, IFI70 and IFI71 presented proteolytic activity versus both caseinate and gelatin (Table 2). The holes on the plates were considerably larger than the lipolytic haloes. The only strain that did not present proteolytic activity versus the substrates was IFI58. The largest halo was produced by strain IFI61 on gelatin agar (33 mm).

Only two of the 15 strains isolated from Serrano hams were capable of growth at $A_w = 0{\cdot}949$ (Table 2). All strains showed growth at 0·976 and none at 0·928. All presented

Table 1. Microbiological counts of spoiled and control hams (cfu g^{-1})

	Serrano ham counts		Iberian ham counts	
	Spoiled	Control	Spoiled	Control
Total aerobes	$6{\cdot}5 \times 10^3$a	$3{\cdot}8 \times 10^4$a	$3{\cdot}2 \times 10^4$a	$8{\cdot}2 \times 10^3$a
Micrococcaceae	$3{\cdot}5 \times 10^4$a	$6{\cdot}6 \times 10^4$a	$8{\cdot}3 \times 10^4$a	$2{\cdot}8 \times 10^4$a
Enterobacteriaceae	$2{\cdot}5 \times 10^3$a	< 10	$3{\cdot}9 \times 10^2$a	< 10
Lactic acid bacteria	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$

(Data in the same row with different letters differ significantly: $P < 0{\cdot}05$).

Table 2. Characterization of enterobacterial strains isolated from spoiled Serrano hams

	Lipolysis[a]			Proteolysis[a]		A_w				
Strain number	T40	T80	TRI	CAS	GEL	0·976	0·949	0·928	4°C[b]	Species name
IFI57	1	–	1	–	15	+	–	–	8	*Serratia liquefaciens*
IFI58	3	–	–	–	–	+	–	–	10	*Serratia liquefaciens*
IFI59	–	–	2	–	23	+	–	–	9	*Serratia liquefaciens*
IFI60	3	–	1	–	13	+	–	–	8	*Serratia liquefaciens*
IFI61	–	–	–	30	33	+	+	–	6	*Serratia liquefaciens*
IFI62	2	–	1	–	15	+	–	–	10	*Serratia liquefaciens*
IFI63	–	–	2	–	11	+	–	–	10	*Serratia liquefaciens*
IFI64	2	–	2	–	9	+	–	–	10	*Serratia liquefaciens*
IFI65	1	–	1	6	21	+	+	–	10	*Serratia liquefaciens*
IFI66	–	–	2	2	13	+	–	–	10	*Serratia liquefaciens*
IFI67	3	–	2	3	27	+	–	–	9	*Serratia liquefaciens*
IFI68	2	–	1	–	12	+	–	–	13	*Serratia liquefaciens*
IFI69	3	1	–	–	16	+	–	–	7	*Serratia liquefaciens*
IFI70	1	1	–	7	17	+	–	–	6	*Serratia liquefaciens*
IFI71	2	1	–	28	14	+	–	–	7	*Serratia liquefaciens*

[a]Data given in mm.
[b]Data given in days.

Table 3. Characterization of enterobacterial strains isolated from spoiled Iberian hams

	Lipolysis[a]			Proteolysis[a]		A_w				
Strain number	T40	T80	TRI	CAS	GEL	0·976	0·949	0·928	4°C[b]	Species name
IFI72	–	–	–	7	28	+	+	–	–	*Proteus vulgaris*
IFI73	–	–	–	27	16	+	+	–	13	*Proteus vulgaris*
IFI76	–	–	–	30	30	+	+	–	–	*Proteus vulgaris*
IFI77	–	–	–	30	33	+	+	–	14	*Proteus vulgaris*
IFI82	–	–	–	22	24	+	+	–	–	*Proteus vulgaris*
IFI90	–	–	–	35	60	+	+	–	–	*Proteus vulgaris*
IFI73b	–	–	–	15	26	+	+	–	–	*Proteus vulgaris*
IFI78b	–	–	–	7	28	+	+	–	–	*Proteus vulgaris*
IFI80b	–	–	–	6	26	+	+	–	–	*Proteus vulgaris*
IFI81b	–	–	–	10	24	+	+	–	–	*Proteus vulgaris*
IFI83b	–	–	–	17	30	+	+	–	–	*Proteus vulgaris*
IFI84b	–	–	–	45	22	+	+	–	–	*Proteus vulgaris*
IFI85b	–	–	–	26	29	+	+	–	–	*Proteus vulgaris*
IFI86b	–	–	–	6	30	+	+	–	13	*Proteus vulgaris*
IFI87b	–	–	–	6	23	+	+	–	–	*Proteus vulgaris*

[a]Data given in mm.
[b]Data given in days.

growth at 4°C in less than 14 days incubation; IFI61 and IFI70 did so in only 6 days. The 15 strains isolated from Serrano hams were classified as *Serratia liquefaciens* (Table 2).

The results of the characterization of the 15 strains from Fam. Enterobacteriaceae isolated in spoiled Iberian hams are summarized in Table 3. No lipolytic activity was detected in any of the isolated strains by the methods used, either vs Tween 40, Tween 80 or tributyrin. The reverse was true in the case of proteolytic activity: all strains presented activity vs caseinate and gelatin. The proteolytic haloes were maximum for strain IFI84b vs caseinate (45 mm) and for strain IFI90 vs gelatin (60 mm). This last was the largest of the entire study.

All the Enterobacteriaceae strains isolated from Iberian hams presented growth at both $A_w = 0{\cdot}976$ and 0·949, and no growth at 0·928 (Table 3). Only strains IFI73, IFI77 and IFI86b

Table 4. pH and water activity (A_w) of spoiled and control hams

	Serrano hams		Iberian hams	
	Spoiled	Control	Spoiled	Control
pH	5·85a	6·09a	5·94a	5·92a
A_w	0·895a	0·909a	0·889a	0·888a

(Data in the same row with different letters differ significantly: $P < 0{\cdot}05$).

grew at 4°C before the ellapse of 14 days incubation. All the enterobacteria strains isolated from Iberian hams were identified as *Proteus vulgaris*.

The results of the analysis of physicochemical parameters are shown in Table 4. The values of pH and A_w were very similar for control and spoiled hams. In the case of A_w, the values were slightly higher ($P > 0{\cdot}05$) in the Serrano hams.

Discussion

The predominant group of micro-organisms in the deep tissues at the end of processing was Micrococcaceae in both Serrano and Iberian hams. This was deduced from a comparison between the total aerobe count and the count for these microbe groups. This comparison and the detection of lactic acid bacteria agree with data reported by other authors for similar products (Baldini et al. 1977, Langlois et al. 1979, Molina et al. 1989, Rodríguez et al. 1994). Our results further showed that this also occurs in Serrano and Iberian hams with deep putrefaction.

The results for pathogenic micro-organisms confirmed once more that Serrano and Iberian hams, even when affected with deep putrefaction, lacked such microorganisms at levels that could cause epidemiological risk, and therefore they are similar to cooked ham in terms of sanitary health quality (Marín et al. 1994, Sanabria et al. 1997).

The enterobacteria count was higher in the spoiled hams with deep putrefaction (10^2–10^3 cfu g^{-1}) than in the controls (<10 cfu g^{-1}). Other authors have reported similar enterobacteria counts in cured hams (Arnau et al. 1987, Simonetti et al. 1983, Cantoni et al. 1994, Papa 1994). The concentration of enterobacteria in the hams with deep putrefaction was similar to that found on the surface of fresh Serrano hams and greater than that found in the interior of these hams during processing (Marín et al. 1996).

Only 75% of the strains isolated on the VRBG plate counts belonged to Fam. Enterobacteriaceae, as reported in previous studies of Serrano ham (Marín et al. 1996). The isolated strains presented high proteolytic capacity and low lipolytic capacity versus the substrates assayed, unlike the strains of *Staphylococcus xylosus*, which predominate during the curing of ham (Carrascosa and Cornejo 1991).

Given that putrefaction consists broadly of the decomposition of proteins by microorganisms resulting in the formation of gas and bad smells (Pelczar et al. 1981), and given high proteolytic capacity of the strains of Enterobacteriaceae isolated in Serrano and Iberian hams affected with deep putrefaction where major aroma defects were detected as well as gas, these strains could have caused the spoilage known as deep putrefaction. The direct action of Enterobacteriaceae on myoglobin could also be the cause of the serious colour defects in the areas affected by such spoilage.

In the estimation of the minimum A_w, the strains of Enterobacteriaceae isolated from spoiled hams did not grow at $A_w = 0{\cdot}928$, but many did so 0·949. These values are much higher than those found in the hams with deep putrefaction and the controls in the present study. We therefore suppose that these strains grew until their high proteolytic activity caused deep putrefaction in stages of processing where the values of A_w were such as to permit their growth.

During processing, A_w in the interior of hams decreased continuously. Moreover, this

decrease was not homogeneous. In Serrano hams values of 0·93 were attained in *semimembranosus* muscle at the end of post-salting, and 0·95 in the *biceps femoris* muscle (Marín et al. 1996). In Iberian ham the pattern was the same but at the beginning of post-salting (Sanabria et al. 1997). Depending on the values of A_w, we assume that the strains could have grown only up to the end of post-salting or a little after, as long as the processing temperature so permitted.

The Enterobacteriaceae strains isolated from Serrano ham were more psychrotrophic than those from Iberian ham. In ham processing the temperature is kept at about 4°C during salting. It increases following post-salting and can exceed 30°C during drying (Rodríguez et al. 1994, Marín et al. 1996). The isolated strains could therefore have begun growing during salting and carried on growing until prevented by A_w during post-salting or drying. This occurs first in the *semimembranosus* muscle and only later in the *biceps femoris* muscle (Marín et al. 1996, Sanabria et al. 1997) where deep putrefaction is normally located. The occurrence of deep putrefaction would be favoured by faulty salting that delayed the reduction of A_w in the interior of the hams.

Taxonomic determination showed that all the strains of Enterobacteriaceae isolated in Serrano hams belonged to the species *S. liquefaciens*, and that the ones isolated in Iberian hams belonged to the species *P. vulgaris*. Both species are considered to be proteolytic and non-pathogenic (Brenner 1994) and have never produced food poisoning (ICMSF 1996).

The enterobacteria species found in this study were the same as those isolated at the early stages of curing of white pig hams (Marín et al. 1996), and in similar spoiled meat products (Simonetti et al. 1983, Cantoni et al. 1994, Papa 1994). Stiles (1981) reported that *Serratia* and *Proteus* were the genera most commonly present on working surfaces in the meat processing industry.

The pH and A_w registered in spoiled and unspoiled hams were quite similar to those recorded in Italian (Baldini et al. 1977) and American (Langlois et al. 1979) hams.

If we have to accept the hypothesis that the strains of *S. liquefaciens* and *P. vulgaris* isolated from hams presenting deep putrefaction are the cause of this spoilage, we must also accept that these strains could have reached the interior of the hams in the area where the spoilage is detected.

This would be possible during processing only if there were deep cracks in the musculature, given that deep putrefaction betrayed no signs of proteolytic activity in the surface tissues at any time during processing.

It is virtually impossible to accept that this could occur during processing, since the micro-organisms would have to be capable of carrying on proteolytic activity to be able to penetrate the internal musculature during salting and post-salting, when the surface A_w is $<0{\cdot}90$ (Marín et al. 1994, Sanabria et al. 1997). Moreover, only a very small number of micro-organisms belonging to Fam. Micrococcaceae can reach the deep musculature from the surface (Cornejo et al. 1992).

The only possibility is that the strains reached the deep musculature during slaughter and cutting through contamination of faecal origin transmitted by the circulatory flow, as some authors have shown can occur (Troeger and Woltersdorf 1986).

In such an event, the strains of *S. liquefaciens*, being clearly psychrotrophic, would grow slowly during the salting and post-salting stages; in the event of faulty salting, once drying began they would grow faster given processing temperatures of up to 35°C, until spoilage occurred, which would not be detected until the end of the process. In the case of *P. vulgaris*, given that these micro-organisms are less psychrotrophic but more halotolerant, these would grow as a result either of inadequate chilling during cutting and transport, which is conceivable given the low incidence of spoilage, or else after post-salting as a result of high temperatures and faulty salting which slow down the reduction of A_w to inhibiting levels.

The results make it possible to differentiate deep putrefaction from other kinds of meat spoilage like bone taint. Whereas gas production is clearly detectable in deep putrefaction, it is uncommon in bone taint of fresh meat, while isolation of *Clostridium* spp. is common (Rosset 1982). Both surface and deep spoilage in fresh meat are normally associated with

slow chilling of carcasses, particularly where the animal has a relatively thick subcutaneous fat layer. The appearance of gas in the interior of the meat is also associated with higher temperatures (25–40°C) (Rosset 1982).

To our knowledge, the Enterobacteriaceae levels and species found in the spoiled hams studied are harmless to man and are therefore of minor interest strictly from the point of view of health. Faulty curing could explain the presence of banal saprophytic enterobacterial species present in fresh hams, which may sometimes cause spoilage in cured hams, as has also been described in the case of other cured meat products (Hechelmann et al. 1980, Campanini and Casolari 1983, Lenges 1986).

The results of this study seems to indicate that certain Enterobacteriaceae could cause spoilage in dry hams of Iberian and Serrano types. To definitively confirm this hypothesis, some of the isolated strains should be inoculated in unspoiled fresh hams, ascertain that deep putrefaction does occur, and isolate the spoiled tissues containing these strains, just as must be done with human infectious agents according to the Postulates of Koch. If confirmed, these organisms should be considered as a quality related hazard in any hazard analysis done as part of introducing HACCP in dry ham processing lines.

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